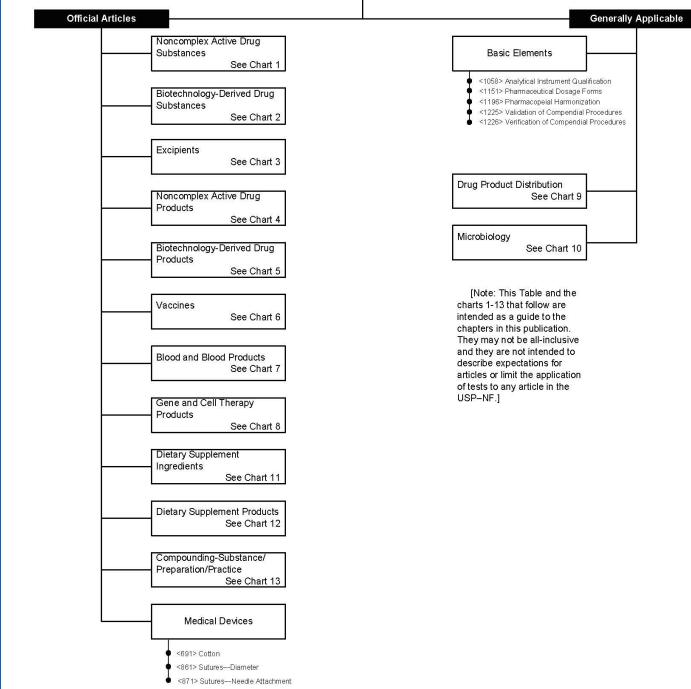


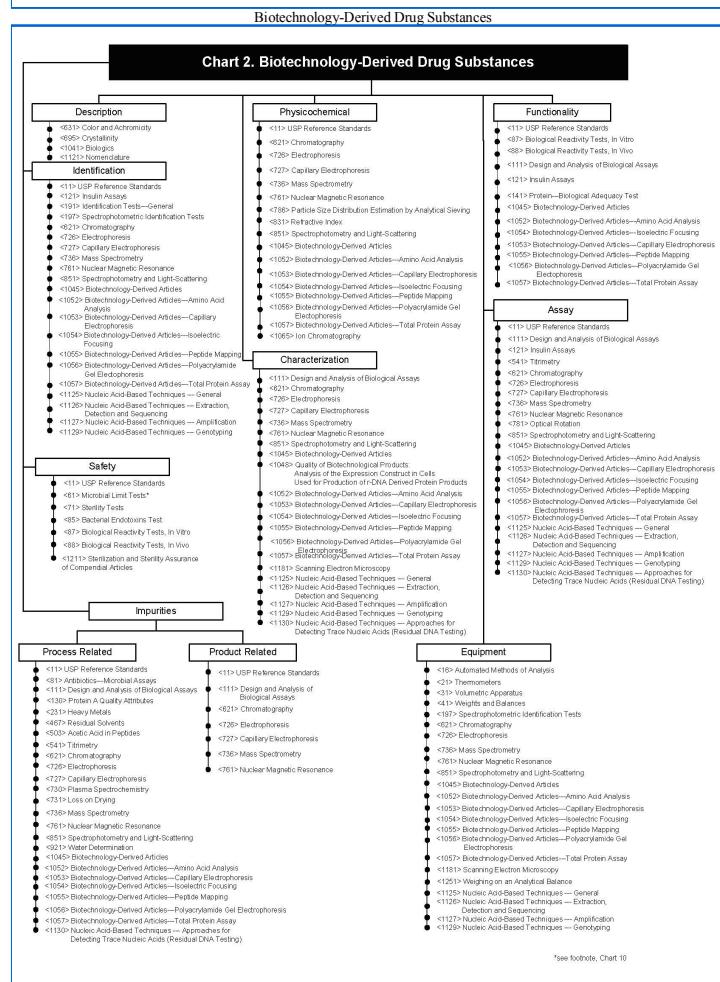
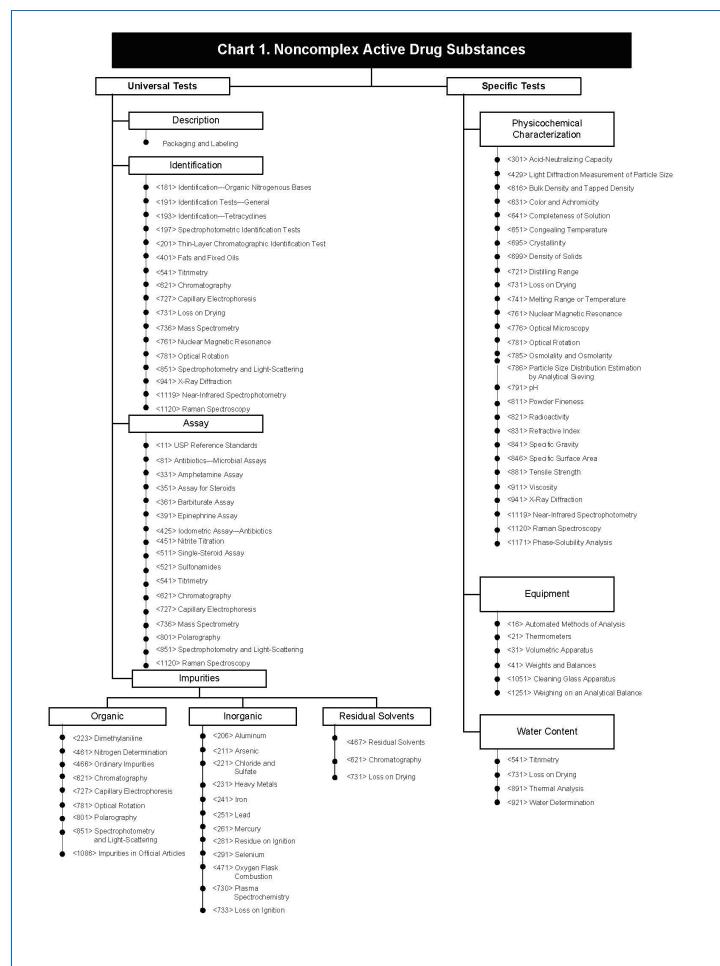


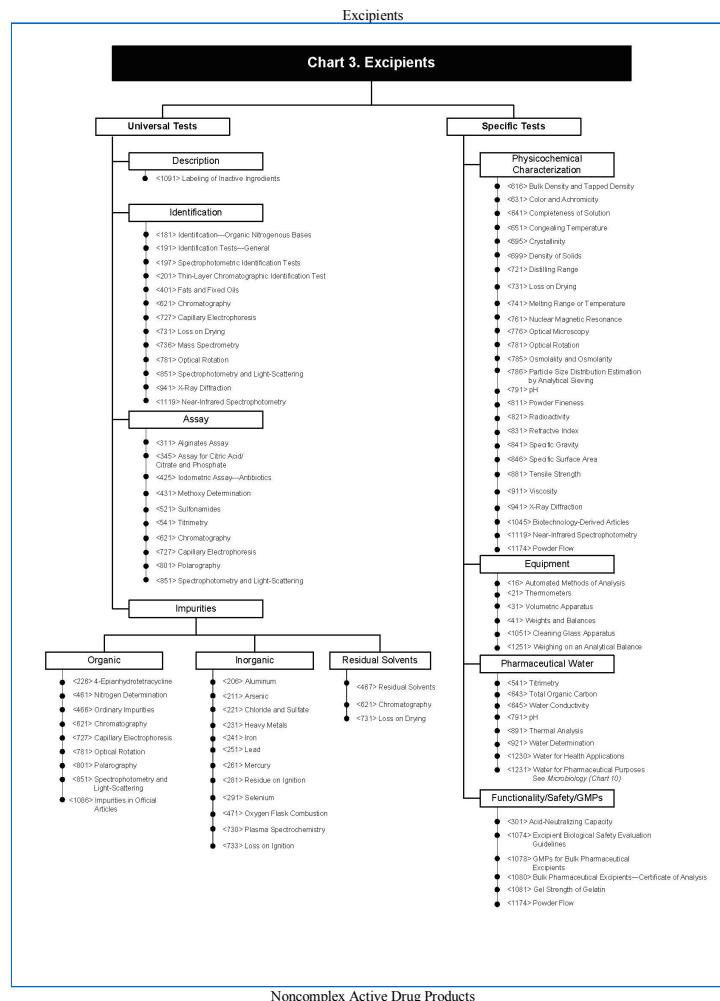
## USP General Chapters - Chart Guide

## Chart Guide

## USP General Chapters



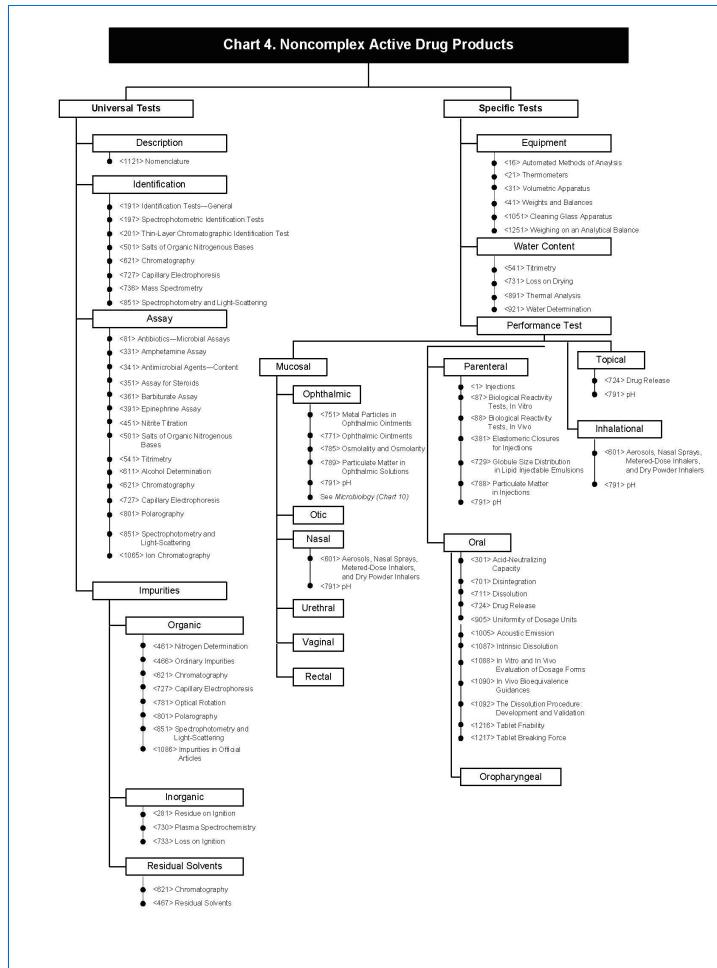




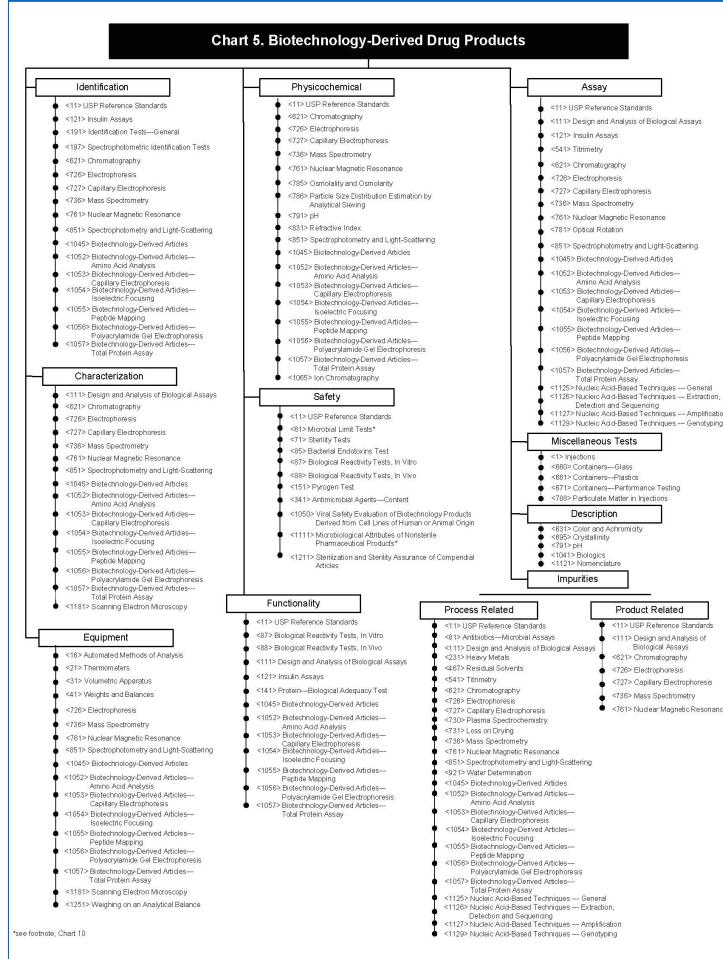
Noncomplex Active Drug Products



**Chart 4. Noncomplex Active Drug Products**



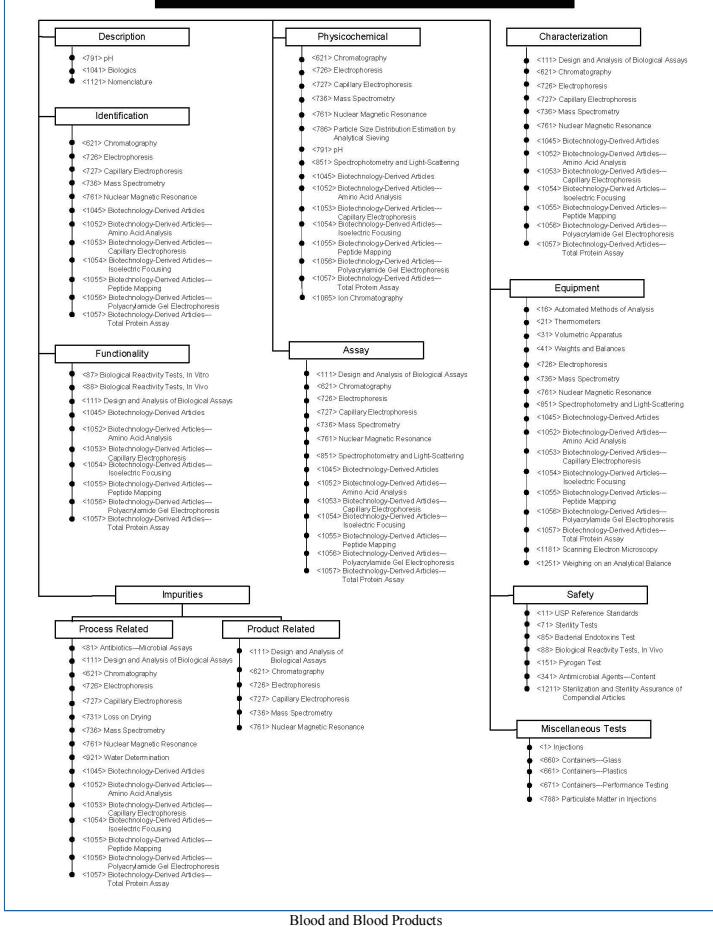
## Biotechnology-Derived Drug Product



\*see footnote, Chart 1

## Vaccines

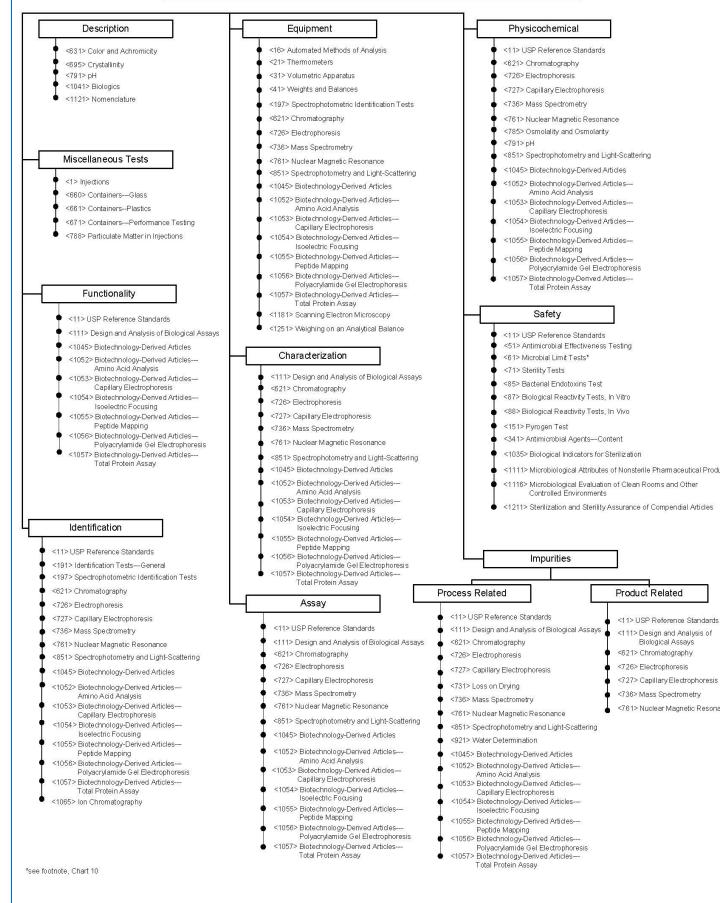
## Chart 6. Vaccines



## Blood and Blood Products



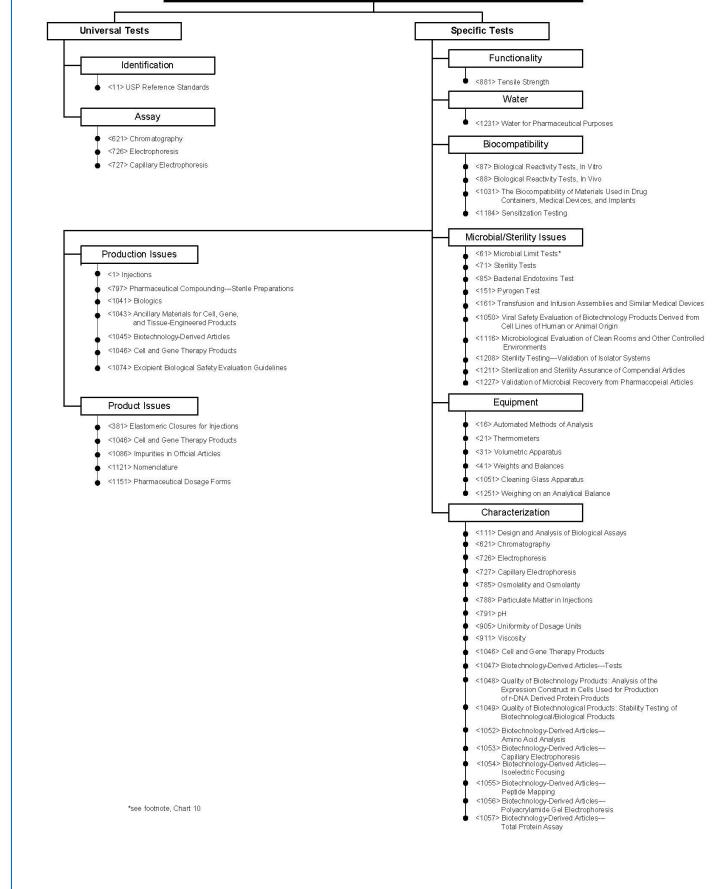
#### Chart 7. Blood and Blood Product



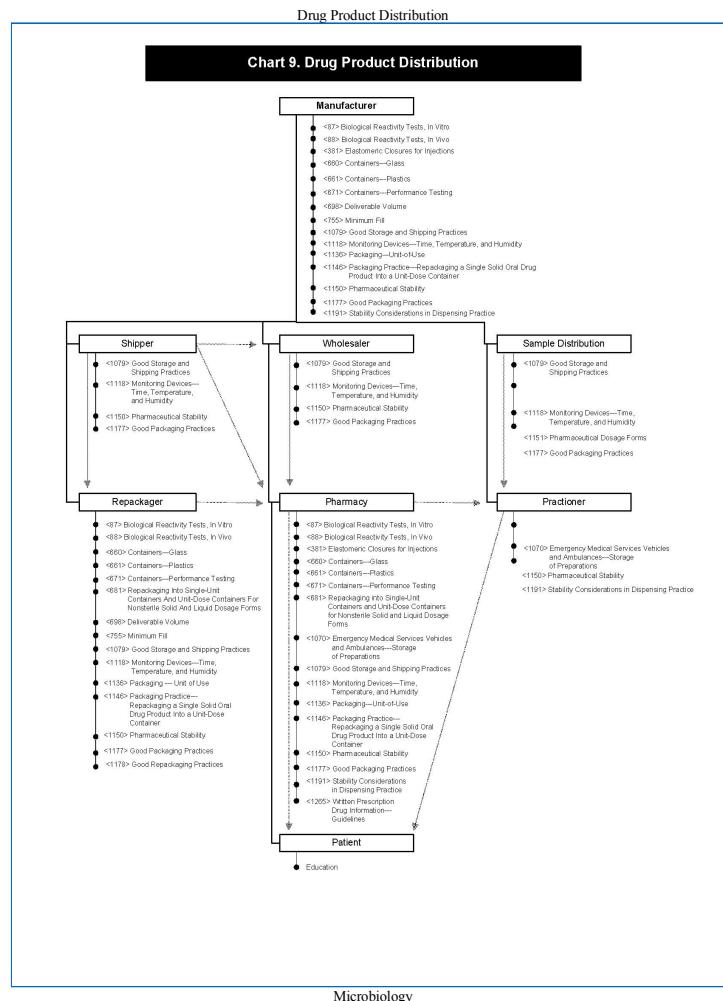
\*see footnote, Chart 1

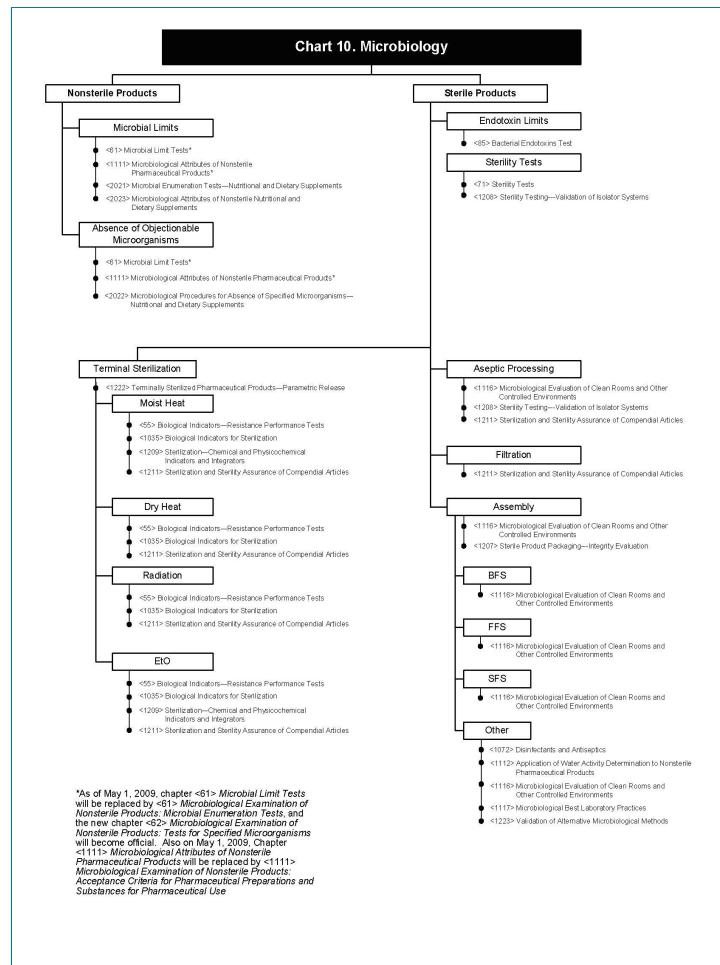
Gene and Cell Therapy Products

**Chart 8. Gene and Cell Therapy Product**

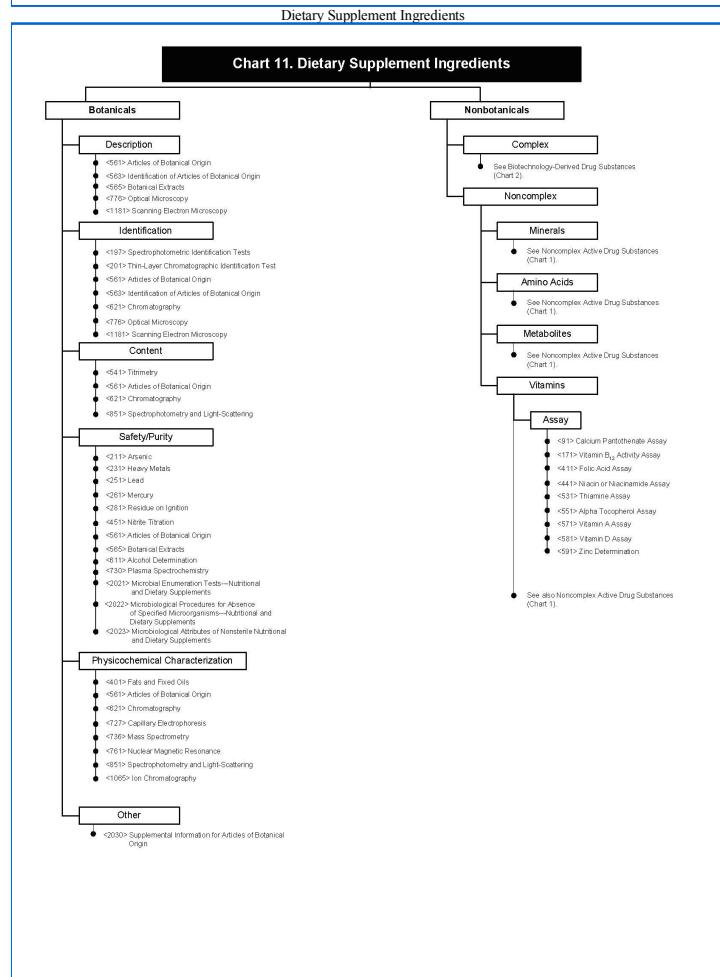


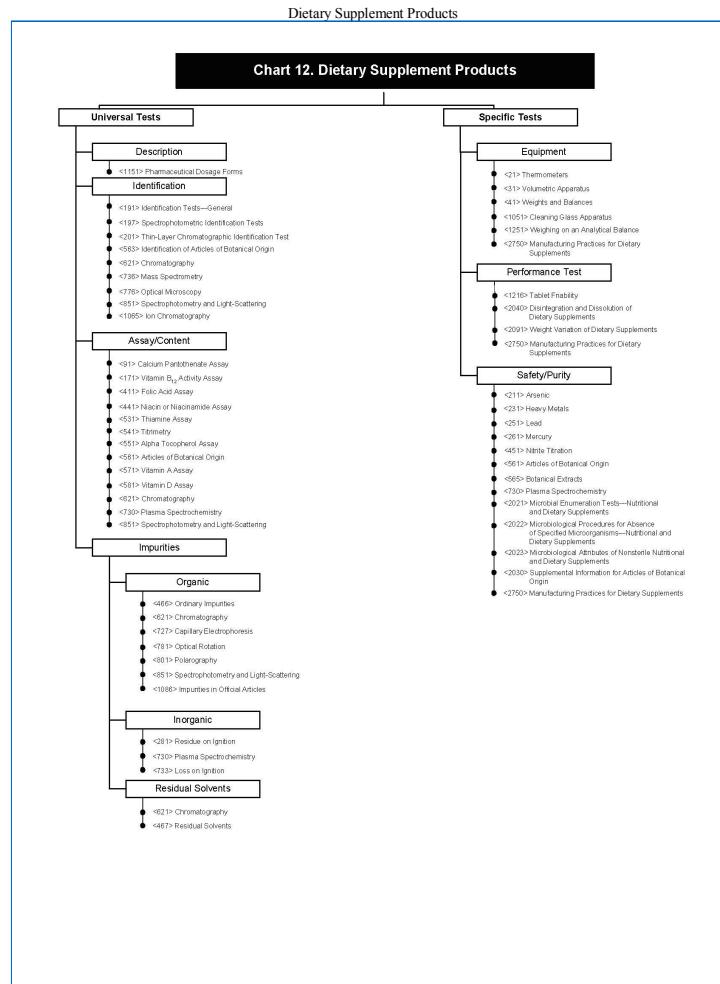
Two Institute Chart 10

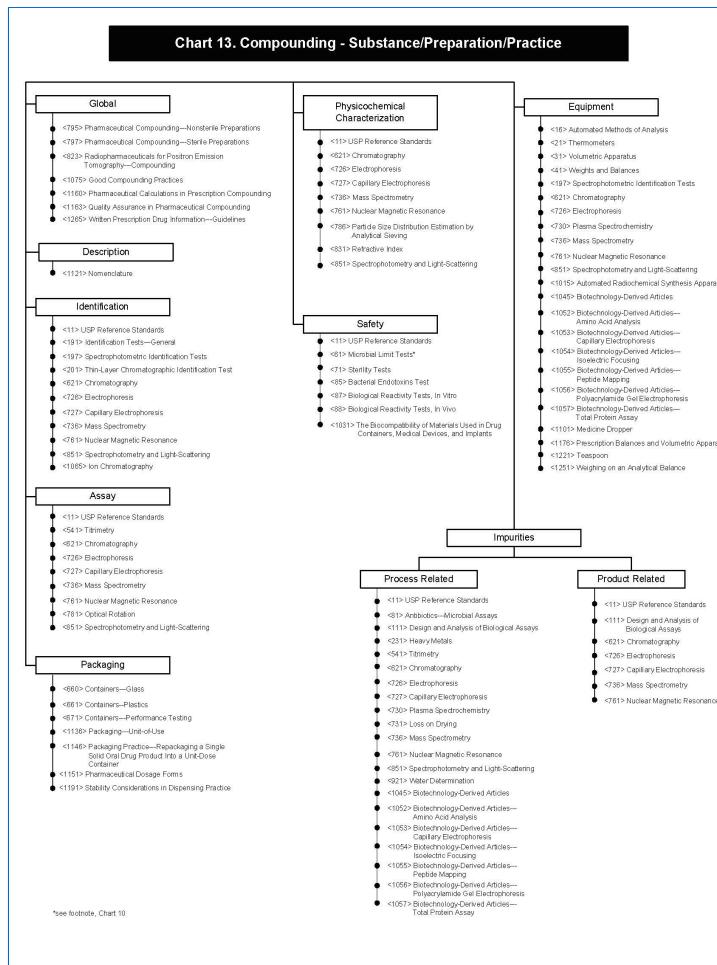




\*As of May 1, 2003, chapter <81> Microbial Limit Tests will be replaced by <81> Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests, and the new chapter <82> Microbiological Examination of Nonsterile Products: Microbiological Attributes of Nonsterile Pharmaceutical Products. Chapter <1111> Microbiological Attributes of Nonsterile Pharmaceutical Products—Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use will become official. Also on May 1, 2003, Chapter <1111> Microbiological Attributes of Nonsterile Pharmaceutical Products—Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use







\*see footnote, Chart 10

## 1 INJECTIONS

### INTRODUCTION

Parenteral articles are preparations intended for injection through the skin or other external boundary tissue, rather than through the alimentary canal, so that the active substances they contain are administered, using gravity or force, directly into a blood vessel, organ, tissue, or lesion. Parenteral articles are prepared scrupulously by methods designed to ensure that they meet Pharmacopeial requirements for sterility, pyrogens, particulate matter, and other contaminants, and, where appropriate, contain inhibitors of the growth of microorganisms. An Injection is a preparation intended for parenteral administration and/or for constituting or diluting a parenteral article prior to administration.

### NOMENCLATURE AND DEFINITIONS

#### Nomenclature\*

The following nomenclature pertains to five general types of preparations, all of which are suitable for, and intended for, parenteral administration. They may contain buffers, preservatives, or other added substances.

1. [DRUG] Injection—Liquid preparations that are drug substances or solutions thereof.
2. [DRUG] for Injection—Dry solids that, upon the addition of suitable vehicles, yield solutions conforming in all respects to the requirements for Injections.
3. [DRUG] Injectable Emulsion—Liquid preparations of drug substances dissolved or dispersed in a suitable emulsion medium.
4. [DRUG] Injectable Suspension—Liquid preparations of solids suspended in a suitable liquid medium.
5. [DRUG] for Injectable Suspension—Dry solids that, upon the addition of suitable vehicles, yield preparations conforming in all respects to the requirements for Injectable Suspensions.

#### Definitions

##### pharmacy bulk package

A Pharmacy bulk package is a container of a sterile preparation for parenteral use that contains many single doses. The contents are intended for use in a pharmacy admixture program and are restricted to the preparation of admixtures for infusion or, through a sterile transfer device, for the filling of empty sterile syringes.

The closure shall be penetrated only one time after constitution with a suitable sterile transfer device or dispensing set which allows measured dispensing of the contents. The Pharmacy bulk package is to be used only in a suitable work area such as a laminar flow hood (or an equivalent clean air compounding area).

Designation as a Pharmacy bulk package is limited to preparations from Nomenclature categories 1, 2, or 3 as defined above. Pharmacy bulk packages, although containing more than one single dose, are exempt from the multiple-dose container volume limit of 30 mL and the requirement that they contain a substance or suitable mixture of substances to prevent the growth of microorganisms.

Where a container is offered as a Pharmacy bulk package, the label shall (a) state prominently "Pharmacy Bulk Package—Not for direct infusion," (b) contain or refer to information on proper techniques to help assure safe use of the product, and (c) bear a statement limiting the time frame in which the container may be used once it has been entered, provided it is held under the labeled storage conditions.

#### large- and small-volume injections

Where used in this Pharmacopeia, the designation Large-volume intravenous solution applies to a single-dose injection that is intended for intravenous use and is packaged in containers labeled as containing more than 100 mL. The designation Small-volume Injection applies to an Injection that is packaged in containers labeled as containing 100 mL or less.



## biologics

The Pharmacopeial definitions for sterile preparations for parenteral use generally do not apply in the case of the biologics because of their special nature and licensing requirements (see [Biologics \(1041\)](#)).

### INGREDIENTS

#### Vehicles and Added Substances

**Aqueous Vehicles**— The vehicles for aqueous Injections meet the requirements of the [Pyrogen Test \(151\)](#) or the [Bacterial Endotoxins Test \(85\)](#), whichever is specified. Water for Injection generally is used as the vehicle, unless otherwise specified in the individual monograph. Sodium chloride may be added in amounts sufficient to render the resulting solution isotonic; and Sodium Chloride Injection, or Ringer's Injection, may be used in whole or in part instead of Water for Injection, unless otherwise specified in the individual monograph. For conditions applying to other adjuvants, see Added Substances in this chapter.

**Other Vehicles**— Fixed oils used as vehicles for nonaqueous Injections are of vegetable origin, are odorless or nearly so, and have no odor suggesting rancidity. They meet the requirements of the test for Solid paraffin under Mineral Oil, the cooling bath being maintained at  $10^{\circ}$ , have a Saponification Value between 185 and 200 (see [Fats and Fixed Oils \(401\)](#)), have an Iodine Value between 79 and 141 (see [Fats and Fixed Oils \(401\)](#)), and meet the requirements of the following tests.

**Unsaponifiable Matter**— Reflux on a steam bath 10 mL of the oil with 15 mL of sodium hydroxide solution (1 in 6) and 30 mL of alcohol, with occasional shaking until the mixture becomes clear. Transfer the solution to a shallow dish, evaporate the alcohol on a steam bath, and mix the residue with 100 mL of water: a clear solution results.

**Free Fatty Acids**— The free fatty acids in 10 g of oil require for neutralization not more than 2.0 mL of 0.020 N sodium hydroxide (see [Fats and Fixed Oils \(401\)](#)).

Synthetic mono- or diglycerides of fatty acids may be used as vehicles, provided they are liquid and remain clear when cooled to  $10^{\circ}$  and have an Iodine Value of not more than 140 (see [Fats and Fixed Oils \(401\)](#)).

These and other nonaqueous vehicles may be used, provided they are safe, in the volume of Injection administered, and also provided they do not interfere with the therapeutic efficacy of the preparation or with its response to prescribed assays and tests.

**Added Substances**— Suitable substances may be added to preparations intended for injection to increase stability or usefulness, unless proscribed in the individual monograph, provided they are harmless in the amounts administered and do not interfere with the therapeutic efficacy or with the responses to the specified assays and tests. No coloring agent may be added, solely for the purpose of coloring the finished preparation, to a solution intended for parenteral administration (see also Added Substances under General Notices and [Antimicrobial Effectiveness Testing \(51\)](#)).

Observe special care in the choice and use of added substances in preparations for injection that are administered in a volume exceeding 5 mL. The following maximum limits prevail unless otherwise directed: for agents containing mercury and the cationic, surface-active compounds, 0.01%; for chlorobutanol, cresol, phenol, and similar types of substances, 0.5%; and for sulfur dioxide, or an equivalent amount of the sulfite, bisulfite, or metabisulfite of potassium or sodium, 0.2%.

A suitable substance or mixture of substances to prevent the growth of microorganisms must be added to preparations intended for injection that are packaged in multiple-dose containers, regardless of the method of sterilization employed, unless one of the following conditions prevails: (1) there are different directions in the individual monograph; (2) the substance contains a radionuclide with a physical half-life of less than 24 hours; and (3) the active ingredients are themselves antimicrobial. Such substances are used in concentrations that will prevent the growth of or kill microorganisms in the preparations for injection. Such substances also meet the requirements of [Antimicrobial Effectiveness Testing \(51\)](#) and [Antimicrobial Agents—Content \(341\)](#). Sterilization processes are employed even though such substances are used (see also [Sterilization and Sterility Assurance of Compendial Articles \(1211\)](#)). The air in the container may be evacuated or be displaced by a chemically inert gas. Where specified in a monograph, information regarding sensitivity of the article to oxygen is to be provided in the labeling.

### LABELS AND LABELING

#### Labeling

**note**—See definitions of "label" and "labeling" in Labeling in the section Preservation, Packaging, Storage, and Labeling of the General Notices and Requirements.

The label states the name of the preparation; in the case of a liquid preparation, the percentage content of drug or amount of drug in a specified volume; in the case of a dry preparation, the amount of active ingredient; the route of administration; a statement of storage conditions and an expiration date; the name and place of business of the manufacturer, packer, or distributor; and an identifying lot number. The lot number is capable of yielding the complete manufacturing history of the specific package, including all manufacturing, filling, sterilizing, and labeling operations.

Where the individual monograph permits varying concentrations of active ingredients in the large-volume parenteral, the concentration of each ingredient named in the official title is stated as if part of the official title, e.g., Dextrose Injection 5%, or Dextrose (5%) and Sodium Chloride (0.2%) Injection.

The labeling includes the following information if the complete formula is not specified in the individual monograph: (1) In the case of a liquid preparation, the percentage content of each ingredient or the amount of each ingredient in a specified volume, except that ingredients added to adjust to a given pH or to make the solution isotonic may be declared by name and a statement of their effect; and (2) in the case of a dry preparation or other preparation to which a diluent is intended to be added before use, the amount of each ingredient, the composition of recommended diluent(s) [the name(s) alone, if the formula is specified in the individual monograph], the amount to be used to attain a specific concentration of active ingredient and the final volume of solution so obtained, a brief description of the physical appearance of the constituted solution, directions for proper storage of the constituted solution, and an expiration date limiting the period during which the constituted solution may be expected to have the required or labeled potency if it has been stored as directed.

Containers for Injections that are intended for use as dialysis, hemofiltration, or irrigation solutions and that contain a volume of more than 1 L are labeled to indicate that the contents are not intended for use by intravenous infusion.

Injections intended for veterinary use are labeled to that effect.

The container is so labeled that a sufficient area of the container remains uncovered for its full length or circumference to permit inspection of the contents.

strength and total volume for single- and multiple-dose injectable drug products

For single-dose and multiple-dose injectable drug products, the strength per total volume should be the primary and prominent expression on the principal display panel of the label, followed in close proximity by strength per mL enclosed by parentheses. For containers holding a volume of less than 1 mL, the strength per fraction of a mL should be the only expression of strength. Strength per single mL should be expressed as mg/mL, not mg/1 mL.

The following formats are acceptable for contents of greater than 1 mL:

Total strength/total volume: 500 mg/10 mL

Strength/mL: 50 mg/mL

or

Total strength/total volume: 25,000 Units/5 mL

Strength/mL: 5,000 Units/mL

The following format is acceptable for contents of less than 1 mL: 12.5 mg/0.625 mL

There are, however, some exceptions to expressing strength per total volume. In certain cases, the primary and prominent expression of the total drug content per container would not be effective in preventing medication errors (e.g., insulin). An example is the use of lidocaine or other similar drugs used as a local anesthetic where the product is ordered and



...nistered by percentage (e.g., 1%, 2%) or a local anesthetic in combination with epinephrine that is expressed as a ratio (e.g., 1:100,000). In such cases, the total strength should be expressed: for example, 1% (100 mg/10 mL). Dry solids, which need to be reconstituted, should follow the same format, with the exception that only the total strength of the drug should be listed, not the strength/total volume or strength/mL.

(Official February 1, 2009)

Aluminum in Large-Volume Parenterals (LVPs), Small-Volume Parenterals (SVPs), and Pharmacy Bulk Packages (PBPs) Used in Total Parenteral Nutrition (TPN) Therapy

- a. The aluminum content of LVPs used in TPN therapy must not exceed 25 µg per L (µg/L).
- b. The package insert of LVPs used in TPN therapy must state that the drug product contains no more than 25 µg of aluminum per L. This information must be contained in the "Precautions" section of the labeling of all LVPs used in TPN therapy.
- c. If the maximum amount of aluminum in SVPs and PBPs is 25 µg per L (µg/L) or less, instead of stating the exact amount of aluminum that each contains, as in paragraph (d), the immediate container label for SVPs and PBPs used in the preparation of TPN parenterals (with exceptions as noted below) may state: "Contains no more than 25 µg/L of aluminum". If the SVP or PBP is a lyophilized powder, the immediate container label may state the following: "When reconstituted in accordance with the package insert instructions, the concentration of aluminum will be no more than 25 µg/L".
- d. The maximum level of aluminum at expiry must be stated on the immediate container label of all SVPs and PBPs used in the preparation of TPN parenterals and injectable emulsions. The aluminum content must be stated as follows: "Contains no more than \_\_\_\_ µg/L of aluminum". The immediate container label of all SVPs and PBPs that are lyophilized powder used in the preparation of TPN solutions must contain the following statement: "When reconstituted in accordance with the package insert instructions, the concentration of aluminum will be no more than \_\_\_\_ µg/L." This maximum amount of aluminum must be stated as the highest one of the following three levels:
  1. The highest level for the batches produced during the last three years
  2. The highest level for the latest five batches
  3. The maximum level in terms of historical levels, but only until completion of production of the first five batches after July 26, 2004.

The package insert for all LVPs, SVPs, and PBPs used in the preparation of TPN products must contain a warning statement. This warning must be contained in the "Warning" section of the labeling and must state the following: "WARNING: This product contains aluminum that may be toxic. Aluminum may reach toxic levels with prolonged parenteral administration if kidney function is impaired. Premature neonates are particularly at risk because their kidneys are immature, and they require large amounts of calcium and phosphate solutions that contain aluminum. Research indicates that patients with impaired kidney function, including premature neonates, who receive parenteral levels of aluminum at greater than 4 to 5 µg per kg per day accumulate aluminum at levels associated with central nervous system and bone toxicity. Tissue loading may occur at even lower rates of administration of TPN products."

## PACKAGING

### Containers for Injections

Containers, including the closures, for preparations for injections do not interact physically or chemically with the preparations in any manner to alter the strength, quality, or purity beyond the official requirements under the ordinary or customary conditions of handling, shipment, storage, sale, and use. The container is made of material that permits inspection of the contents. The type of glass preferable for each parenteral preparation is usually stated in the individual monograph. Unless otherwise specified in the individual monograph, plastic containers may be used for packaging injections (see [Containers—Plastics \(661\)](#)).

For definitions of single-dose and multiple-dose containers, see [Containers in the General Notices and Requirements](#). Containers meet the requirements under [Containers—Glass \(660\)](#) and [Containers—Plastics \(661\)](#).

Containers are closed or sealed in such a manner as to prevent contamination or loss of contents. Validation of container integrity must demonstrate no penetration of microbial contamination or chemical or physical impurities. In addition, the solutes and the vehicle must maintain their specified total and relative quantities or concentrations when exposed to anticipated extreme conditions of manufacturing and processing, and storage, shipment, and distribution. Closures for multiple-dose containers permit the withdrawal of the contents without removal or destruction of the closure. The closure permits penetration by a needle and, upon withdrawal of the needle, closes at once, protecting the container against contamination. Validation of the multiple-dose container integrity must include verification that such a package prevents microbial contamination or loss of product contents under anticipated conditions of multiple entry and use.

Piggyback containers are usually intravenous infusion containers used to administer a second infusion through a connector of some type or an injection port on the administration set of the first fluid, thereby avoiding the need for another injection site on the patient's body. Piggyback containers are also known as secondary infusion containers.

### Potassium Chloride for Injection Concentrate

The use of a black closure system on a vial (e.g., a black flip-off button and a black ferrule to hold the elastomeric closure) or the use of a black band or series of bands above the constriction on an ampul is prohibited, except for Potassium Chloride for Injection Concentrate.

### Neuromuscular Blocking and Paralyzing Agents

All injectable preparations of neuromuscular blocking agents and paralyzing agents must be packaged in vials with a cautionary statement printed on the ferrules or cap overseals. Both the container cap ferrule and the cap overseal must bear in black or white print (whichever provides the greatest color contrast with the ferrule or cap color) the words: "Warning: Paralyzing Agent" or "Paralyzing Agent" (depending on the size of the closure system). Alternatively, the overseal may be transparent and without words, allowing for visualization of the warning labeling on the closure ferrule.

### Containers for Sterile Solids

Containers, including the closures, for dry solids intended for parenteral use do not interact physically or chemically with the preparation in any manner to alter the strength, quality, or purity beyond the official requirements under the ordinary or customary conditions of handling, shipment, storage, sale, and use.

A container for a sterile solid permits the addition of a suitable solvent and withdrawal of portions of the resulting solution or suspension in such manner that the sterility of the product is maintained.

Where the Assay in a monograph provides a procedure for the Assay preparation, in which the total withdrawable contents are to be withdrawn from a single-dose container with a hypodermic needle and syringe, the contents are to be withdrawn as completely as possible into a dry hypodermic syringe of a rated capacity not exceeding three times the volume to be withdrawn and fitted with a 21-gauge needle not less than 2.5 cm (1 inch) in length, with care being taken to expel any air bubbles, and discharged into a container for dilution and assay.

### Volume in Container

Each container of an injection is filled with sufficient excess of the labeled "size" or that volume which is to be withdrawn. See [Injections](#) under [Pharmaceutical Dosage Forms \(1151\)](#). determination of volume of injection in containers

Suspensions and emulsions must be shaken before withdrawal of the contents and before the determination of the density. Oily and viscous preparations may be warmed according to the instructions on the label, if necessary, and thoroughly shaken immediately before removing the contents. The contents are then cooled to 20°–25°C before measuring the volume.

Single-Dose Containers— Select 1 container if the volume of the container is 10 mL or more, 3 containers if the nominal volume is more than 3 mL and less than 10 mL, or 5 containers if the nominal volume is 3 mL or less. Take up individually the total contents of each container selected into a dry syringe of a capacity not exceeding three times the volume to be measured and fitted with a 21-gauge needle not less than 2.5 cm (1 inch) in length. Expel any air bubbles from the syringe and needle, and then discharge the contents of the syringe, without emptying the needle, into a standardized, dry cylinder (graduated to contain rather than to deliver the designated volumes) of such size that the volume to be measured occupies at least 40% of its graduated volume. Alternatively, the volume of the contents in mL may be calculated as the mass, in g, divided by the density. For containers with a nominal volume of 2 mL or less, the contents of a sufficient number of containers may be pooled to obtain the volume required for the measurement, provided that a separate,



... syringe assembly is used for each container. The contents of containers holding 10 mL or more may be determined by means of opening them and emptying the contents directly into the graduated cylinder or tared beaker.

The volume is not less than the nominal volume in the case of containers examined individually or, in the case of containers with a nominal volume of 2 mL or less, is not less than the sum of the nominal volumes of the containers taken collectively.

**Multi-Dose Containers**— For Injections in multiple-dose containers labeled to yield a specific number of doses of a stated volume, select 1 container, and proceed as directed for single-dose containers, using the same number of separate syringe assemblies as the number of doses specified. The volume is such that each syringe delivers not less than the stated dose.

**Injections in Cartridges or Prefilled Syringes**— Select 1 container if the volume is 10 mL or more, 3 containers if the nominal volume is more than 3 mL and less than 10 mL, or 5 containers if the nominal volume is 3 mL or less. If necessary, fit the containers with the accessories required for their use (needle, piston, syringe) and transfer the entire contents of each container without emptying the needle into a dry tared beaker by slowly and constantly depressing the piston. Determine the volume in mL, calculated as the mass, in g, divided by the density.

The volume measured for each of the containers is not less than the nominal volume.

**Large-Volume Intravenous Solutions**— For intravenous solutions, select 1 container. Transfer the contents into a dry measuring cylinder of such a capacity that the volume to be determined occupies at least 40% of the nominal volume of the cylinder. Measure the volume transferred.

The volume is not less than the nominal volume.

#### Labeling on Ferrules and Cap Overseals

Only cautionary statements are to appear on the top (circle) surface of the ferrule or cap overseal of a vial containing an injectable product. A cautionary statement is one intended to prevent an imminent life-threatening situation if the injectable drug is used inappropriately. Examples of such statements include but are not limited to the following: "Warning", "Dilute Before Using", "Paralyzing Agent", "I.M. Use Only", and "Chemotherapy".

The text must be in contrasting color and conspicuous under ordinary conditions of use. The cautionary statement may appear solely on the ferrule, provided the cap overseal is constructed so as to allow the cautionary statement beneath the cap to be readily legible.

Identifying numbers or letters, such as code numbers, lot numbers, etc., may appear on the side (skirt) surface of the ferrule on vials containing injectable products. The appearance of such identifying data on the skirt surface of the ferrule, placed where it does not detract from, or interfere with, the cautionary statement on the top surface, should be considered to be a beneficial attribute of the in-process quality control of a product throughout the manufacturing process. Any anticounterfeiting scheme must not detract from or interfere with the cautionary statements.

Under no circumstances would advertising such as company names, logos, or product names be permitted to appear on the top (circle) surface of any ferrule or cap overseal.

(Official February 1, 2009)  
Packaging and Storage

The volume of injection in single-dose containers provides the amount specified for parenteral administration at one time and in no case is more than sufficient to permit the withdrawal and administration of 1 L.

Preparations intended for intraspinal, intracisternal, or peridural administration are packaged only in single-dose containers.

Unless otherwise specified in the individual monograph, a multiple-dose container contains a volume of Injection sufficient to permit the withdrawal of not more than 30 mL.

The following injections are exempt from the 1-L restriction of the foregoing requirements relating to packaging:

1. Injections packaged for extravascular use as irrigation solutions or peritoneal dialysis solutions
2. Injections packaged for extravascular use as parenteral nutrition or as replacement or substitution fluid to be administered continuously during hemofiltration

Injections packaged for intravascular use that may be used for intermittent, continuous, or bolus replacement fluid administration during hemodialysis or other procedures, unless excepted above, must conform to the 1-L restriction.

Injections labeled for veterinary use are exempt from packaging and storage requirements concerning the limitation to single-dose containers and the limitation on the volume of multiple-dose containers.

#### FOREIGN AND PARTICULATE MATTER

All articles intended for parenteral administration shall be prepared in a manner designed to exclude particulate matter as defined in [Particulate Matter in Injections \(788\)](#) and other foreign matter. Each final container of all parenteral preparations shall be inspected to the extent possible for the presence of observable foreign and particulate matter (hereafter termed "visible particulates") in its contents. The inspection process shall be designed and qualified to ensure that every lot of all parenteral preparations is essentially free from visible particulates. Qualification of the inspection process shall be performed with reference to particulates in the visible range of a type that might emanate from the manufacturing or filling process. Every container whose contents shows evidence of visible particulates shall be rejected. The inspection for visible particulates may take place when inspecting for other critical defects, such as cracked or defective containers or seals, or when characterizing the appearance of a lyophilized product.

Where the nature of the contents or the container-closure system permits only limited capability for the inspection of the total contents, the 100% inspection of a lot shall be supplemented with the inspection of constituted (e.g., dried) or withdrawn (e.g., dark amber container) contents of a sample of containers from the lot.

All large-volume Injections for single-dose infusion and small-volume Injections are subject to the light obscuration or microscopic procedures and limits for subvisible particulate matter set forth in [Particulate Matter in Injections \(788\)](#), unless otherwise specified in the individual monograph. An article packaged as both a large-volume and a small-volume Injection meets the requirements set forth for small-volume Injections where the container is labeled as containing 100 mL or less, if the individual monograph states a test for [Particulate Matter \(788\)](#); it meets the requirements set forth for large-volume Injections for single-dose infusion where the container is labeled as containing more than 100 mL. Injections administered exclusively by the intramuscular or subcutaneous route or packaged and labeled for use as irrigating solutions are exempt from requirements for [Particulate Matter \(788\)](#).

#### STERILITY

**Sterility Tests**— Preparations for injection meet the requirements under [Sterility Tests \(71\)](#).

#### CONSTITUTED SOLUTIONS

Dry solids from which constituted solutions are prepared for injection bear titles of the form [DRUG] for Injection. Because these dosage forms are constituted at the time of use by the health care practitioner, tests and standards pertaining to the solution as constituted for administration are not included in the individual monographs on sterile dry solids or liquid concentrates. However, in the interest of assuring the quality of injection preparations as they are actually administered, the following nondestructive tests are provided for demonstrating the suitability of constituted solutions when they are prepared just prior to use.

**Completeness and Clarity of Solution**— Constitute the solution as directed in the labeling supplied by the manufacturer for the sterile dry dosage form.  
A: The solid dissolves completely, leaving no visible residue as undissolved matter.



B: The constituted solution is not significantly less clear than an equal volume of the diluent or of Purified Water contained in a similar vessel and examined similarly.

Particulate Matter—Constitute the solution as directed in the labeling supplied by the manufacturer for the sterile dry dosage form: the solution is essentially free from particles of foreign matter that can be observed on visual inspection.

\* This nomenclature has been adopted by the USP Drug Nomenclature Committee for implementation by supplemental revisions of USP 23-NF 18. For currently official monograph titles in the form Sterile [DRUG] that have not yet been revised, the following nomenclature continues in use in this Pharmacopeia: (1) medicaments or solutions or emulsions thereof suitable for injection, bearing titles of the form [DRUG] Injection; (2) dry solids or liquid concentrates containing no buffers, diluents, or other added substances, and which, upon the addition of suitable solvents, yield solutions conforming in all respects to the requirements for injections, and which are distinguished by titles of the form Sterile [DRUG]; (3) preparations the same as those described under (2) except that they contain one or more buffers, diluents, or other added substances, and which are distinguished by titles of the form [DRUG] for Injection; (4) solids which are suspended in a suitable fluid medium and which are not to be injected intravenously or into the spinal canal, distinguished by titles of the form Sterile [DRUG] Suspension; and (5) dry solids which, upon the addition of suitable vehicles, yield preparations conforming in all respects to the requirements for Sterile Suspensions, and which are distinguished by titles of the form Sterile [DRUG] for Suspension.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Desmond G. Hunt, Ph.D.</a> Scientist 1-301-816-8341	(PPI05) Parenteral Products-Industrial 05

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#### 11 USP REFERENCE STANDARDS

USP Reference Standards are highly characterized specimens of drug substances, excipients, reportable impurities, degradation products, compendial reagents, and performance calibrators.

They are explicitly required in many Pharmacopeial assays and tests and are provided solely for such use. Assessment of the suitability for use in other application(s) rests with the purchaser.

#### AUTHORITY FOR ESTABLISHMENT AND RELEASE

USP Reference Standards are established and released under the authority of the USPC Board of Trustees upon recommendation of the USP Reference Standards Expert Committee, which approves each lot as being suitable for use in its compendial applications. For some Reference Standards a preliminary review and approval is sought from other Expert Committees of the Council of Experts.

The distribution of controlled substances is subject to the regulations and licensing provisions of the Drug Enforcement Administration of the Department of Justice.

Industry Advisory Panels and other expert groups (such as Project Teams) may be assembled to advise USP on various aspects of the Reference Standards Program.

#### HISTORY

Future availability of the first USP Reference Standards was announced in 1926 (USP X) "... in order to facilitate the adoption of the biological assay standards of the Pharmacopoeia, and to provide a greater degree of uniformity in their application." The list of USP Reference Standards that in 1936 comprised 6 items has grown to almost 1650 in 2004, and the collection has tracked the progress in pharmaceutical sciences: The first vitamins (Cod Liver Oil) and the first enzyme (Pepsin) in 1936; the first sulfonamide (Sulfanilamide) and the first hormones (Insulin; Posterior Pituitary) in 1942; the first performance standards (Melting Point Standards) in 1947; the first penicillin (Penicillin G Sodium) in 1950; the first recombinant-DNA technology protein (Insulin Human) in 1985, etc.

The continuous increase in the number of USP Reference Standards (over 100 new standards are being developed yearly) reflects not only the increase in the number of monographs and General Chapters, but also the development and extensive use of modern analytical methodology (such as chromatography, spectrophotometry, biological and biochemical assays, etc.) which require measurements relative to a reference standard.

#### NOMENCLATURE

Standards designated as USP Reference Standards (USP RS) are, with a few exceptions, required for use in USP-NF monographs or General Chapters. The exceptions include current lots of USP and NF Reference Standards for which uses are no longer specified in the current USP or NF but for which sufficient demand remains (upon depletion of the current lots, future lots will be designated as Authentic Substances). Reference Standards specified in monographs developed by USP that are not intended for publication in the USP—NF, Reference Standards specified in the current edition of the Food Chemicals Codex (labeled with an additional designation "FCC"), and Fluoride Dentifrices (evaluated and distributed by agreement with the FDA and the Cosmetics, Toiletries, and Fragrances Association). A USP Reference Standard required in a monograph or General Chapter proposed in Pharmacopeial Forum may be released in advance of the official date of the proposed PF revision.

Reference Standards currently labeled as "NF Reference Standards" will eventually all be designated and labeled as "USP Reference Standards" pursuant to the consolidation of USP and NF within the USPC as of January 2, 1975. Meanwhile, where a USP Reference Standard is called for, the corresponding substance labeled as an "NF Reference Standard" may be used.

As a service, the USPC tests and distributes additional Authentic Substances (designated by AS) not currently required for use in a USP monograph or General Chapter. These also are provided under the supervision of the USP Reference Standards Expert Committee. They are highly characterized samples of chemicals, including substances of abuse, which are collaboratively tested and made available as a service primarily to analytical, clinical, pharmaceutical, and research laboratories. Such materials may be used for identification, method development, evaluation of method performance, or other applications as found suitable and validated by the user.

#### Authentic Visual References

Unlike chemical reference standards, Authentic Visual References (AVRs) are not used in chemical analyses. Instead, the AVRs are visual images used by analysts to compare certain test articles to ensure that they meet compendial requirements and are incorporated by reference into the monograph. Approval of AVRs for use in a monograph is the decision of the Expert Committee that approves the specific monograph.

#### DIVERSITY AND IMPLICATIONS

The USP Reference Standards collection is very diverse in terms of appearance, chemical structure, composition, and uses. This diversity has significant implications for the way the materials are tested, packaged, stored, and utilized.

The USP Reference Standards may be crystalline or amorphous powders, volatile or viscous liquids, solutions or suspensions, gels or pastes, plastic sheets, etc. In chemical structure they vary from simple inorganic salts to proteins produced by recombinant DNA technology. Some are highly-purified single components, while others are complex mixtures (in most cases extracted from plant or animal sources).



## USES OF USP REFERENCE STANDARDS

The official and authorized uses of USP Reference Standards are specified in the USP monographs and General Chapters and they include the following:

- quantitative uses in assays for drug substances and formulations, limit tests, or blanks and controls
- qualitative uses, such as identification tests, system suitability tests, chromatographic peak markers, etc.
- performance standards and calibrators, such as dissolution calibrators, melting point standards, the particle count set, etc.

As discussed under Nomenclature, USP also establishes and distributes standards not specified for use in a USP monograph or General Chapter.

The most frequent applications of USP Reference Standards (USP RS) are in chromatographic and spectroscopic methodologies. However, they are also widely used in biological and biochemical applications, such as microbial assays for antibiotics, enzymatic reactions, cell-culture tests, whole-animal tests; in thermal analysis for polymers; and in titration, etc.

Some of the most frequently used USP RS are those utilized in General Chapters tests such as [Dissolution](#) (711), [Bacterial Endotoxins Test](#) (85), [Total Organic Carbon](#) (643), and [Particulate Matter in Injections](#) (788).

## STEPS IN ESTABLISHING A USP REFERENCE STANDARD

The establishment of a new USP Reference Standard is triggered by the proposal of a new monograph or of a revision of an existing monograph by the inclusion of a test requiring a new USP RS. The need for a new lot of an existing USP Reference Standard is identified when its inventory reaches a pre-established threshold. The new lot is designated as a "replacement lot" if a new bulk material is to be procured or as a "continuation lot" if the candidate material is another portion of the bulk used for the existing official lot.

USP scientists generate a set of documents including procurement specifications and a testing protocol. A bulk material is obtained, generally from a major manufacturer of the article. The material is tested and characterized in an inter-laboratory collaborative study organized according to the protocol designed at USP Headquarters. The results are evaluated by USP Staff, additional testing or investigations are performed when necessary, and a report is compiled and presented for review and approval to the USP Reference Standards Expert Committee. After approval the material is subdivided (if not packaged prior to the collaborative study), labeled, quality checks are performed, and the standard is made available for distribution. If a candidate material is found to be unsuitable by USP scientific staff or by the Reference Standards Committee, a new bulk is procured and tested.

## COLLABORATIVE STUDY FOR THE EVALUATION OF A USP RS CANDIDATE MATERIAL

The goals of the evaluation study are to confirm the identity and assess the purity of the material, to determine its suitability for use in the official applications, to provide the user with all the necessary information and directions for use, and to acquire time-zero information for future continued-suitability-for-use studies.

USP scientists design a detailed testing protocol that includes the following elements: types of tests, number of tests, number of collaborators, number of replicates, and references to the procedures to be used.

The following factors are considered when designing the study protocol: the compendial status of the standard, its official uses, the history of the standard, its composition and complexity, the characteristics of the methodology, and the availability of material and of competent laboratories.

The testing protocol may comprise visual and microscopic evaluation; identification tests (more elaborate for first-time standards); determination of physical-chemical constants (e.g., melting range, specific rotation, refractive index, specific gravity, etc); chromatographic and electrophoretic purity tests; inorganic contaminants determination; volatile tests (water, solvents); functional group analysis (such as titrations, UV absorptivity, elemental analysis); thermal analysis; and assays against another well-characterized standard (a previous lot, an international standard, etc). Specialized testing is implemented where appropriate, such as for dissolution calibrators and the particle count set, for standards that define an attribute (negative and positive bioreaction, ion-exchange capacity, permeability diameter), and for biological standards that define a Unit of activity (heparin, endotoxin, enzymes, complex antibiotics). Vapor sorption analysis may also be performed to assist in determining packaging and storage conditions, and directions for use. For lyophilized single-use USP RS, acceptable vial content reproducibility and stability of the lyophilized form are demonstrated.

The number of collaborators is generally not less than three (two outside of USP); but it can increase significantly, especially when the methodology is complex or does not have a high level of precision or when potential users express an interest in participating in the evaluation of the candidate. (Participation in all evaluation studies is open to all competent, interested parties.) Where appropriate, statistical control is exercised in the design of the evaluation study and in the analysis of the results. The USP Reference Standards Laboratory and the FDA laboratories participate in almost all evaluation studies. Other collaborators include Health Canada, the USP Research and Development Laboratory, and industrial and academic laboratories from the United States and from abroad.

## BIOLOGICAL REFERENCE STANDARDS

The World Health Organization, an agency of the United Nations, manages a program providing International Standards for biological materials.

USP collaborates closely with the WHO in the harmonization of analytical methodology, in the definition of the units of potency, and in some cases to share in the preparation of a reference standard. In many cases the USP Units and the International Units of potency are identical.

## SUITABILITY FOR USE AND PURITY ASSIGNMENT

The data collected in the collaborative evaluation study are analyzed to determine whether the material is suitable for its monograph-designated use. Characterization data and results must be considered as a whole when evaluating suitability for intended use, assignment strategy, and assigned value. For Reference Standards used in quantitative applications, this includes the determination of a calculation value to be used in the compendial utilization of the standard.

The method of choice in computing the assigned value is a mass balance analysis using independently determined components such as moisture, solvent residues, inorganic residues, chromatographic impurities, and ion content. The assay results against a previous lot or against another validated standard and the results of the functional group analysis are for confirmatory purposes only. Exceptions to the mass balance approach include many biological Reference Standards, especially those which define the Unit of activity.

The number of significant figures in the labeled calculation value is a function of the use of the standard and the number of significant figures in the acceptance range or limit. Generally, Reference Standards used in assays are labeled with three significant figures and standards used in limit tests with two significant figures. Reference Standards having multiple applications in different methodologies may require separate assay-specific assignments.

The assigned value is labeled without any associated uncertainty. However, for calibration standards, the labeled value is a range, determined by a statistical analysis of the results. Previous approaches used a purity threshold above which the content was no longer labeled, and the analyst was directed to use a default value of 100.0%. This approach is no longer in use, but older lots of standards have not been re-labeled, and users should continue to apply the default 100.0% value for compendial quantitative applications.

For antibiotics, the designation "µg/mg" is sometimes used as a unit of biological activity, and values greater than 1000 µg/mg may be assigned to some of these standards. This can happen when the first standard is assigned a value higher than its actual purity and subsequent standards of higher purity are defined relative to the previous lot. A relatively overstated assigned value can also result when less selective separation techniques are replaced with more selective modern methodologies. As a result, the original content might have been assumed to be higher than the actual level.

No value is assigned to standards having only qualitative applications.

A report compiling the results of the evaluation study and including the proposed label text is submitted for review and approval to the USP Reference Standards Expert Committee.

## LABEL TEXT



The label text is designed to provide the user with all the information needed for the correct storage and usage of the Reference Standard in monograph application(s). The label includes directions for use, safety warnings, required information for controlled substances, and a calculation value for standards with quantitative applications. For calibrators, acceptance ranges are provided. Where necessary, USP Reference Standards are accompanied by additional documentation, such as Technical Sheets or Typical Chromatograms. USP generally does not provide Certificates of Analysis because all the information that the user needs for the official or authorized applications of the standard is provided in the label text and, where necessary, in the additional documentation provided.

Directions for use are lot-specific, and they take precedence over any other indication in the compendium.

Material Safety Data Sheets are generated for every standard that USP distributes. They are available on the USP website.

#### USP REFERENCE STANDARDS EXPERT COMMITTEE

The USP Reference Standards Expert Committee comprises professionals from industry, government agencies and academia from the United States and abroad. It is organized in groups and may be assisted in the review of the evaluation studies by an Industry Advisory Panel. The approval of the evaluation report has to be unanimous.

#### PACKAGING

The USP Reference Standard production process operates under a registered ISO 9001:2000 Quality System and appropriate cGMP principles.

USP Reference Standards are packaged in individual units designed to maintain the integrity of the contained Reference Standard material. The packaging and storage conditions for USP Reference Standards provide protection for all materials even though the material may not need such exceptional protection due to its inherent stability. The most common packaging configurations are vials for solid materials and ampuls for liquids. The packaging environment is determined by the sensitivity of the material to light, oxidation, or atmospheric humidity, and by its toxicity. Where appropriate, containers are filled in a glove box under inert gas and in conditions of controlled low residual humidity. (The need to store such standards under inert gas protection is indicated on the label.) They may also be sealed in a foil bag as an added protective barrier. Ampuls are filled and sealed on an automated device and are typically purged with an inert gas. The most common ampul sizes are 2 mL and 5 mL. Vials may be filled by manual, semi-automated, or fully automated operations. Vials may be of different sizes depending upon the amount of material. The amount of material per individual container depends on the compendial application of the standard and is generally sufficient for several replicates. Larger amounts are provided when additional experiments are required (such as a titrimetric determination of the water content at the time of use). In general, Reference Standard containers are slightly over-filled so that the user can retrieve the labeled, nominal amount of material. Vials are closed with Teflon-lined stoppers and secured with aluminum crimps and a USP-logo tamper-evident seal. Lots using prior vial closure configurations may still be in distribution.

Various considerations may determine the need to provide the standard in single-use containers, mainly for materials with significant handling issues or for those that are available only in small amounts. Such single-use containers are generally filled by lyophilization, and their content is labeled in mass or activity Units per container. If so labeled, the content of the container is to be reconstituted in its entirety without any additional weighing. Instructions for reconstitution are given either on the label or in the monographs where the standard is being used.

#### IMPURITY REFERENCE STANDARDS

The topic of impurities is addressed in several sections of USP, such as General Notices, General Chapters such as [Ordinary Impurities](#) (466), [Impurities in Official Articles](#) (1086), etc. In addition, most of the monographs for drug substances and many of those for formulations include specific tests for the identification or quantitation of impurities. Such tests generally require an official Reference Standard. The development of these impurity Reference Standards is one of the reasons for the continuous accelerated growth of the USP Reference Standards.

In many cases, the materials for impurity Reference Standards are expensive and difficult to procure. Only a limited amount of material may be available—procurement might require custom synthesis—and it may be of lesser quality than the Reference Standard for the official article, requiring purification. The limited amount of material available can affect the testing protocol and the packaging. Impurity Reference Standards might be available as purified single-component materials, solutions, or solid dispersions, or mixtures of more than one impurity. Other options include samples of the official article with a labeled content of impurity(ies), the in-situ generation of the impurity from the official article by a validated specified procedure, the use of relative chromatographic mobilities and relative response factors, or of theoretical values such as UV absorptivities at selective wavelengths.

In earlier editions of the compendium, the impurities were designated by their chemical names. For ease of indexing and searching, these have been gradually replaced with the designation "X Related Compound Y RS," where X is the name of the official article and Y is a sequential alphabetical letter. Reference Standard impurity mixtures might be designated by their use, such as "X System Suitability RS". The conventional names and the chemical names are cross-referenced in the final section of this chapter and in a special section of the Official USP Reference Standards Catalog.

#### CONTINUED SUITABILITY FOR USE PROGRAM

To ensure that the Reference Standards maintain the properties determined at the initial evaluation, USP maintains a Continued Suitability for Use Program. The retesting intervals and protocols are a function of the uses and properties of the standard and of the information available about its stability. Abbreviated protocols use the stability-indicating methodology employed in the initial characterization of the material to confirm the consistency of attributes such as appearance, chromatographic purity, or volatiles content.

#### PROPER USE

Neither the Reference Standards nor the Authentic Substances are intended for use as drugs or as medical devices.

USP Reference Standards do not carry an expiration date as long as they are in distribution. A lot of USP RS may be used in its official applications as long as it is listed as "Current Lot" in the current (most recent) Official USP Reference Standards Catalog. Upon depletion, the lot is designated in the catalog as "Previous Lot" and a "Valid Use Date" is assigned. USP publishes the Official Catalog of Reference Standards (which also includes Authentic Substances) bimonthly as a separate brochure\*. An updated version of the catalog can be found on the USP website at [www.usp.org](http://www.usp.org). It is the responsibility of the user to ascertain that a particular supply of USP Reference Standard has official status either as a "Current Lot" or as a "Previous Lot" within the valid use date.

Many Pharmacopeial tests and assays are based on comparison of a test specimen with a USP Reference Standard. In such cases, measurements are made on preparations of both the test specimen and the Reference Standard. Where it is directed that a Standard solution or a Standard preparation be prepared for a quantitative determination by stepwise dilution or otherwise, it is intended that the Reference Standard substance shall be accurately weighed (see [Weights and Balances](#) (41) and [Volumetric Apparatus](#) (31)). Due account should also be taken of the relatively large errors associated with weighing small masses.

The label text provides the user with directions on the proper use of a Reference Standard. The directions include one of the following options. A Reference Standard may be used as follows:

- As-is, i.e., without any prior treatment or correction for volatiles. This is the preferred option, and it is selected whenever validated data show that the volatiles content is constant over time.
- Immediately after a prior drying under stated conditions. Drying should not be performed in the original container. A portion of the material should be transferred to a separate drying vessel.
- With a correction for the water content or the loss on drying determined on a separate portion of material. Where the titrimetric determination of water is required at the time a Reference Standard is to be used, proceed as directed for Method I under [Water Determination](#) (921). Instrumental or microanalytical methods are acceptable for this purpose. When using typical amounts (about 50 mg of the Reference Standard), titrate with a two- to five-fold dilution of the reagent. Where the determination of the loss on drying on a separate portion of USP Reference Standard is required, proceed as directed on the label. Smaller sample sizes than those required in General Chapter [Loss on Drying](#) may be used.



Driving [731](#) may be used for a USP Reference Standard, provided that the user can obtain a sufficiently accurate result.

Whenever the labeled directions for use require a preliminary drying or a correction for volatiles, it should be performed "at the time" of use. Further experimental details should be controlled by the user's Standard Operating Procedures and good laboratory practices.

#### STORAGE

To serve its intended purpose, each USP Reference Standard must be properly stored, handled, and used. Generally, Reference Standards should be stored in their original stoppered containers away from heat and protected from light. Avoid humid storage areas in particular. Where special storage conditions are necessary, directions are given on the label.

#### RELATIONSHIPS WITH OTHER STANDARDS-SETTING ORGANIZATIONS

USP maintains continuous contact with other organizations that establish Reference Materials for compendial and other purposes, such as the European and the Japanese Pharmacopoeias (through the Pharmacopeial Discussion Group), the World Health Organization, the National Institute for Science and Technology, the Reference Materials Committee of ISO (REMCO), etc.

The specific nature of pharmacopeial reference substances has been officially recognized by ISO-REMCO in the introduction of the ISO Guide 34—General requirements for the competence of reference material producers (Second Edition 2000): "Pharmacopeial standards and substances are established and distributed by pharmacopeial authorities following the general principle of this guide. It should be noted, however, that a different approach is used by the pharmacopeial authorities to give the user the information provided by certificate of analysis and the expiration dates. Also, the uncertainty of their assigned values is not stated since it is negligible in relation to the defined limits of the method-specific assays of the pharmacopoeias for which they are used."

The USP Reference standards section of an individual USP or NF monograph or general chapter names each USP Reference Standard required for assay and test procedures and refers to this chapter for additional information and instructions. It is especially important to refer to the current Supplement to USP and to NF for official revisions listed in the following section.

#### USP REFERENCE STANDARDS SPECIFIED IN USP AND NF MONOGRAPHS AND GENERAL CHAPTERS

note—Consult the latest Supplement or Interim Revision Announcement pertaining to USP and to NF for revisions, additions, or deletions.

Revisions, additions, and deletions of individual USP Reference Standards are listed cumulatively in each Supplement to USP–NF. As a consequence, therefore, it is necessary to consult only the current edition of USP–NF and the latest Supplement for the complete list of USP Reference Standards currently specified in USP–NF monographs and general chapters. The list provides up-to-date and complete names and applicable chemical information for the USP Reference Standards that are in distribution as of the official date of that Supplement.

Revisions of this chapter are implemented continuously via the Interim Revision Announcements that are published in Pharmacopeial Forum. Those interim revisions of USP Reference Standards are cumulatively included in the next USP–NF Supplement.

The alphabetical list that follows constitutes an index of all revisions to this chapter. Thus, it is unnecessary to name repetitively the revised Reference Standards in the general index to the Supplement.

In the list that follows, chemical names are given for many substances (e.g., related compounds) that are not USP or NF monograph articles. Following the name of such a chemical substance RS, the empirical formula and molecular weight, separated by the ♦ symbol, may be given in parentheses if those data are available.

\* For nonsubscribers, the most recent Official Catalog is available from: U.S. Pharmacopeial Convention, Inc., Reference Standards Order Department, 12601 Twinbrook Parkway, Rockville, MD 20852. Telephone 1-301-881-0666. FAX 1-301-816-8148. Toll-free telephone 1-800-227-USPC or access the Catalog on USP's website [www.usp.org/dsd/refstd](http://www.usp.org/dsd/refstd).

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">P. Steven Lane</a> Director, Quality Assurance 1-301-816-8337	(HDQ) USP Headquarters

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USP Acebutolol Hydrochloride RS [\[PDF\]](#).

USP Acepromazine Maleate RS [\[PDF\]](#).

USP Acesulfame Potassium RS [\[PDF\]](#).

USP Acetaminophen RS [\[PDF\]](#).

USP Acetanilide Melting Point RS [\[PDF\]](#).

USP Acetazolamide RS [\[PDF\]](#).

USP Glacial Acetic Acid RS [\[PDF\]](#).

USP Acetoheexamide RS [\[PDF\]](#).

USP Acetohydroxamic Acid RS [\[PDF\]](#).

USP Acetylcholine Chloride RS.



USP Acetylcysteine RS

USP Acetyltributyl Citrate RS

USP Acetyltriethyl Citrate RS

USP Actein RS.

USP Acitretin RS.

USP Acitretin Related Compound A RS [(2Z,4E,6E,8E)-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethylnona-2,4,6,8-tetraenoic acid] (C<sub>21</sub>H<sub>26</sub>O<sub>3</sub>  $\Delta$  326.43).

USP Acitretin Related Compound B RS [(ethyl (all-E)-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethylnona-2,4,6,8-tetraenoate)] (C<sub>23</sub>H<sub>30</sub>O<sub>3</sub>  $\Delta$  354.48).

USP Acyclovir RS

USP Ademethionine Disulfate Tosylate RS.

USP Adenine RS

USP Adenosine RS

USP Adipic Acid RS

Add the following:

▲USP Agar RS. ▲USP32

USP Agigenin RS .

USP Agnuside RS .

USP Air–Helium RS.

USP I-Alanine RS

USP Beta Alanine RS [3-aminopropionic acid].

USP Albendazole RS

Add the following:

▲USP rAlbumin Human RS (C<sub>29</sub>H<sub>46</sub>24N<sub>7</sub>S<sub>4</sub>  $\Delta$  66,438). ▲USP32

USP Albuterol RS

USP Albuterol Sulfate RS .

USP Alclometasone Dipropionate RS .

USP Alcohol RS

USP Dehydrated Alcohol RS

USP Alcohol Determination—Acetonitrile RS .

USP Alcohol Determination—Alcohol RS .

USP Alendronate Sodium RS

USP Alfentanil Hydrochloride RS.

Add the following:



▲USP Alfuzosin Hydrochloride RS. ▲USP32

Add the following:

▲USP Alfuzosin System Suitability Mixture RS [Alfuzosin Hydrochloride containing approximately 0.4% Impurity A (N-[3-[(4-amino-6,7-dimethoxyquinazolin-2-yl)(methyl)amino]propyl]furan-2-carboxamide); and about 0.4% Impurity D (N-(4-amino-6,7-dimethoxyquinazolin-2-yl)-N-methylpropane-1,3-diamine)]. ▲USP32

USP Allantoin RS

USP Alliin RS .

USP Allopurinol RS

USP Allopurinol Related Compound A RS [3-amino-4-carboxamidopyrazole hemisulfate] (C4H6N4O)2·H2SO4  $\Delta$  350.32 .

USP Allopurinol Related Compound B RS [5-(formylamino)-1H-pyrazole-4-carboxamide] (C5H6N4O2  $\Delta$  154.13).

USP Allopurinol Related Compound C RS [N-(4H-1,2,4-triazol-4-yl)-1H-pyrazole-4-carboxamide] (C6H6N6O  $\Delta$  178.15).

USP Allopurinol Related Compound D RS [ethyl 5-amino-1H-pyrazole-4-carboxylate] (C6H9N3O2  $\Delta$  155.15).

USP Allopurinol Related Compound E RS [ethyl 5-(formylamino)-1H-pyrazole-4-carboxylate].

USP Allopurinol Related Compound F RS [ethyl 3-(2-carbethoxy-2-cyanoethenyl)amino-1H-pyrazole-4-carboxylate].

USP S-Allyl-L-Cysteine RS .

USP Alprazolam RS

USP Alprazolam Related Compound A RS [2-(2-acetylhydrazino)-7-chloro-5-phenyl-3H-1,4-benzodiazepine].

USP Alprostadil RS

USP Alteplase RS.

USP Altretamine RS

USP Dried Aluminum Hydroxide Gel RS .

USP Amantadine Hydrochloride RS .

USP Amcinonide RS

USP Amifostine RS .

USP Amifostine Disulfide RS [1,3-propanediamine, N,N-(dithiodi-2,1-ethanediyl)bis, tetrahydrochloride] (C10H30N4S2Cl4  $\Delta$  412.32) .

USP Amifostine Thiol RS [ethanethiol, 2-[(3-aminopropyl)amino]-, dihydrochloride] (C5H16N2SCl2  $\Delta$  207.17) .

USP Amikacin RS

USP Amiloride Hydrochloride RS .

USP Amiloxate RS [isoamyl methoxycinnamate].

USP Aminobenzoate Potassium RS .

USP Aminobenzoic Acid RS

USP Aminobutanol RS (C4H11NO  $\Delta$  89.14) .

USP Aminocaproic Acid RS



USP N-(Aminocarbonyl)-N-[(5-nitro-2-furanyl)-methylene]-glycine RS

USP 2-Amino-5-chlorobenzophenone RS (C13H10ClNO  $\Delta$  231.68).

USP 3-Amino-2,4,6-triiodobenzoic Acid RS (C7H4I3NO2  $\Delta$  514.83).

USP 5-Amino-2,4,6-triiodo-N-methylisophthalamic Acid RS (C9H7I3N2O3  $\Delta$  571.88).

USP Aminoglutethimide RS .

USP m-Aminoglutethimide RS .

USP Aminohippuric Acid RS .

USP 5-Aminoimidazole-4-carboxamide Hydrochloride RS (C4 H6N4O·HCl  $\Delta$  162.58).

USP Amino Methacrylate Copolymer RS.

USP Aminopentamide Sulfate RS.

USP 4-Aminophenol RS.

USP m-Aminophenol RS .

USP Aminosalicylic Acid RS .

USP Amitraz RS .

USP Amitriptyline Hydrochloride RS .

USP Amitriptyline Related Compound A RS (also known as Dibenzosuberone) [10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5-one] (C15H12O  $\Delta$  208.26).

USP Amitriptyline Related Compound B RS (also known as Amitriptynol) [5-[3-(Dimethylamino)propyl]-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5-ol] (C20H25O  $\Delta$  295.42).

USP Amlodipine Besylate RS.

USP Ammonio Methacrylate Copolymer, Type A RS.

USP Ammonio Methacrylate Copolymer, Type B RS.

USP Ammonium Chloride RS.

USP Amobarbital RS .

USP Amodiaquine Hydrochloride RS.

USP Amoxapine RS .

USP Amoxicillin RS.

USP Amphotericin B RS .

USP Ampicillin RS.

USP Ampicillin Sodium RS .

USP Ampicillin Trihydrate RS .

USP Amprolium RS .

USP Ane cortave Acetate RS. 

USP Ane cortave Acetate Related Compound A RS. 

USP 1,6-Anhydro-d-glucose RS. 

USP Anileridine Hydrochloride RS.

USP Antazoline Phosphate RS. 

USP Anthralin RS.

USP Antipyrine RS. 

Add the following:

▲USP Apigenin RS. ▲USP32

USP Apigenin-7-Glucoside RS.

USP Apomorphine Hydrochloride RS.

USP Apraclonidine Hydrochloride RS.

USP Aprotinin RS.

USP Aprotinin System Suitability RS.

USP I-Arabinitol RS.  [I-arabinitol, 1,2,3,4,5-pentanepentol] (C5H12O5  $\Delta$  152.15).

USP I-Arginine RS. 

USP Arginine Hydrochloride RS. 

USP Arsanilic Acid RS. 

USP Ascorbic Acid RS. 

USP Asparagine Anhydrous RS. 

USP Asparagine Monohydrate RS. 

USP Aspartame RS.

USP Aspartame Acesulfame RS. 

USP Aspartame Related Compound A RS.  [5-benzyl-3,6-dioxo-2-piperazineacetic acid] (C13H14N2O4  $\Delta$  262.27).

USP Aspartic Acid RS. 

USP Aspirin RS. 

USP Astemizole RS. 

USP Atenolol RS. 

USP Atovaquone RS. 

USP Atovaquone Related Compound A RS.  [cis-2[4-(4-chlorophenyl)cyclohexyl]-3-hydroxy-1,4-naphthoquinone].

USP Atracurium Besylate RS. 

USP Atropine Sulfate RS .

USP Aurothioglucose RS  .

USP Avobenzone RS  .

USP Azaerythromycin A RS .

USP Azaperone RS  .

USP Azatadine Maleate RS .

USP Azathioprine RS  .

USP Azithromycin RS .

USP Azithromycin Identity RS  [A mixture of azithromycin, 3'-(N,N-dimethyl-3'-N-formyl-azithromycin, 3'-N-demethyl-3'-N-formylazithromycin (Rotamer 1), 3'-N-demethyl-3'-N-formylazithromycin (Rotamer 2), 3'-de(dimethylamino)-3'-oxoazithromycin, 2-desethyl-2-propylazithromycin, 3-deoxyazithromycin and 3'-N-demethyl-3'-N-[(4-methylphenyl)sulfonyl]azithromycin].

USP Azithromycin N-Oxide RS.

USP Azo-aminoglutethimide RS .

USP Aztreonam RS  .

USP Aztreonam E-Isomer RS  .

USP Open Ring Aztreonam RS  (C18H19N5O9S2  $\Delta$  453.46) .

USP Bacampicillin Hydrochloride RS .

USP Bacitracin Zinc RS .

USP Baclofen RS  .

USP Baclofen Related Compound A RS  [4-(4-chlorophenyl)-2-pyrrolidinone] (C10H10ClNO  $\Delta$  195.65) .

USP Beclomethasone Dipropionate RS  .

USP Bemotrizinol RS  .

USP Benazepril Hydrochloride RS  .

USP Benazepril Related Compound A RS  [(3R) 3-[(1R) 1-(ethoxycarbonyl)-3-phenylpropyl]amino]-2,3,4,5-tetrahydro-2-oxo-1H-1-benzazepine-1-acetic acid, monohydrochloride] (C24H28N2O5·HCl  $\Delta$  460.95) .

USP Benazepril Related Compound B RS  [(3S) 3-[(1R) 1-(ethoxycarbonyl)-3-phenylpropyl]amino]-2,3,4,5-tetrahydro-2-oxo-1H-1-benzazepine-1-acetic acid, monohydrochloride] (C24H28N2O5·HCl  $\Delta$  460.95) .

USP Benazepril Related Compound C RS  [3-(1-carboxy-3-phenyl-1(S)-propyl)amino-2,3,4,5-tetrahydro-2-oxo-1H-1-(3S)-benzazepine-1-acetic acid] (C22H24N2O5  $\Delta$  396.44) .

USP Benazepril Related Compound D RS  [(3-(1-ethoxycarbonyl-3-cyclohexyl-(1S)-propyl)amino-2,3,4,5-tetrahydro-2-oxo-1H-1-(3S)-benzazepine)-1-acetic acid, monohydrochloride] (C24H34N2O5·HCl  $\Delta$  467.00) .

USP Benazepril Related Compound E RS  [3-amino-2,3,4,5-tetrahydro-2-oxo-1H-1-(3S)-benzazepine-1-acetic acid] .

USP Benazepril Related Compound F RS  [tert-butyl-3-amino-2,3,4,5-tetrahydro-2-oxo-1H-1-(3S)-benzazepine-1-acetic acid] .

USP Benazepril Related Compound G RS  [(3-(1-ethoxycarbonyl-3-phenyl-(1S)-propyl)amino-2,3,4,5-tetrahydro-2-oxo-1H-1-(3S)-benzazepine)-1-acetic acid ethyl ester] .



USP Bendoflumethiazide RS

USP Benoxinate Hydrochloride RS .

USP Benzalkonium Chloride RS

USP Benzocaine RS

USP Benzoic Acid RS

USP Benzonatate RS

USP 1,4-Benzoquinone RS

USP Benzothiadiazine Related Compound A RS [4-amino-6-chloro-1,3-benzenedisulfonamide] (C<sub>6</sub>H<sub>8</sub>CIN<sub>3</sub>O<sub>4</sub>S<sub>2</sub>  $\Delta$  285.73) .

USP Benztropine Mesylate RS .

USP Benzyl Alcohol RS

USP Benzyl Benzoate RS

USP 1-Benzyl-3-methyl-5-aminopyrazole Hydrochloride RS (C<sub>11</sub>H<sub>13</sub>N<sub>3</sub>·HCl  $\Delta$  223.71) .

USP Betahistine Hydrochloride RS .

USP Betaine Hydrochloride RS .

USP Betamethasone RS

USP Betamethasone Acetate RS

USP Betamethasone Benzoate RS

USP Betamethasone Dipropionate RS

USP Betamethasone Sodium Phosphate RS .

USP Betamethasone Valerate RS

USP Betaxolol Hydrochloride RS .

USP Bethanechol Chloride RS .

USP Bicalutamide RS

USP Bicalutamide Related Compound B RS [(RS)-N-(4-cyano-3-(trifluoromethyl)phenyl)-3-(3-fluorophenylsulfonyl)-2-hydroxy-2-methylpropanamide] (C<sub>18</sub>H<sub>14</sub>F<sub>4</sub>N<sub>2</sub>O<sub>4</sub>S  $\Delta$  430.37) .

USP Powdered Bilberry Extract RS.

USP Bile Salts RS .

USP Positive Bioreaction RS .

USP Biotin RS

USP Biperiden RS

USP Biperiden Hydrochloride RS .



USP Bisacodyl RS

Add the following:

▲USP Bisdesmethoxycurcumin RS. ▲USP32

USP Bis(2-ethylhexyl) Maleate RS (C20H36O4  $\Delta$  340.51).USP 4,4'-Bis[1,2,3,6-tetrahydro-4-(2-oxo-1-benzimidazolinyl)-1-pyridyl]butyrophenone RS (C34H34N6O3  $\Delta$  574.69).

USP Bismuth Citrate RS

USP Bismuth Subsalsalate RS .

USP Bisoctrizole RS.

USP Bisoctrizole Related Compound A RS.

USP Bisoctrizole Resolution Mixture RS.

USP Bisoprolol Fumarate RS .

USP Bleomycin Sulfate RS .

USP Bretylium Tosylate RS .

USP Brinzolamide RS .

USP Brinzolamide Related Compound A RS [brinzolamide (S)-isomer] (C12H21N3O5S3  $\Delta$  383.52).USP Brinzolamide Related Compound B RS [(R-4-amino)-2,3-dihydro-2-(3-methoxypropyl)-4H-thieno[3,2,-e]-thiazine-6-sulfonamide-1,1-dioxide ethandioate 1:1] (C10H17N3O5S3-C2H2O4  $\Delta$  445.49).

USP Bromocriptine Mesylate RS .

USP Bromodiphenhydramine Hydrochloride RS .

USP 8-Bromotheophylline RS [8-bromo-3,7-dihydro-1,3-dimethyl-1H-purine-2,6-dione] (C7H7N4O2Br  $\Delta$  259.06).

USP Brompheniramine Maleate RS .

USP Bumetanide RS .

USP Bumetanide Related Compound A RS [3-amino-4-phenoxy-5-sulfamoylbenzoic acid] (C13H12N2O5S  $\Delta$  308.31).USP Bumetanide Related Compound B RS [3-nitro-4-phenoxy-5-sulfamoylbenzoic acid] (C13H10N2O7S  $\Delta$  338.29).

USP Bupivacaine Hydrochloride RS .

USP Buprenorphine Hydrochloride RS .

USP Buprenorphine Related Compound A RS [21-[3-(1-propenyl)-7 $\alpha$ -[(S)-1-hydroxy-1,2,2-trimethylpropyl]-6,14-endo-ethano-6,7,8,14-tetrahydrooripavine] (C29H41NO4  $\Delta$  467.65).

USP Bupropion Hydrochloride RS .

USP Bupropion Hydrochloride Related Compound A RS [2-(tert-butylamino)-4'-chloropropiophenone hydrochloride] (C13H18ClNO-HCl  $\Delta$  276.21).USP Bupropion Hydrochloride Related Compound B RS [2-(tert-butylamino)-3'-bromopropiophenone hydrochloride] (C13H18BrNO-HCl  $\Delta$  320.66).USP Bupropion Hydrochloride Related Compound C RS [1-(3-chlorophenyl)-2-hydroxy-1-propanone] (C9H9O2Cl  $\Delta$  184.62).



USP Bupropion Hydrochloride Related Compound F RS [1-(3-chlorophenyl)-1-hydroxy-2-propanone] (C9H9O2  $\Delta$  184.62).

USP Buspirone Hydrochloride RS.

USP Butabarbital RS.

USP Butalbital RS.

USP Butamben RS.

USP Butoconazole Nitrate RS.

USP Butorphanol Tartrate RS.

USP 2-tert-Butyl-4-hydroxyanisole RS [C11H16O2  $\Delta$  180.25].

USP 3-tert-Butyl-4-hydroxyanisole RS [C11H16O2  $\Delta$  180.25].

USP Butyl 3-(butylamino)-4-phenoxy-5-sulfamoylbenzoate RS [C21H28N2O5S  $\Delta$  420.53].

USP Butylparaben RS.

Add the following:

▲USP Cabergoline RS. ▲USP32

USP Caffeine RS.

USP Caffeine Melting Point RS.

USP Calcifediol RS.

USP Calcitonin Salmon RS.

USP Calcitonin Salmon Related Compound A RS [N-acetyl-cys1-calcitonin] (C146H243N44O49S2  $\Delta$  3463).

USP Calcitriol RS.

USP Calcitriol Solution RS.

USP Calcium Ascorbate RS.

USP Calcium Gluceptate RS.

USP Calcium Lactate RS.

USP Calcium Lactobionate RS.

USP Calcium Pantothenate RS.

USP Calcium Saccharate RS.

USP Candelilla Wax RS.

USP Capecitabine RS.

USP Capecitabine Related Compound A RS.

USP Capecitabine Related Compound B RS.

USP Capecitabine Related Compound C RS.



USP Capreomycin Sulfate RS .

USP Caprylic Acid RS.

USP Caprylocaproyl Polyoxyglycerides RS .

USP Capsaicin RS .

USP Captopril RS .

USP Captopril Disulfide RS .

USP Carbachol RS .

USP Carbamazepine RS .

USP Carbamazepine Related Compound A RS [10, 11-dihydrocarbamazepine].

USP Carbamazepine Related Compound B RS [iminostilbene].

USP Carbenicillin Indanyl Sodium RS .

USP Carbenicillin Monosodium Monohydrate RS .

USP Carbidopa RS .

USP Carbidopa Related Compound A RS [3-O-methylcarbidopa] (C11H16N2O4  $\Delta$  240.26) .

USP Carbinoxamine Maleate RS .

USP Urea C 13 RS .

USP Carboplatin RS .

USP Carboprost Tromethamine RS.

USP Carisoprodol RS .

USP Carprofen RS.

USP Carprofen Related Compound A RS [carbazole] (C12H9N  $\Delta$  167.21).

USP Carteolol Hydrochloride RS .

USP Carvedilol RS.

USP Carvedilol Related Compound A RS [1-[[9-[2-hydroxy-3-[[2-(2-methoxyphenoxy)ethyl]amino]propyl]-9H-carbazol-4-yl]oxy]-3-[[2-(2-methoxyphenoxy)ethyl]amino]propan-2-ol] .

USP Carvedilol Related Compound B RS [1,1'-[[2-(2-methoxyphenoxy)ethyl]nitrilo]bis[3-(9H-carbazol-4-yloxy)propan-2-ol] .

USP Carvedilol Related Compound C RS [(2RS)-1-[benzyl[2-(2-methoxyphenoxyethyl]amino]-3-(9H-cabazol-4-yloxy)propan-2-ol] .

USP Carvedilol Related Compound D RS [4-(2,3-epoxypropoxy)carbazole] .

USP Carvedilol Related Compound E RS [2-(2-methoxyphenoxy)ethylamine] .

USP Casticin RS .

USP Powdered Cat's Claw Extract RS.



USP Cathinone Hydrochloride RS [ $\alpha$ -aminopropiophenone hydrochloride] (C9H11NO·HCl  $\Delta$  185.65).

USP Cefaclor RS .

USP Cefaclor Delta-3 Isomer RS

USP Cefadroxil RS

USP Cefamandole Lithium RS .

USP Cefamandole Nafate RS .

USP Cefazolin RS

Add the following:

▲USP Cefdinir RS ▲USP32

Add the following:

▲USP Cefdinir Related Compound A RS [(2R)-2-[(Z)-2-(2-aminothiazol-4-yl)-2-(hydroxyimino)acetamido]-2-[(2RS,5RS)-5-methyl-7-oxo-2,4,5,7-tetrahydro-1H-furo[3,4-d][1,3]thiazin-2-yl]acetic acid (other three stereo isomers are also present in this RS)] (C14H15N5O6S2  $\Delta$  413.43). ▲USP32

Add the following:

▲USP Cefdinir Related Compound B RS [(6R,7R)-7-[2-(2-amino-4-thiazolyl)acetamido]-8-oxo-3-vinyl-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid] (C14H13N4O4S2  $\Delta$  365.41). ▲USP32

USP Cefepime Hydrochloride RS

USP Cefepime Hydrochloride System Suitability RS— This is a mixture of cefepime hydrochloride related compound A (6R-[6 $\alpha$ ,7 $\beta$ (E)]-1-[[7-[(2-amino-4-thiazolyl) (methoxyimino) acetyl]amino]-2-carboxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl]methyl]-1-methylpyrrolidinium chloride, monohydrate; (C19H25ClN6O5S2·HCl·H<sub>2</sub>O  $\Delta$  571.50); cefepime related compound B ([6R-trans]-7-[[2-[(2-amino-4-thiazolyl)(methoxyimino)acetyl]amino]-4-thiazolyl](methoxyimino)acetyl]amino)-3-(1-methylpyrrolidinium-1-yl) methyl]-5-thia-1-azabicyclo[4.2.0]oct-3-ene-2-carboxylic acid, inner salt; (C25H29N9O7S3  $\Delta$  663.75); and cefepime hydrochloride.

USP Cefixime RS

USP Cefmenoxime Hydrochloride RS .

USP Cefmetazole RS

USP Cefonidic Sodium RS

USP Cefoperazone Dihydrate RS

USP Ceforanide RS

USP Cefotaxime Sodium RS .

USP Cefotetan RS

USP Cefotiam Hydrochloride RS .

USP Cefoxitin RS

USP Cefpiramide RS

USP Cefpodoxime Proxetil RS .

USP Cefprozil (E)-Isomer RS

USP Cefprozil (Z)-Isomer RS

USP Ceftazidime Delta-3-Isomer RS



USP Ceftazidime Pentahydrate RS

USP Ceftrizoxime RS

USP Ceftriaxone Sodium RS .

USP Ceftriaxone Sodium E-Isomer RS

USP Cefuroxime Axetil RS .

USP Cefuroxime Axetil Delta-3 Isomers RS

USP Cefuroxime Sodium RS

USP Cellaburate RS .

USP Cellacefate RS .

USP F<sub>1</sub> Cells for Cell Proliferation Test RS (ATCC cell line FDCP-F1 shipped with approval from USP).

USP Cellulose Acetate RS .

USP Cephaeline Hydrobromide RS

USP Cephalexin RS .

USP Cephalothin Sodium RS .

USP Cephapirin Benzathine RS

USP Cephapirin Sodium RS .

USP Cephradine RS

USP Cetyl Alcohol RS .

USP Cetyl Palmitate RS

USP Cetylpyridinium Chloride RS .

USP Chlorambucil RS

USP Chloramphenicol RS

USP Chloramphenicol Palmitate RS

USP Chloramphenicol Palmitate Nonpolymorph A RS .

USP Chloramphenicol Palmitate Polymorph A RS .

USP Chlordiazepoxide RS

USP Chlordiazepoxide Related Compound A RS [7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one 4-oxide] (C<sub>15</sub>H<sub>11</sub>CIN<sub>2</sub>O<sub>2</sub> 286.72) .

USP Chlordiazepoxide Hydrochloride RS .

USP Chlorhexidine RS

USP Chlorhexidine Acetate RS .



USP Chlorhexidine Related Compounds RS .

USP Chlorobutanol RS .

USP 2-Chloro-3,5-dimethylphenol RS (C8H9ClO  $\Delta$  156.61) .

USP Chlorogenic Acid RS.

USP  $\beta$ -Chlorogenin RS .

USP Chlorophyllin Copper Complex Sodium RS.

USP Chloroprocaine Hydrochloride RS .

USP Chloroquine Phosphate RS .

USP Chlorothiazide RS .

USP Chloroxylene RS .

USP Chloroxylene Related Compound A RS [2-chloro-3,5-dimethylphenol] .

USP Chlorpheniramine Extended-Release Tablets RS (Drug Release Calibrator, Single Unit) .

USP Chlorpheniramine Maleate RS .

USP Chlorpromazine Hydrochloride RS .

USP Chlorpropamide RS .

USP (E)-Chlorprothixene RS .

USP Chlortetracycline Hydrochloride RS .

USP Chlorthalidone RS .

USP Chlorthalidone Related Compound A RS [4'-Chloro-3'-sulfamoyl-2-benzophenone carboxylic acid] .

USP Chlorzoxazone RS .

USP Chlorzoxazone Related Compound A RS [2-amino-4-chlorophenol] (C6H6ClNO  $\Delta$  143.57) .

USP Cholecalciferol RS .

USP  $\Delta^{4,6}$ -Cholestadienol RS [cholesta-4,6-dien-3 $\beta$ -ol] (C27H44O  $\Delta$  384.64) .

USP Cholesteryl Caprylate RS (C35H60O2  $\Delta$  512.86) .

USP Cholestyramine Resin RS .

USP Choline Bitartrate RS .

USP Choline Chloride RS .

USP Chondroitin Sulfate Sodium RS .

USP Chromium Picolinate RS .

USP Chymotrypsin RS .

USP Ciclopirox RS .



USP Ciclopirox Related Compound A RS [3-cyclohexyl-4,5-dihydro-5-methyl-5-isoxazolyl acetic acid].

USP Ciclopirox Related Compound B RS [6-cyclohexyl-4-methyl-2-pyrone].

USP Ciclopirox Olamine RS .

USP Cilastatin Ammonium Salt RS .

USP Cilostazol RS .

USP Cilostazol Related Compound A RS [6-hydroxy-3,4-dihydro-1H-quinolin-2-one] (C9H9NO2  $\Delta$  163.17).

USP Cilostazol Related Compound B RS [6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)-butoxy]-1H-quinolin-2-one] (C20H25N5O2  $\Delta$  367.45).

USP Cilostazol Related Compound C RS [1-(4-(5-cyclohexyl-1H-tetrazol-1-yl)butyl)-6-(4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy)-3,4-dihydroquinolin-2(1H)-one] (C31H43N9O3  $\Delta$  589.73).

USP Cimetidine RS .

USP Cimetidine Hydrochloride RS .

USP Cinoxacin RS .

USP Ciprofloxacin RS .

USP Ciprofloxacin Ethylenediamine Analog RS [1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-[(2-aminoethyl)amino]-3-quinolinecarboxylic acid hydrochloride] (C15H16FN3O3·HCl  $\Delta$  341.77).

USP Ciprofloxacin Hydrochloride RS .

USP Cisplatin RS .

USP Citalopram Hydrobromide RS. .

USP Citalopram Related Compound A RS [1-(3-dimethylaminopropyl)-1-(4'-fluorophenyl)-1,3-dihydroisobenzofuran-5-carboxamide] (C20H23FN2O2  $\Delta$  342.22).

USP Citalopram Related Compound B RS [1-(3-dimethylaminopropyl)-1-(4-fluorophenyl)-3-hydroxy-1,3-dihydroisobenzofuran-5-carbonitrile] (C20H21FN2O2  $\Delta$  340.22).

USP Citalopram Related Compound C RS [3-(3-N,N-dimethylamino)-1-(4-fluorophenyl)-6-cyano-1(3H)-isobenzofuranone] (C20H19FN2O2  $\Delta$  338.22).

USP Citalopram Related Compound D RS [1-(4-fluorophenyl)-1-(3-(methylamino)propyl)-1,3-dihydroisobenzofuran-5-carbonitrile hydrochloride] (C19H19FN2O·HCl  $\Delta$  346.83).

USP Citalopram Related Compound E RS [1-(3-dimethylaminopropyl)-1-(4-fluorophenyl)-1,3-dihydrobenzofuran-5-carbonitrile-N-oxide] (C20H21FN2O2  $\Delta$  340.22).

USP Citalopram Related Compound F RS [dimethyl-(1-methyl-3,3-diphenylallyl)amine hydrochloride] (C18H21NHCl  $\Delta$  286.64).

USP Citalopram Related Compound G RS [1-(4'-fluorophenyl)-1-(3-dimethylaminopropyl)-5-chlorophthalane hydrobromide] (C19H21FNNOCl·HBr  $\Delta$  414.74).

USP Citalopram Related Compound H RS [1-(4'-fluorophenyl)-1-(3-dimethylaminopropyl)-5-bromophthalane hydrobromide] (C19H21FNNOBr·HBr  $\Delta$  459.1).

USP Citric Acid RS .

USP Clarithromycin RS .

USP Clarithromycin Identity RS. .

USP Clarithromycin Related Compound A RS [6,11-di-O-methylerythromycin A] (C39H71NO13  $\Delta$  762.00).

USP Clavam-2-Carboxylate Potassium RS .

USP Clavulanate Lithium RS .

USP Clemastine Fumarate RS .

USP Clidinium Bromide RS .

USP Clidinium Bromide Related Compound A RS  [3-hydroxy-1-methylquinuclidinium bromide] (C8H16BrNO  $\Delta$  222.13) .

USP Clindamycin Hydrochloride RS .

USP Clindamycin Palmitate Hydrochloride RS .

USP Clindamycin Phosphate RS .

USP Clioquinol RS .

USP Clobetasol Propionate RS .

USP Clobetasol Propionate Related Compound A RS  [9 $\alpha$ -fluoro-11 $\beta$ -hydroxy-16 $\beta$ -methyl 3-oxo-androsta-1,4-diene-17(R)-spiro-2'-[4'-chloro-5'-ethylfuran-3'(2'H)-one]] (C25H30ClFO4  $\Delta$  448.96) .

USP Clocortolone Pivalate RS .

USP Clofazimine RS .

USP Clofibrate RS .

USP Clomiphene Citrate RS .

USP Clomiphene Related Compound A RS  [(E,Z)-2-[4-(1,2-diphenylethoxy)phenoxy]-N,N-diethylethanamine hydrochloride] (C26H29NO·HCl  $\Delta$  407.98) .

USP Clomipramine Hydrochloride RS .

USP Clonazepam RS .

USP Clonazepam Related Compound A RS  [3-amino-4-(2-chlorophenyl)-6-nitrocarbostyryl] (C15H10ClN3O3  $\Delta$  315.72) .

USP Clonazepam Related Compound B RS  [2-amino-2'-chloro-5-nitrobenzophenone] (C13H9ClN2O3  $\Delta$  276.68) .

USP Clonazepam Related Compound C RS  [2-bromo-2'-(2-chlorobenzoyl)-4'-nitroacetanilide] .

USP Clonidine RS .

USP Clonidine Hydrochloride RS .

USP Clonidine Related Compound B RS  [2-[(E)-2,6-dichlorophenylimino]-1-(1-[(E)-2,6-dichlorophenylimino]-imidazolidin-1-yl)ethyl]imidazolidine] (C20H20Cl4N6  $\Delta$  486.23) .

USP Clopidogrel Bisulfate RS.

USP Clopidogrel Related Compound A RS  [(+)-(S)-(o-chlorophenyl)-6,7-dihydrothieno[3,2-c]pyridine-5(4H)-acetic acid].

USP Clopidogrel Related Compound B RS  [methyl ( $\pm$ )-(o-chlorophenyl)-4,5-dihydrothieno[2,3-c]pyridine-6(7H)-acetate, hydrochloride].

USP Clopidogrel Related Compound C RS  [methyl (R)-(o-chlorophenyl)-6,7-dihydrothieno[3,2-c]pyridine-5(4H)-acetate, hydrogen sulfate].

USP Clorazepate Dipotassium RS .

USP Clorsulon RS .

USP Clotrimazole RS .



USP Clotrimazole Related Compound A RS [(o-chlorophenyl)diphenylmethanol] (C<sub>19</sub>H<sub>15</sub>ClO  $\Delta$  294.78).

USP Powdered Red Clover Extract RS .

USP Cloxacillin Benzathine RS .

USP Cloxacillin Sodium RS .

USP Clozapine RS .

USP Clozapine Resolution Mixture RS —Contains the following components:

Clozapine: 11, 11'-(piperazine-1,4-diy)bis(8-chloro-5H-dibenzo(b,e)(1,4)diazepine.

Impurity A: 8-chloro-5,10-dihydro-11H-dibenzo(b,e)(1,4)diazepin-11-one.

Impurity B: 11,11'-(piperazine-1,4-diy)bis(8-chloro-5H-dibenzo(b,e)(1,4)diazepine.

Impurity C: 8-chloro-11-(piperazin-1-yl)-5H-dibenzo(b,e)(1,4)diazepine.

Impurity D: 4-chloro-N1-(2-(4-methylpiperazin-1-yl)carbonyl)phenyl)benzene-1,2-diamine.

USP Cocaine Hydrochloride RS .

USP Cod Liver Oil RS.

USP Codeine N-Oxide RS (C<sub>18</sub>H<sub>21</sub>NO<sub>4</sub>  $\Delta$  315.37) .

USP Codeine Phosphate RS .

USP Codeine Sulfate RS .

USP Powdered Black Cohosh Extract RS .

USP Colchicine RS .

USP Colestipol Hydrochloride RS .

USP Colistimethate Sodium RS .

USP Colistin Sulfate RS .

USP Copovidone RS .

USP Corticotropin RS .

USP Cortisone Acetate RS .

USP Creatinine RS .

USP Cromolyn Sodium RS .

USP Crospovidone RS .

USP Crotamiton RS .

Add the following:

▲USP Curcumin RS. ▲USP32

Add the following:

▲USP Curcuminoids RS. ▲USP32

USP Cyanidin Chloride RS.

USP Cyanidin-3-O-glucoside Chloride RS.



USP Cyanocobalamin RS

USP Cyclandelate RS

USP Cyclizine Hydrochloride RS .

USP Cyclobenzaprine Hydrochloride RS .

USP Alpha Cyclodextrin RS

USP Beta Cyclodextrin RS

USP Gamma Cyclodextrin RS

USP Cyclomethicone 4 RS

USP Cyclomethicone 5 RS

USP Cyclomethicone 6 RS

USP Cyclopentolate Hydrochloride RS .

USP Cyclophosphamide RS .

USP 2-Cyclopropylmethylamino-5-chlorobenzophenone RS

USP Cycloserine RS

USP Cyclosporine RS

USP Cyclosporine Resolution Mixture RS [This material is a 100:1 mixture of cyclosporine and cyclosporine U.] .

USP Cyproheptadine Hydrochloride RS .

USP Cyromazine RS.

USP I-Cysteine Hydrochloride RS .

USP Cytarabine RS

USP Cytosine RS.

USP Dacarbazine RS

USP Dacarbazine Related Compound A RS [5-aminoimidazole-4-carboxamide hydrochloride] .

USP Dacarbazine Related Compound B RS [2-azahypoxanthine] (C4H3N5O  $\Delta$  137.10) .

USP Dactinomycin RS

Add the following:

▲USP Daidzein RS. ▲USP32

Add the following:

▲USP Daidzin RS. ▲USP32

USP Danazol RS

USP Danthron RS



USP Dantrolene RS.

USP Dantrolene Related Compound A RS [5-(4-nitrophenyl)-2-furaldehyde azine] (C22H14N4O6).

USP Dantrolene Related Compound B RS [5-(4-nitrophenyl)-2-furaldehyde-2-carboxymethyl semicarbazone] (C14H12N4O6).

USP Dantrolene Related Compound C RS [5-(4-nitrophenyl)-2-furancarboxyaldehyde] (C11H7NO4).

USP Dantrolene Sodium RS.

USP Dapsone RS .

USP Daunorubicin Hydrochloride RS .

USP Deacetylorgestimate RS [mixture of syn-17-deacetylorgestimate and anti-17-deacetylorgestimate] .

USP Decoquinate RS.

USP Deferoxamine Mesylate RS .

USP Dehydroacetic Acid RS.

USP Dehydrocortefol Hydrochloride RS [5-(3-tert-butylamino-2-hydroxy)-propoxycarbostyryl hydrochloride] (C16H22N2O3·HCl  $\Delta$  326.82) .

USP Dehydrocholic Acid RS .

USP Demecarium Bromide RS .

USP Demeclocycline Hydrochloride RS .

USP Denatonium Benzoate RS .

USP Bovine Acellular Dermal Matrix Reference Photomicrographs.

USP Cryopreserved Human Fibroblast-Derived Dermal Substitute Reference Photomicrographs VRS.

USP Desacetyl Diltiazem Hydrochloride RS (C20H24N2O3S·HCl  $\Delta$  408.95) .

USP Desflurane RS .

USP Desflurane Related Compound A RS [bis-(1,2,2,2-tetrafluoroethyl)ether] (C4H2F8O  $\Delta$  218.05) .

USP Desipramine Hydrochloride RS .

USP Deslanoside RS .

Add the following:

▲USP Desmethoxycurcumin RS. ▲USP32

USP Desmopressin Acetate RS.

USP Desoaminylazithromycin RS .

USP Desogestrel RS.

USP Desogestrel Related Compound A RS [13-ethyl-11-methylene-18, 19-dinor-5 $\alpha$ , 17 $\alpha$ -pregn-3-en-20-yn-17-ol, desogestrel  $\Delta$ 3-isomer] (C22H30O  $\Delta$  310.48).USP Desogestrel Related Compound B RS [3-hydroxy-desogestrel] (C22H30O2  $\Delta$  326.48).USP Desogestrel Related Compound C RS [3-keto-desogestrel] (C22H28O2  $\Delta$  324.46) .



USP Desoximetasone RS

USP Desoxycorticosterone Acetate RS

USP Desoxycorticosterone Pivalate RS .

USP Dexamethasone RS

USP Dexamethasone Acetate RS .

USP Dexamethasone Phosphate RS

USP Dexbrompheniramine Maleate RS .

USP Dexchlorpheniramine Maleate RS .

USP Dexpanthenol RS

USP Dextran 1 RS .

USP Dextran 4 Calibration RS .

USP Dextran 10 Calibration RS .

USP Dextran 40 RS.

USP Dextran 40 Calibration RS .

USP Dextran 40 System Suitability RS.

USP Dextran 70 RS.

USP Dextran 70 Calibration RS .

USP Dextran 70 System Suitability RS.

USP Dextran 250 Calibration RS .

USP Dextran T-10 RS .

USP Dextran Vo Marker RS.

USP Dextroamphetamine Sulfate RS .

USP Dextromethorphan RS

USP Dextromethorphan Hydrobromide RS .

USP Dextrose RS

USP Diacetylated Monoglycerides RS .

USP Diacetylfluorescein RS (C<sub>24</sub>H<sub>16</sub>O<sub>7</sub> ♦ 416.39) .

USP Diatrizoic Acid RS .

USP Diatrizoic Acid Related Compound A RS [5-acetamido-3-amino-2,4,6-triiodobenzoic acid] (C<sub>9</sub>H<sub>7</sub>I<sub>3</sub>N<sub>2</sub>O<sub>3</sub> ♦ 571.88) .

USP Diazepam RS



USP Diazepam Related Compound A RS [2-methylamino-5-chlorobenzophenone] (C14H12ClNO  $\Delta$  245.71).

USP Diazepam Related Compound B RS [3-amino-6-chloro-1-methyl-4-phenylcarboxylic acid] (C16H13ClN2O  $\Delta$  284.74).

USP Diazoxide RS.

USP Dibucaine Hydrochloride RS.

USP Dibutyl Phthalate RS.

USP Dichloralphenazone RS.

USP 2,4-Dichlorophenol RS.

USP Dichlorphenamide RS.

USP Diclofenac Potassium RS.

USP Diclofenac Sodium RS.

USP Diclofenac Related Compound A RS [N-(2,6-dichlorophenyl)indolin-2-one] (C14H9Cl2NO  $\Delta$  278.14).

USP Dicloxacillin Sodium RS.

USP Dicyclomine Hydrochloride RS.

USP Didanosine RS.

USP Didanosine Related Compound A RS [hypoxanthine].

USP Didanosine Related Compound B RS [2',3'-dideoxyadenosine].

USP Dienestrol RS.

USP Diethanolamine RS.

USP Diethylcarbamazine Citrate RS.

USP Diethylene Glycol RS.

USP Diethylene Glycol Monoethyl Ether RS.

USP Diethyl Phthalate RS.

USP Diethylpropion Hydrochloride RS.

USP Diethylstilbestrol RS.

Delete the following:

▲USP Diethylstilbestrol Diphosphate RS. ▲USP32

USP Diethyltoluamide RS.

USP Diflorasone Diacetate RS.

USP Diflunisal RS.

USP Digitalis RS.

USP Digitoxin RS.



USP Digoxin RS

USP Dihydrocapsaicin RS .

USP Dihydrocodeine Bitartrate RS .

USP 17 $\alpha$ -Dihydroequilin RS (C18H22O<sub>2</sub>) 270.37 .

USP Dihydroergotamine Mesylate RS .

USP Dihydrostreptomycin Sulfate RS .

USP Dihydrotachysterol RS

USP Dihydroxyacetone RS .

USP Diloxanide Furoate RS

USP Diltiazem Hydrochloride RS .

USP Dimenhydrinate RS

USP Dimethyl Sulfoxide RS

USP Dinoprostone RS

USP Dinoprost Tromethamine RS

USP Dioxybenzone RS

USP Diphenhydramine Citrate RS .

USP Diphenhydramine Hydrochloride RS

USP Diphenoxylate Hydrochloride RS .

USP Dipivefrin Hydrochloride RS

USP Dipyridamole RS

USP Dirithromycin RS

USP Disopyramide Phosphate RS .

USP 2,4-Disulfamyl-5-trifluoromethylaniline RS (C7H8F3N3O4S<sub>2</sub>) 319.29 .

USP Disulfiram RS

USP Divalproex Sodium RS [sodium hydrogen bis(2-propylvalerate), oligomer; pentanoic acid, 2-propyl-, sodium salt (2:1)] [(C<sub>16</sub>H<sub>31</sub>NaO<sub>4</sub>)<sub>n</sub>] 310.41.

USP Dobutamine Hydrochloride RS .

USP Docosahexaenoic Acid Ethyl Ester RS [all cis-4,7,10,13,16,19-docosahexaenoic ethyl ester] (C<sub>24</sub>H<sub>36</sub>O<sub>2</sub>) 356.55.

USP Docusate Calcium RS .

USP Docusate Potassium RS .

USP Docusate Sodium RS .

USP Dolasetron Mesylate RS .



USP Dolasetron Mesylate Related Compound A RS [hexahydro-8-hydroxy-2,6-methano-2H-quinolizin-3 (4H)-one, hydrochloride] .

USP Dopamine Hydrochloride RS .

USP Dorzolamide Hydrochloride RS .

USP Dorzolamide Hydrochloride Related Compound A RS [(4R,6R)-4-(ethylamino)-5,6-dihydro-6-methyl-4H-thieno[2,3-b]thiopyran-2-sulfonamide-7,7-dioxide, monohydrochloride] (C10H16N2O4S3·HCl 360.91) .

USP Doxapram Hydrochloride RS .

USP Doxazosin Mesylate RS.

USP Doxazosin Related Compound A RS [N-1,4-benzodioxane-2-carbonyl piperazine] (C13H16N2O3 248.28).

USP Doxazosin Related Compound B RS [6,7-dimethoxyquinazoline-2,4-dione] (C10H10N2O4 222.20).

USP Doxazosin Related Compound C RS [2-chloro-4-amino-6,7-dimethoxyquinazoline] (C10H10ClN3O2 239.66).

USP Doxazosin Related Compound D RS [1,4-benzodioxane-2-carboxylic acid] (C9H8O5 196.16).

USP Doxazosin Related Compound E RS [2,4-dichloro-6,7-dimethoxyquinazoline] (C10H8Cl2N2O2 259.09).

USP Doxazosin Related Compound F RS [N,N'-bis(1,4-benzodioxane-2-carbonyl)piperazine] (C22H22N2O6 410.42).

USP Doxepin Hydrochloride RS .

USP Doxepin Related Compound A RS [5-(4-nitrophenyl)-2-furaldehyde-2-carboxymethyl semicarbazone] (C14H10O2 210.23).

USP Doxepin Related Compound B RS [11(RS)-(3-(dimethylamino)propyl)-6,11-dihydrodibenzo[b,e]oxepin-11-ol] (C19H23NO2 297.39).

USP Doxepin Related Compound C RS [(E,Z)-3-(dibenzo[b,e]oxepin-11(6H)-ylidene)-N-methylpropan-1-amine] (C18H19NO·HCl 301.81).

USP Doxorubicin Hydrochloride RS .

USP Doxycycline Hyclate RS .

USP Doxylamine Succinate RS .

USP Droperidol RS .

USP Drospirenone RS.

USP Dyclonine Hydrochloride RS .

USP Dydrogesterone RS .

USP Dyphylline RS .

USP Powdered Echinacea angustifolia Extract RS .

USP Powdered Echinacea pallida Extract RS.

USP Powdered Echinacea purpurea Extract RS .

USP Econazole Nitrate RS .

USP Eddate Calcium Disodium RS .

USP Eddate Disodium RS .



USP Edetic Acid RS

USP Edrophonium Chloride RS .

USP Eicosapentaenoic Acid Ethyl Ester RS [all cis-5,8,11,14,17-eicosapentaenoic ethyl ester] (C22H34O2  $\Delta$  330.51).

USP Powdered Eleuthero Extract RS .

USP Emedastine Difumarate RS

USP Emetine Hydrochloride RS .

USP Enalapril Maleate RS .

USP Enalaprilat RS .

USP Endotoxin RS .

USP Enflurane RS

USP Enoxaparin Sodium RS.

USP Enoxaparin Sodium Solution for Bioassay RS.

USP Enoxaparin Sodium Molecular Weight RS.

USP Enoxaparin Sodium Molecular Weight Calibrant A RS.

USP Enoxaparin Sodium Molecular Weight Calibrant B RS.

USP Enzacamene RS.

USP Ephedrine Sulfate RS .

USP 4-Epianhydrotetracycline Hydrochloride RS .

USP (–)-Epigallocatechin-3-O-gallate RS.

USP Epilactose RS

USP Epinephrine Bitartrate RS .

USP Eprinomectin RS.

USP Equilin RS

USP Ergocalciferol RS

USP Ergoloid Mesylates RS .

USP Ergonovine Maleate RS .

USP Ergosterol RS (C28H44O  $\Delta$  396.66) .

USP Ergotamine Tartrate RS .

USP Ergotaminine RS .

Add the following:

▲USP Erythorbic Acid RS. ▲USP32



USP Erythritol RS [meso-erythritol, 1,2,3,4-butanetetrol] (C4H10O4 ⚡ 122.12) .

USP Erythromycin RS .

USP Erythromycin B RS .

USP Erythromycin C RS .

USP Erythromycin Related Compound N RS [N-demethylerythromycin A] (C36H65NO13 ⚡ 719.91) .

USP Erythromycin Estolate RS .

USP Erythromycin Ethylsuccinate RS .

USP Erythromycin Gluceptate RS .

USP Erythromycin Lactobionate RS .

USP Erythromycin Stearate RS .

USP Escin RS.

USP Esomeprazole Magnesium RS .

USP Estradiol RS .

USP Estradiol Benzoate RS .

USP Estradiol Cypionate RS .

USP Estradiol Valerate RS .

USP Estriol RS .

USP Estrone RS .

USP Estropipate RS .

USP Ethacrynic Acid RS .

USP Ethambutol Hydrochloride RS .

USP Ethchlorvynol RS .

USP Ethinyl Estradiol RS .

USP Ethionamide RS .

USP Ethopabate RS .

USP Ethopabate Related Compound A RS [methyl-4-acetamido-2-hydroxybenzoate] (C10H11NO4 ⚡ 209.20) .

USP Ethosuximide RS .

USP Ethotoin RS .

USP Ethyl Acetate RS .

USP Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion RS .



USP Ethylcellulose RS .

USP Ethylene Glycol RS .

USP Ethylparaben RS  .

USP Ethyl Vanillin RS  .

USP Ethynodiol Diacetate RS .

USP Etidronate Disodium RS .

USP Etidronate Disodium Related Compound A RS [sodium phosphite dibasic pentahydrate] ( $\text{Na}_2\text{HPO}_3 \cdot 5\text{H}_2\text{O}$   $\Delta$  216.04  $\Delta$  CAS-13708-85-5) .

USP Etidronic Acid Monohydrate RS  ( $\text{C}_2\text{H}_8\text{O}_7\text{P}_2\text{H}_2\text{O}$   $\Delta$  224.04) .

USP Etodolac RS  .

USP Etodolac Related Compound A RS  [( $\pm$ )-8-ethyl-1-methyl-1,3,4,9-tetrahydropyrano [3,4-b]-indole-1-acetic acid] ( $\text{C}_{16}\text{H}_{19}\text{NO}_3$   $\Delta$  273.33) .

USP Etoposide RS  .

USP Etoposide Resolution Mixture RS .

Delete the following:

▲USP Eucatropine Hydrochloride RS. ▲USP32

USP Famotidine RS  .

USP Felodipine RS  .

USP Felodipine Related Compound A RS  [ethyl methyl 4-(2,3-dichlorophenyl)-2,6-dimethylpyridine-3,5-dicarboxylate] ( $\text{C}_{18}\text{H}_{17}\text{Cl}_2\text{NO}_4$   $\Delta$  382.24) .

USP Fenbendazole RS  .

USP Fenbendazole Related Compound A RS  [methyl (1H-benzimidazole-2-yl)carbamate] ( $\text{C}_9\text{H}_9\text{N}_3\text{O}_2$   $\Delta$  191.19) .

USP Fenbendazole Related Compound B RS  [methyl [5(6)-chlorobenzimidazole-2-yl]carbamate] ( $\text{C}_9\text{H}_8\text{ClN}_3\text{O}_2$   $\Delta$  225.63) .

USP Fenofibrate RS. 

USP Fenofibrate Related Compound A RS  [(4-chlorophenyl)(4-hydroxyphenyl)methanone].

USP Fenofibrate Related Compound B RS  [2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoic acid, or fenofibric acid].

USP Fenofibrate Related Compound C RS  [1-methylethyl 2-[[2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoyl]oxy]-2-methylpropanoate].

USP Fenoldopam Mesylate RS .

USP Fenoldopam Related Compound A RS  [1-methyl-3-benzazepine-7,8-diol, 6-chloro-2,3,4,5-tetrahydro-1-(4-hydroxyphenyl)-, methanesulfonate (salt)] ( $\text{C}_{17}\text{H}_{18}\text{ClNO}_3 \cdot \text{CH}_4\text{SO}_3$   $\Delta$  415.89) .

USP Fenoldopam Related Compound B RS  [1H-3-benzazepine-7,8-diol, 2,3,4,5-tetrahydro-1-(4-hydroxyphenyl)-, methanesulfonate (salt)] ( $\text{C}_{16}\text{H}_{16}\text{NO}_3 \cdot \text{CH}_4\text{SO}_3$   $\Delta$  366.42) .

USP Fenoprofen Calcium RS .

USP Fenoprofen Sodium RS  .

USP Fentanyl Citrate RS .

USP Fexofenadine Hydrochloride RS .



USP Fexofenadine Related Compound A RS [benzeneacetic acid, 4-[1-oxy-4-[4-(hydroxydiphenylmethyl)-1-piperidinyl]butyl]- $\alpha$ , $\alpha$ -dimethyl] (C32H37NO4  $\Delta$  499.65).

USP Fexofenadine Related Compound B RS [3-[1-hydroxy-4-[4-(hydroxydiphenylmethyl)-1-piperidinyl]butyl]- $\alpha$ , $\alpha$ -dimethyl benzeneacetic acid hydrochloride] (C32H39NO4·HCl  $\Delta$  538.12).

USP Finasteride RS .

USP Fish Oil RS.

Add the following:

▲USP Flavoxate Hydrochloride RS. ▲USP32

Add the following:

▲USP Flavoxate Related Compound A RS [3-methylflavone-8-carboxylic acid] (C17H12O4  $\Delta$  280.27). ▲USP32

USP Flecainide Acetate RS.

USP Flecainide Related Compound A RS [3-[2,5-bis(2,2,2-trifluoroethoxy)phenyl]-1,5,6,7,8,8a-hexahydroimidazo-[1,5a]pyridine hydrochloride] (C17H18F6N2O2·HCl  $\Delta$  432.8).

USP Flouxuridine RS .

USP Fluconazole RS .

USP Fluconazole Related Compound A RS [2-[2-fluoro-4-(1H-1,2,4-triazol-1-yl)phenyl]-1,3-bis(1H-1,2,4-triazol-1-yl)-propan-2-ol].

USP Fluconazole Related Compound B RS [2-(4-fluorophenyl)-1,3-bis(1H-1,2,4-triazol-1-yl)-propan-2-ol].

USP Fluconazole Related Compound C RS [1,1'-(1,3-phenylene)di(1H-1,2,4-triazole)].

USP Flucytosine RS .

USP Fludarabine Phosphate RS .

USP Fludeoxyglucose RS .

USP Fludeoxyglucose Related Compound A RS [4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8.]hexacosane] (C18H36N2O6  $\Delta$  376.49).

USP Fludeoxyglucose Related Compound B RS (C6H11ClO5  $\Delta$  198.60).

USP Fludrocortisone Acetate RS .

USP Flumazenil RS .

USP Flumazenil Related Compound A RS [8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazol-[1,5-a][1,4]benzodiazepine-3-carboxylic acid] (C13H10FN3O3  $\Delta$  275.24).

USP Flumazenil Related Compound B RS [Ethyl 8-hydroxy-5,6-dihydro-5-methyl-6-oxo-4H-imidazol-[1,5-a][1,4]benzodiazepine-3-carboxylate] (C15H15N3O4  $\Delta$  301.30).

USP Flumazenil Related Compound C RS [N,N-dimethylformamide diethyl acetal].

USP Flumethasone Pivalate RS .

USP Flunisolide RS .

USP Flunixin Meglumine RS .

USP Fluocinolone Acetonide RS .

USP Fluocinonide RS .

USP Fluorescein RS .



USP I-Fluorodopa RS

USP Fluorometholone RS .

USP Fluorometholone Acetate RS .

USP Fluoroquinolonic Acid RS .

USP Fluorouracil RS .

USP Fluoxetine Hydrochloride RS .

USP Fluoxetine Related Compound A RS [N-methyl-3-phenyl-3-[( $\alpha$ , $\alpha$ , $\alpha$ -(trifluoro-m-tolyl)oxy]propylamine hydrochloride] (C17H18F3NO·HCl  $\Delta$  345.79).USP Fluoxetine Related Compound B RS [N-methyl-3-phenylpropylamine] (C10H15N  $\Delta$  149.24).USP Fluoxetine Related Compound C RS [N-methyl-N-[3-phenyl-3-(4-trifluoromethyl-phenoxy)-propyl]-succinamic acid] (C21H22F3NO4  $\Delta$  409.40).

USP Fluoxymesterone RS .

USP Fluphenazine Decanoate Dihydrochloride RS .

USP Fluphenazine Enanthate Dihydrochloride RS (C29H38F3N3O2S·2HCl  $\Delta$  622.63).

USP Fluphenazine Hydrochloride RS .

USP Flurandrenolide RS .

USP Flurazepam Hydrochloride RS .

USP Flurazepam Related Compound C RS [5-chloro-2-(2-diethylaminoethyl)(amino)-2'-fluorobenzophenone hydrochloride] (C19H22ClFN2O·HCl  $\Delta$  385.31).USP Flurazepam Related Compound F RS [7-chloro-5-(2-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one] (C15H10ClFN2O  $\Delta$  288.71).

USP Flurbiprofen RS .

USP Flurbiprofen Related Compound A RS [2-(4-biphenyl)propionic acid] (C15H14O2  $\Delta$  226.28).

USP Flurbiprofen Sodium RS .

USP Flutamide RS .

USP o-Flutamide RS [2-methyl-N-[6-nitro-3-(trifluoromethyl)phenyl]propanamide] (C11H11F3N2O3  $\Delta$  276.22).USP Fluticasone Propionate RS [S-(fluoromethyl)6 $\alpha$ ,9 $\alpha$ -difluoro-11 $\beta$ ,17-dihydroxy-16 $\alpha$ -methyl-3-oxoandrosta-1,4-diene-17 $\beta$ -carbothioate, 17-propionate] (C25H31F3O5S  $\Delta$  500.6).

USP Fluticasone Propionate Nasal Spray Resolution Mixture RS— This Reference Standard is a mixture of fluticasone propionate and fluticasone propionate related compound D, and the chemical names for both are given below:

Fluticasone propionate: S-Fluoromethyl 6 $\alpha$ ,9 $\alpha$ -difluoro-11 $\beta$ -hydroxy-16 $\alpha$ -methyl-3-oxo-17 $\alpha$ -propionyloxyandrosta-1,4-diene-17 $\beta$ -carbothioate.Fluticasone propionate related compound D: S-methyl-6 $\alpha$ ,9 $\alpha$ -difluoro-11 $\beta$ -hydroxy-16 $\alpha$ -methyl-3-oxo-17 $\alpha$ -propionyloxy-androsta-1,4-diene-17 $\beta$ -carbothioate.

USP Fluticasone Propionate Related Compounds Mixture RS— This Reference Standard is a mixture of fluticasone propionate and fluticasone propionate related compounds D and F, and the chemical names for all are given below:

Fluticasone propionate: S-Fluoromethyl 6 $\alpha$ ,9 $\alpha$ -difluoro-11 $\beta$ -hydroxy-16 $\alpha$ -methyl-3-oxo-17 $\alpha$ -propionyloxyandrosta-1,4-diene-17 $\beta$ -carbothioate.Fluticasone propionate related compound D: S-methyl 6 $\alpha$ ,9 $\alpha$ -difluoro-11 $\beta$ -hydroxy-16 $\alpha$ -methyl-3-oxo-17 $\alpha$ -propionyloxy-androsta-1,4-diene-17 $\beta$ -carbothioate.Fluticasone propionate related compound F: [6 $\alpha$ ,9 $\alpha$ -difluoro-11 $\beta$ ,17 $\alpha$ -dihydroxy-16 $\alpha$ -methyl-3-oxoandrosta-1,4-diene-17 $\beta$ -carboxylic acid].

USP Fluticasone Propionate System Suitability Mixture RS— It is a mixture of



[USP Fluticasone Propionate RS](#) and fluticasone propionate related compounds B, C, and D.

Fluticasone propionate related compound A [6 $\alpha$ ,9 $\alpha$ -difluoro-11 $\beta$ -hydroxy-16 $\alpha$ -methyl-3-oxo-17 $\alpha$ -propionyloxyandrosta-1,4-diene-17 $\beta$ -carboxylsulfenic acid].

Fluticasone propionate related compound B [6 $\alpha$ ,9 $\alpha$ -difluoro-11 $\beta$ -hydroxy-16 $\alpha$ -methyl-2',3,4'-trioxo-17 $\alpha$ -spiro(androsta-1,4-diene-17,5'-(1,3)oxathiolane)].

Fluticasone propionate related compound C [S-fluoromethyl 17 $\alpha$ -acetoxy-6 $\alpha$ ,9 $\alpha$ -difluoro-11 $\beta$ -hydroxy-16 $\alpha$ -methyl-3-oxo-androsta-1,4-diene-17 $\beta$ -carbothioate].

Fluticasone propionate related compound D [S-methyl 6 $\alpha$ ,9 $\alpha$ -difluoro-11 $\beta$ -hydroxy-16 $\alpha$ -methyl-3-oxo-17 $\alpha$ -propionyloxy-androsta-1,4-diene-17 $\beta$ -carbothioate].

Fluticasone propionate related compound E [6 $\alpha$ ,9 $\alpha$ -difluoro-11 $\beta$ ,17 $\alpha$ -dihydroxy-16 $\alpha$ -methyl-3-oxo-androsta-1,4-diene-17 $\beta$ -carboxylic acid 6 $\alpha$ ,9 $\alpha$ -difluoro-17 $\beta$ -(fluoromethylthio)carbonyl-11 $\beta$ -hydroxy-16 $\alpha$ -methyl-3-oxo-androsta-1,4-dien-17 $\alpha$ -yl ester].

USP Fluvastatin Sodium RS.

USP Fluvastatin Related Compound B RS

USP Fluvastatin for System Suitability RS [fluvastatin sodium and fluvastatin sodium anti-isomer].

USP Fluvoxamine Maleate RS

USP Folic Acid RS

USP Folic Acid Related Compound A RS [calcium formyltetrahydrofolate].

Add the following:

▲USP Formoterol Fumarate RS ▲USP32

Add the following:

▲USP Formoterol Resolution Mixture RS— This standard is a mixture of formoterol and formoterol fumarate impurity I. Impurity I is N-[2-hydroxy-5-[(1RS)-1-hydroxy-2-[(1SR)-2-(4-methoxyphenyl)-1-methylethyl]amino]ethyl]phenyl]formamide fumarate salt (2:1)(diastereoisomer). ▲USP32

Add the following:

▲USP Formoterol Fumarate System Suitability Mixture RS —It is a mixture of USP Formoterol Fumarate RS and formoterol related compounds A, B, C, D, E, F, G, and H.

Formoterol related compound A [1-(3-amino-4-hydroxyphenyl)-2-[[2-(4-methoxyphenyl)-1-methylethyl]amino]ethanol].

Formoterol related compound B [N-[2-hydroxy-5-[(1RS)-1-hydroxy-2-[[2-(4-methoxyphenyl)ethyl]amino]ethyl]phenyl]formamide].

Formoterol related compound C [N-[2-hydroxy-5-[(1RS)-1-hydroxy-2-[[2-(4-methoxyphenyl)-1-methylethyl]amino]ethyl]phenyl]acetamide].

Formoterol related compound D [N-[2-hydroxy-5-[(1RS)-1-hydroxy-2-[[2-(4-methoxyphenyl)-1-methylethyl]amino]ethyl]phenyl]formamide].

Formoterol related compound E [N-[2-hydroxy-5-[(1RS)-1-hydroxy-2-[[2-(4-methoxyphenyl)-1-methylethyl]amino]ethyl]phenyl]formamide].

Formoterol related compound F [N-[2-hydroxy-5-[(1RS)-1-hydroxy-2-[[2-(4-methoxyphenyl)-1-methylethyl]amino]ethyl]phenyl]amino]-2-[[2-(4-methoxyphenyl)-1-methylethyl]amino]ethyl]phenyl]formamide].

Formoterol related compound G [(2RS)-1-(4-methoxyphenyl)propan-2-amine].

Formoterol related compound H [N-[5-[(1RS)-2-[benzyl]((1RS)-2-(4-methoxyphenyl)-1-methylethyl)amino]-1-hydroxyethyl]-2-hydroxyphenyl]formamide (monobenzyl analogue)].▲USP32

USP Formononetin RS .

USP 10-Formylfolic Acid RS .

Add the following:

▲USP Foscarnet Sodium RS. ▲USP32

Add the following:

▲USP Foscarnet Related Compound B RS [disodium (ethoxyxidophosphoryl)formate] (C3H5Na2O5P  $\Delta$  198.02). ▲USP32

Add the following:

▲USP Foscarnet Related Compound D RS [O,O-diethyl ethoxycarbonylphosphonate] (C7H15O5P  $\Delta$  210.16). ▲USP32

USP Fosinopril Sodium RS.

USP Fosinopril Related Compound A RS [(4S)-4-cyclohexyl-[(4-phenylbutyl)phosphinyl]acetyl-l-proline] (C23H34NO5P  $\Delta$  435.49).

USP Fosinopril Related Compound B RS [(4S)-4-cyclohexyl-1-[(R)-[(S)-1-hydroxy-2-methylpropoxy](4-phenylbutyl)phosphinyl]acetyl-d-proline propionate (ester), hemibarium salt, sesquihydrate] (C30H45NO7P· $\frac{1}{2}$ Ba· $\frac{1}{2}$ H2O  $\Delta$  658.34).

USP Fosinopril Related Compound C RS [(4S)-4-cyclohexyl-1-[(RS)-[(RS)-1-hydroxy-2-methylpropoxy](4-phenylbutyl)phosphinyl]acetyl-l-proline propionate (ester), sodium salt]

(C30H45NNaO7P  $\Delta$  585.64).

USP Fosinopril Related Compound D RS  [(4R)-4-cyclohexyl-1-[(R)-[(S)-1-hydroxy-2-methylpropoxy](4-phenylbutyl)phosphinyl]acetyl-l-proline propionate (ester), sodium salt] (C30H45NNaO7P  $\Delta$  585.64).

USP Fosinopril Related Compound E RS  [(4S)-4-phenyl-1-[(R)-[(S)-1-hydroxy-2-methylpropoxy](4-phenylbutyl)phosphinyl]acetyl-l-proline propionate (ester), sodium salt] (C30H39NNaO7P  $\Delta$  579.60).

USP Fosinopril Related Compound F RS  [(4S)-4-cyclohexyl-1-[(R)-[(S)-1-hydroxypropoxy](4-phenylbutyl)phosphinyl]acetyl-l-proline propionate (ester), sodium salt] (C29H43NNaO7P  $\Delta$  571.62).

USP Fosinopril Related Compound G RS  [(4-phenylbutyl)phosphinylacetic acid, disodium salt] (C12H15Na2O4P  $\Delta$  300.20).

USP Fosinopril Related Compound H RS  [4-phenylbutyl phosphonic acid] (C10H15O3P  $\Delta$  214.20).

USP Fosphenytoin Sodium RS .

USP Fructose RS .

USP Fulvestrant RS. .

USP Fulvestrant System Suitability Mixture RS —Contains fulvestrant isomer A, fulvestrant isomer B, and fulvestrant  $\beta$ -isomer.

USP Fumaric Acid RS .

USP Furazolidone RS .

USP Furosemide RS .

USP Furosemide Related Compound A RS  [2-chloro-4-N-furylaminomethyl-5-sulfamoylbenzoic acid] (C12H11ClN2O5S  $\Delta$  330.74).

USP Furosemide Related Compound B RS  [4-chloro-5-sulfamoylthiophene-2-carboxylic acid] (C7H7ClN2O4S  $\Delta$  250.66).

USP Gabapentin RS .

USP Gabapentin Related Compound A RS  [2-aza-spiro[4.5]decan-3-one] (C9H15NO  $\Delta$  153.22).

USP Gabapentin Related Compound B RS  [(1-cyano-cyclohexyl)-acetic acid] (C9H13NO2  $\Delta$  167.21).

USP Gabapentin Related Compound D RS  [(1-(3-oxo-2-aza-spiro[4.5]dec-2-ylmethyl)-cyclohexyl)-acetic acid] (C18H29NO3  $\Delta$  307.43).

USP Gabapentin Related Compound E RS  [carboxymethyl-cyclohexanecarboxylic acid] (C9H14O4  $\Delta$  186.21).

USP Gadodiamide RS .

USP Gadodiamide Related Compound A RS  [gadolinium sodium diethylenetriamine pentaacetic acid monomethylamide] (C15H22GdN4NaO9  $\Delta$  582.60).

USP Gadodiamide Related Compound B RS  [gadolinium disodium diethylenetriamine pentaacetic acid] (C14H18GdN3Na2O10  $\Delta$  591.54).

USP Gadopentetate Monomeglumine RS. .

USP Gadoteridol RS. .

USP Gadoteridol Related Compound A RS  [10-(2-hydroxypropyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid] (C17H32N4O7  $\Delta$  404.46).

USP Gadoteridol Related Compound B RS  [1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid gadolinium salt].

USP Gadoteridol Related Compound C RS  [1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid].

USP Gadoversetamide RS .



USP Gadoversetamide Related Compound A RS [hydrogen [8, 11,14-tris(carboxymethyl)-6-oxo-2-oxa-5,8,11,14-tetraazahexadecan-16-oato(4-)]gadolinium].

USP Galactitol RS [dulcitol] (C6H14O6 ₩ 182.17) .

USP Galactose RS .

USP Galantamine Hydrobromide RS. [

USP Galantamine Hydrobromide Related Compounds Mixture RS— Contains galantamine hydrobromide, 6 $\beta$ -hexahydrogalantamine, 6 $\beta$ -octahydrogalantamine, 6 $\alpha$ -hexahydrogalantamine, and tetrahydrogalantamine.

USP Galantamine Hydrobromide Racemic RS— A 50:50 mixture of 4S,8S and 4R,8R isomers.

USP Gallamine Triethiodide RS .

USP Ganciclovir RS .

USP Ganciclovir Related Compound A RS [(RS)-2-amino-9-(2,3-dihydroxy-propoxymethyl)-1,9-dihydro-purin-6-one] .

USP Gemcitabine Hydrochloride RS .

USP Gemfibrozil RS .

USP Gemfibrozil Related Compound A RS [2,2-dimethyl-5-[2,5-dimethyl-4-(propene-1-yl)phenoxy]valeric acid] (C18H26O3 ₩ 290.40) .

Add the following:

▲USP Genistein RS ▲USP32

Add the following:

▲USP Genistin RS ▲USP32

USP Gentamicin Sulfate RS .

USP Gentian Violet RS .

USP Ginger Constituent Mixture RS.

USP Powdered Ginger RS .

USP Powdered Ginkgo Extract RS.

USP Ginkgolic Acids RS.

USP Ginkgo Terpene Lactones RS.

USP Powdered American Ginseng Extract RS.

USP Asian Ginseng Extract RS.

USP Powdered Asian Ginseng Extract RS .

USP Gitoxin RS (C41H64O14 ₩ 780.96) .

USP Glimepiride RS. [

USP Glimepiride Related Compound A RS [glimepiride cis-isomer].

USP Glimepiride Related Compound B RS [glimepiride sulfonamide].

USP Glimepiride Related Compound C RS [glimepiride urethane].



USP Glimepiride Related Compound D RS [glimepiride 3-isomer].

USP Glipizide RS .

USP Glipizide Related Compound A RS [N-[2-[(4-aminosulfonyl)phenyl]ethyl]-5-methyl-pyrazinecarboxamide] (C14H16N4O3S  $\Delta$  320.37).

USP Glipizide Related Compound B RS [6-methyl-N-[2-(4-sulfamoylphenyl)ethyl]pyrazine-2-carboxamide] (C14H16N4O3S  $\Delta$  320.37).

USP Glipizide Related Compound C RS [1-cyclohexyl-3-[[4-[2-[(6-methylpyrazin-2-yl)carbonyl]amino]ethyl]phenyl]sulfonylurea] (C21H27N5O4S  $\Delta$  445.54).

USP Glucagon RS .

USP Glucosamine Hydrochloride RS .

USP Glutamic Acid RS .

USP Glutamine RS .

USP  $\gamma$ -Glutamyl-(S)-Alanyl-Cysteine RS .

USP Glyburide RS .

USP Glyburide Related Compound A RS [(4-[2-(5-chloro-2-methoxybenzamido)ethyl]benzenesulfonamide].

USP Glycerin RS .

USP Glyceryl Behenate RS .

USP Glyceryl Distearate RS .

USP Glyceryl Monolinoleate RS .

Add the following:

▲USP Glyceryl Monoleate 40% RS. ▲USP32

Change to read:

USP Glyceryl Monooleate ▲90%▲USP32RS. .

USP Glycine RS .

Add the following:

▲USP Glycitein RS. ▲USP32

Add the following:

▲USP Glycitin RS ▲USP32

USP Glycopyrrolate RS .

USP Glycyrrhizic Acid RS .

USP Gonadorelin Hydrochloride RS .

USP Gonadorelin Acetate RS [C55H75N17O13·xCH3COOH  $\Delta$  1182.3 (acetate free)].

USP Gonadorelin Acetate Related Compound A RS [gonadorelin free acid] (C55H74N16O14  $\Delta$  1183.3).

USP Chorionic Gonadotropin RS .

USP Goserelin RS .



USP Goserelin Related Compound A RS [4-d-Ser-goserelin].

USP Goserelin Validation Mixture RS.

USP Graftskin Reference Photomicrographs VRS.

USP Gramicidin RS.

Add the following:

▲USP Granisetron Hydrochloride RS. ▲USP32

Add the following:

▲USP Granisetron Related Compound A RS [(2-methyl-N-[(1R,3r,5S)-9-methyl-9-azabicyclo[3.3.1]non-3-yl]-2H-indazole-3-carboxamide)]. ▲USP32

Add the following:

▲USP Granisetron Related Compound B RS [(N-[(1R,3r,5S)-9-methyl-9-azabicyclo[3.3.1]non-3-yl]-1H-indazole-3-carboxamide)]. ▲USP32

Add the following:

▲USP Granisetron Related Compound E RS [((1R,3r,5S)-9-methyl-9-azabicyclo[3.3.1]nonan-3-amine, acetate salt)]. ▲USP32

USP Powdered Decaffeinated Green Tea Extract RS.

USP Griseofulvin RS

USP Griseofulvin Permeability Diameter RS.

USP Guaiacol RS

USP Guaifenesin RS

USP Guanabenz Acetate RS

USP Guanadrel Sulfate RS.

USP Guanethidine Monosulfate RS.

USP Guanfacine Hydrochloride RS.

USP Halcinonide RS

USP Haloperidol RS

USP Haloperidol Related Compound A RS [4,4'-bis[4-p-chlorophenyl)-4-hydroxypiperidino]butyrophenone] (C32H36Cl2N2O3  $\Delta$  567.56).

USP Halothane RS

USP Hemoglobin RS.

USP Heparin Sodium RS

Add the following:

•USP Heparin Sodium System Suitability RS. • (RB 18-Jun-2008)

Add the following:

•USP Heparin Sodium Identification RS. • (RB 18-Jun-2008)

USP Hetacillin RS

USP Heptane RS

USP Hexachlorophene RS



USP Hexacosanol RS.

USP 2E,4E-Hexadienoic Acid Isobutylamide RS

USP Hexylene Glycol RS

USP Hexylresorcinol RS

USP Histamine Dihydrochloride RS .

USP I-Histidine RS .

USP Homatropine Hydrobromide RS .

USP Homatropine Methylbromide RS .

USP Homopolymer Polypropylene RS .

USP Homosalate RS

USP Hyaluronidase RS .

USP Hydralazine Hydrochloride RS .

USP Hydrochlorothiazide RS

USP Hydrocodone Bitartrate RS .

USP Hydrocodone Bitartrate Related Compound A RS

USP Hydrocortisone RS

USP Hydrocortisone Acetate RS .

USP Hydrocortisone Butyrate RS .

USP Hydrocortisone Hemisuccinate RS .

USP Hydrocortisone Phosphate Triethylamine RS (C21H31O8P· C6H15N  $\Delta$  543.64) .

USP Hydrocortisone Valerate RS

USP Hydroflumethiazide RS

USP Hydromorphone Hydrochloride RS .

USP Hydroquinone RS

USP Hydroxyamphetamine Hydrobromide RS .

USP Hydroxychloroquine Sulfate RS .

USP Hydroxyprogesterone Caproate RS .

USP Hydroxypropyl Betadex RS.

USP Hydroxypropyl Cellulose RS .

USP Hydroxyurea RS

USP Hydroxyzine Hydrochloride RS .



USP Hydroxyzine Related Compound A RS [p-chlorobenzhydrylpiperazine].

USP Hydroxyzine Pamoate RS .

USP Hyoscyamine Sulfate RS .

USP Hyoscyamine Related Compound A RS [norhyoscyamine sulfate or (1R,3R,5S)-8-azabicyclo[3.2.1]oct-3-yl(2S)-3-hydroxy-2-phenylpropanoate].

USP Hypericin RS.

USP Hyperoside RS.

USP Hypromellose RS .

USP Hypromellose Phthalate RS .

USP Ibuprofen RS .

USP Idarubicin Hydrochloride RS .

USP Idoxuridine RS .

USP Ifosfamide RS .

USP Imidazole RS .

USP Imidurea RS .

USP Iminodibenzyl RS (C14H13N  $\ddagger$  195.28) .

USP Imipenem Monohydrate RS .

USP Imipramine Hydrochloride RS .

USP Inamrinone RS .

USP Inamrinone Related Compound A RS [5-carboxamide[3,4'-bipyridin]-6(1H)-one] (C11H9N3O2  $\ddagger$  215.21) .

USP Inamrinone Related Compound B RS [N-(1,6-dihydro-6-oxo-(3,4'-bipyridine)-5-yl)-2-hydroxypropanamide] (C13H13N3O3  $\ddagger$  259.3) .

USP Inamrinone Related Compound C RS [1,6-dihydro-6-oxo-(3,4'-bipyridine)-5-carbonitrile] (C11H7N3O  $\ddagger$  197.20) .

USP Indapamide RS .

USP Indotindisulfonate Sodium RS .

USP Indinavir RS .

USP Indinavir System Suitability RS .

USP Indocyanine Green RS .

USP Indomethacin RS .

USP Inositol RS.

USP Insulin RS .

USP Insulin (Beef) RS .



USP Insulin Human RS

USP Insulin (Pork) RS

USP Insulin Lispro RS

USP Iodipamide RS

USP Iodixanol RS

USP Iodixanol Related Compound A RS [5-amino-N,N'-bis(2,3-dihydroxypropyl)-2,4,6-triiodo-1,3-benzenedicarboxamide].

USP Iodixanol Related Compound C RS [5-[acetyl]3-[[3,5-bis[(2,3-dihydroxypropyl)amino]carbonyl]-2,4,6-triiodophenyl]amino]-2-hydroxypropyl]amino]N,N'-bis(2,3-dihydroxypropyl)-2,4,6-triiodo-1,3-benzenedicarboxamide].

USP Iodixanol Related Compound D RS [5-[acetyl(2-hydroxy-3-methoxypropyl)amino]-N,N'-bis(2,3-dihydroxypropyl)-2,4,6-triiodo-1,3-benzenedicarboxamide].

USP Iodixanol Related Compound E RS [5-[[3-[[2,3-dihydroxypropyl]amino]carbonyl]-5-[[amino]carbonyl]-2,4,6-triiodophenyl](acetyllimino)-2-hydroxypropyl]-(acetyllimino)-N,N'-bis(2,3-dihydroxypropyl)-2,4,6-triiodo-1,3-benzenedicarboxamide].

USP o-Iodohippuric Acid RS (C9H8INO3  $\Delta$  305.07).

USP Iodoquinol RS

USP Iohexol RS

USP Iohexol Related Compound A RS [5-(acetylamino)-N,N'-bis(2,3-dihydroxypropyl)-2,4,6-triiodo-1,3-benzenedicarboxamide].

USP Iohexol Related Compound B RS [5-amino-N,N'-bis(2,3-dihydroxypropyl)-2,4,6-triiodo-1,3-benzenedicarboxamide].

USP Iohexol Related Compound C RS [N,N'-bis(2,3-dihydroxypropyl)-5-nitro-1,3-benzenedicarboxamide].

USP Iopamidol RS

USP Iopamidol Related Compound A RS [N,N'-bis-(1,3-dihydroxy-2-propyl)-5-amino-2,4,6-triiodoisophthalamide] (C14H18I3N3O6  $\Delta$  705.03).USP Iopamidol Related Compound B RS [5-glycolamido-N,N'-bis[2-hydroxy-1-(hydroxymethyl)ethyl]-2,4,6-triiodoisophthalamide] (C16H20I3N3O7  $\Delta$  747.07).

Add the following:

▲USP Iopamidol Related Compound C RS [4-chloro-N1, N3-bis(1,3-dihydroxypropan-2-yl)-5-(S)-lactamido-2,6-diiodoisophthalamide] (C17H22ClI2N3O8  $\Delta$  685.63). ▲USP32

USP Iopromide RS

USP Iopromide Related Compound A RS

USP Iopromide Related Compound B RS [5-(acetylamino)-N,N'-bis(2,3-dihydroxypropyl)-2,4,6-triiodo-N-methyl-1,3-benzenedicarboxamide].

USP Iothalamic Acid RS

USP Ioversol RS.

USP Ioversol Related Compound A RS

USP Ioversol Related Compound B RS.

USP Ioxaglic Acid RS.

USP Ioxilan RS

USP Ioxilan Related Compound A RS [5-amino-2,4,6-triiodo-3 N-(2-hydroxyethyl)carbamoyl benzoic acid] (C10H9I3N2O4  $\Delta$  601.90).

USP Ipodate Sodium RS .

USP Ipratropium Bromide RS. 

USP Ipratropium Bromide Related Compound A RS [(1R,3r,5S,8r)-3-hydroxy-8-methyl-8-(1-methylethyl)-8-azoniabicyclo[3.2.1]octane, bromide] (C11H22BrNO  $\Delta$  264.20).

USP Ipratropium Bromide Related Compound B RS [(1R,3r,5S,8s)-3-[(2RS)-3-hydroxy-2-phenylpropanoyl]oxy]-8-methyl-8-(1-methylethyl)-8-azoniabicyclo[3.2.1]octane, bromide] (C20H30BrNO3  $\Delta$  412.36).

USP Ipratropium Bromide Related Compound C RS [(2RS)-3-hydroxy-2-phenylpropanoic acid] (C9H10O3  $\Delta$  166.17).

USP Irbesartan RS .

USP Irbesartan Related Compound A RS  [1-pentanoylamino-cyclopentanecarboxylic acid [2'-(1H-tetrazol-5-yl)-byphenyl-4-ylmethyl]-amide] (C25H30N6O2  $\Delta$  446.54).

USP Isoetharine Hydrochloride RS .

USP Isoflupredone Acetate RS .

USP Isoflurane RS .

USP Isoflurane Related Compound A RS  [1-chloro-2,2,2-trifluoroethylchlorodifluoromethyl ether] (C3HCl2F5O  $\Delta$  219.49) .

USP Isoflurane Related Compound B RS  [2,2,2-trifluoroethyldifluoromethyl ether] (C3H3F5O  $\Delta$  150.05) .

USP I-Isoleucine RS .

USP Isomalathion RS  (C10H19O6PS2  $\Delta$  330.37) .

USP Isometheptene Mucate RS. 

USP Isoniazid RS .

USP Isopropamide Iodide RS .

USP Isopropyl Myristate RS .

USP Isopropyl Palmitate RS .

USP Isoproterenol Hydrochloride RS .

USP Isopterochine RS .

USP Isomalt RS.

USP Isosorbide RS .

USP Diluted Isosorbide Dinitrate RS .

USP Diluted Isosorbide Mononitrate RS. 

USP Diluted Isosorbide Mononitrate Related Compound A RS  [1,4:3,5-dianhydro-d-glucitol 2-nitrate] (C6H9NO6  $\Delta$  191.14).

USP Isotretinoin RS .

USP Isoxsuprine Hydrochloride RS .

USP Isradipine RS .

USP Isradipine Related Compound A RS  [isopropyl methyl 4-(4-benzofurazanyl)-2,6-dimethyl-3,5-pyridinedicarboxylate] (C19H19 N3O5  $\Delta$  369.38) .



USP Ivermectin RS .

USP Kanamycin Sulfate RS .

USP Ketamine Hydrochloride RS .

USP Ketamine Related Compound A RS [1-[(2-chlorophenyl)(methylimino)methyl]cyclopentanol] (C13H16NOCl  $\Delta$  237.73) .

USP Ketoconazole RS .

USP Ketoprofen RS .

USP Ketorolac Tromethamine RS .

USP Labetalol Hydrochloride RS .

USP Lactase RS .

USP Lactitol RS.

USP Anhydrous Lactose RS .

USP Lactose Monohydrate RS .

USP Lactulose RS .

USP Lamivudine RS .

USP Lamivudine Resolution Mixture A RS .

USP Lamivudine Resolution Mixture B RS .

USP Lanolin RS .

USP Lanolin Alcohols RS .

USP Lansoprazole RS (C16H14F3N3O2S  $\Delta$  369.36) .

USP Lansoprazole Related Compound A RS [2-[[3-methyl-4-(2,2,2-trifluoroethoxy)-2-pyridyl]methyl]sulfonyl]benzimidazole] (C16H14F3N3O3S  $\Delta$  385.36) .

USP Lauroyl Polyoxylglycerides RS .

USP Leflunomide RS .

USP Leflunomide Related Compound A RS.

USP Leflunomide Related Compound B RS.

USP Leflunomide Related Compound C RS.

USP Letrozole RS .

USP Letrozole Related Compound A RS [4,4'-(1H-1,3,4-triazol-1-ylmethylene)dibenzonitrile] (C17H11N5  $\Delta$  285.31) .

USP L-Leucine RS .

USP Leucovorin Calcium RS .

USP Leuprolide Acetate RS.



USP Levalbuterol Hydrochloride RS [((R)-1-[(tert-butylamino)methyl]-4-hydroxy-m-xylene-1,2-diol hydrochloride].

USP Levalbuterol Related Compound A RS [4-(2-tert-butylamino-ethyl)-2-hydroxymethyl-phenol].

USP Levalbuterol Related Compound B RS [((1,1-dimethylethyl)amino)methyl]-4-hydroxy-3-methyl-benzenemethanol].

USP Levalbuterol Related Compound C RS [((1,1-dimethylethyl)amino)methyl]-4-hydroxy-3-(methoxymethyl)-benzenemethanol].

USP Levalbuterol Related Compound D RS [5-[2-((1,1-dimethylethyl)amino)-1-hydroxyethyl]-2-hydroxy-benzaldehyde].

USP Levalbuterol Related Compound E RS [((1,1-dimethylethyl)amino)methyl]-3-(ethoxymethyl)-4-hydroxy-benzenemethanol].

USP Levalbuterol Related Compound F RS [((1,1-dimethylethyl)amino)methyl]-4-(phenylmethoxy)-1,3-benzenedimethanol].

USP Levalbuterol Related Compound G RS [((1,1-dimethylethyl)amino)methyl]-4,5-dihydroxy-1,3-benzenedimethanol].

USP Levamisole Hydrochloride RS .

USP Levmetamfetamine RS .

USP Levobunolol Hydrochloride RS .

USP Levocabastine Hydrochloride RS .

USP Levocabastine Related Compound A RS.

USP Levocarnitine RS .

USP Levocarnitine Related Compound A RS [2-propen-1-aminium, 3-carboxy-N,N,N-trimethyl-, chloride] (C7H14ClNO2 179.65) .

USP Levodopa RS .

USP Levodopa Related Compound A RS [3-(3,4,6-trihydroxyphenyl)alanine] (C9H11NO5 213.19) .

USP Levodopa Related Compound B RS [3-methoxytyrosine] (C10H13NO4 211.22) .

USP Levonordefrin RS .

USP Levorphanol Tartrate RS .

USP Levothyroxine RS .

USP Lidocaine RS .

USP Lincomycin Hydrochloride RS .

USP Lindane RS .

USP Linoleoyl Polyoxylglycerides RS .

USP Liothryronine RS .

USP Alpha Lipoic Acid RS. .

USP Lisinopril RS .

Add the following:

▲USP Lisinopril Related Compound A RS [ (S)-2-((3S,8aS)-3-(4-aminobutyl)-1,4-dioxohexahydropyrrolo[1,2-a]pyrazin-2(1H)-yl)-4-phenylbutanoic acid] (C21H29N3O4 387.47)  
▲USP32

USP Lithium Carbonate RS .



USP Loperamide Hydrochloride RS .

USP Loracarbef RS

USP Loracarbef I-Isomer RS .

USP Loratadine RS

USP Loratadine Related Compound A RS [8-chloro-6,11-dihydro-11(4-piperidylidene)-5H-benzo[5,6]cyclohepta[1,2-b] pyridine] (C19H19ClN2  $\Delta$  310.83) .

USP Loratadine Related Compound B RS [8-chloro-6,11-dihydro-11(N-methyl-4-piperinylidene)-5H-benzo[5,6]cyclohepta[1,2-b] pyridine] (C20H21ClN2  $\Delta$  324.88) .

USP Lorazepam RS

USP Lorazepam Related Compound A RS [7-chloro-5-(o-chlorophenyl)-1,3-dihydro-3-acetoxy-2H-1,4-benzodiazepin-2-one] (C17H12 Cl2N2O3  $\Delta$  363.20) .

USP Lorazepam Related Compound B RS [2-amino-2',5-dichlorobenzophenone] (C13H9Cl2NO  $\Delta$  266.13) .

USP Lorazepam Related Compound C RS [6-chloro-4-(o-chlorophenyl)-2-quinazoliniccarboxaldehyde] (C15H8Cl2N2O  $\Delta$  303.15) .

USP Lorazepam Related Compound D RS [6-chloro-4-(o-chlorophenyl)-2-quinazoliniccarboxylic acid] (C15H8Cl2N2O2  $\Delta$  319.15) .

USP Lorazepam Related Compound E RS [6-chloro-4-(o-chlorophenyl)-2-quinazoline methanol] (C15H10Cl2N2O  $\Delta$  305.16) .

USP Lovastatin RS

USP Lovastatin Related Compound A RS [[dihydro-lovastatin] [butanoic acid, 2-methyl-, 1,2,3,4,4a,7,8,8a-octahydro-3,7-dimethyl-8-[2(tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl)-ethyl]-1-naphthalenyl ester, [1S-[1 $\alpha$ (R\*),3 $\alpha$ ,7 $\beta$ ,8 $\beta$ (2S\*,4S\*),-8 $\alpha$  $\beta$ ]]-] (C24 H38O5  $\Delta$  406.56) .

USP Loxapine Succinate RS .

USP Lutein RS .

USP Lycopene RS .

USP Lynestrenol RS [(17 $\alpha$ )-19-Norpregn-4-en-20-yn-17-ol] (C20H28O  $\Delta$  284.42) .

USP I-Lysine Acetate RS

USP I-Lysine Hydrochloride RS .

USP Mafenide Acetate RS

USP Mafenide Related Compound A RS [4-formylbenzenesulfonamide] (C7H7NO3S  $\Delta$  185.20) .

USP Magaldrate RS .

USP Magnesium Salicylate RS .

USP Malathion RS

USP Maleic Acid RS

USP Malic Acid RS

USP Maltitol RS

USP Maltol RS.

USP Maltose RS .

USP Maltose Monohydrate RS .

USP Mangafodipir Trisodium RS  [manganese(II) dipyridoxal diphosphate].

USP Mangafodipir Related Compound A RS  [manganese(II) dipyridoxal monophosphate sodium salt].

USP Mangafodipir Related Compound B RS [manganese(II) dipyridoxal diphosphate mono overalkylated sodium salt].

USP Mangafodipir Related Compound C RS  [manganese(II) dipyridoxal diphosphate sodium salt].

USP Mannitol RS .

USP Maprotiline Hydrochloride RS .

USP Maritime Pine Extract RS .

USP Mazindol RS .

USP Mebendazole RS .

USP Mebrofenin RS .

USP Mecamylamine Hydrochloride RS .

USP Mecamylamine Related Compound A RS  [N,1,7,7-tetramethyl bicyclo [2.2.1] heptan-2-amine] (C<sub>11</sub>H<sub>21</sub>N  $\Delta$  167.29).

USP Mechlorethamine Hydrochloride RS .

USP Medazine Hydrochloride RS .

USP Meclocycline Sulfosalicylate RS .

USP Meclofenamate Sodium RS .

USP Medroxyprogesterone Acetate RS .

USP Medroxyprogesterone Acetate Related Compound A RS  [4,5 $\beta$ -dihydromedroxyprogesterone acetate] (C<sub>24</sub>H<sub>36</sub>O<sub>4</sub>  $\Delta$  388.54) .

USP Mefenamic Acid RS .

USP Mefloquine Hydrochloride RS. .

USP Mefloquine Related Compound A RS  [threo-mefloquine].

USP Megestrol Acetate RS .

USP Melengestrol Acetate RS.

USP Melengestrol Acetate Related Compound A RS  [16-methylene-17 $\alpha$ -hydroxy-4-pregnene-3,20-dione 17-acetate] .

USP Melengestrol Acetate Related Compound B RS  [17 $\alpha$ -hydroxy-6,16-dimethyleneproga-4-ene-3,20-dione 17-acetate] .

USP Meloxicam RS. .

USP Meloxicam Related Compound A RS  [4-hydroxy-2-methyl-2H-1,2-benzothiazine-3-carboxylic acid ethylester 1,1-dioxide].

USP Meloxicam Related Compound B RS  [2-amino-5-methyl-thiazole].



USP Meloxicam Related Compound C RS [isopropyl-4-hydroxy-2-methyl-2H-1,2-benzothiazine-3-carboxylate-1,1-dioxide].

USP Meloxicam Related Compound D RS [4-methoxy-2-methyl-N-(5-methyl-1,3-thiazole-2-yl)-2H-1,2-benzothiazine-3-carboxamide-1,1-dioxide].

USP Melphalan Hydrochloride RS .

USP Menadione RS .

USP Menotropins RS.

USP Menthol RS .

USP Meperidine Hydrochloride RS .

USP Mephobarbital RS .

USP Mepivacaine Hydrochloride RS .

USP Meprednisone RS .

USP Meprobamate RS .

USP Meradimate RS.

USP 3-Mercapto-2-methylpropanoic Acid 1,2-Diphenylethylamine Salt RS (C<sub>4</sub>H<sub>7</sub>O<sub>2</sub>S·C<sub>14</sub>H<sub>16</sub>N  $\Delta$  317.45) .

USP Mercaptopurine RS .

USP Meropenem RS .

USP Mesalamine RS .

USP Mesoridazine Besylate RS .

USP Mestranol RS .

USP Metaproterenol Sulfate RS .

USP Metaraminol Bitartrate RS .

USP Metformin Hydrochloride RS .

USP Metformin Related Compound A RS [1-cyanoguanidine] .

USP Metformin Related Compound B RS [1-methylbiguanide] (C<sub>3</sub>H<sub>9</sub>N<sub>5</sub>  $\Delta$  115.14) .

USP Metformin Related Compound C RS [dimethylmelamine, or N,N-dimethyl-[1,3,5]triazine-2,4,6-triamine] (C<sub>5</sub>H<sub>10</sub>N<sub>6</sub>  $\Delta$  154.17) .

USP Methacrylic Acid Copolymer, Type A RS .

USP Methacrylic Acid Copolymer, Type B RS .

USP Methacrylic Acid Copolymer, Type C RS .

USP Methacycline Hydrochloride RS .

USP Methadone Hydrochloride RS .

USP Methamphetamine Hydrochloride RS .

USP Methazolamide RS .

USP Methdilazine Hydrochloride RS .

USP Methenamine RS .

USP Methenamine Hippurate RS .

USP Methenamine Mandelate RS .

USP Methicillin Sodium RS .

USP Methimazole RS .

USP L-Methionine RS .

USP Methocarbamol RS .

USP Methohexitol RS .

USP Methotrexate RS .

USP Methotriprazine RS .

USP Methoxsalen RS .

USP Methoxyflurane RS .

USP Methoxymethylguanine RS .

USP Methscopolamine Bromide RS .

USP Methylsuximide RS .

USP Methylclothiazide RS .

USP Methylclothiazide Related Compound A RS  [4-amino-6-chloro-N3-methyl-m-benzenedisulfonamide] (C7H10ClN3O4S2  $\text{FW} 299.76$ ) .

USP Methyl Alcohol RS. .

USP Methyl Caprate RS .

USP Methyl Caproate RS .

USP Methyl Caprylate RS .

USP Methyl Laurate RS .

USP Methyl Linoleate RS .

USP Methyl Linolenate RS .

USP Methyl 5-Methyl-3-isoxazolecarboxylate RS .

USP Methyl Myristate RS .

USP Methyl Oleate RS .

USP Methyl Palmitate RS .



USP Methyl Palmitoleate RS

USP Methyl Stearate RS

USP Methyl Tricosanoate RS [tricosanoic acid methyl ester] (C<sub>24</sub>H<sub>48</sub>O<sub>2</sub>  $\Delta$  368.64).

USP Methyldopa RS .

USP Methyldopate Hydrochloride RS .

USP Methylene Blue RS .

USP Methylergonovine Maleate RS .

USP 3-O-Methylmethyldopa RS (C<sub>11</sub>H<sub>15</sub>NO<sub>4</sub>  $\Delta$  225.25) .

USP Methylparaben RS

USP Methylphenidate Hydrochloride RS .

USP Methylphenidate Hydrochloride Erythro Isomer Solution RS This solution contains 0.5 mg of methylphenidate hydrochloride erythro isomer per mL in methanol.

USP Methylphenidate Related Compound A RS [ $\alpha$ -phenyl-2-piperidineacetic acid hydrochloride] (C<sub>13</sub>H<sub>17</sub>NO<sub>2</sub>·HCl  $\Delta$  255.75) .

USP Methylprednisolone RS

USP Methylprednisolone Acetate RS .

USP Methylprednisolone Hemisuccinate RS .

USP Methylsulfonylmethane RS [dimethyl sulfone] (C<sub>2</sub>H<sub>6</sub>O<sub>2</sub>S  $\Delta$  94.13).

USP Methyltestosterone RS

USP Methysergide Maleate RS .

USP Metoclopramide Hydrochloride RS .

USP Metolazone RS

USP Metoprolol Fumarate RS

USP Metoprolol Related Compound A RS [( $\pm$ )1-ethylamino-3-[4-(2-methoxyethyl)phenoxy]-propan-2-ol] (C<sub>14</sub>H<sub>23</sub>NO<sub>3</sub>  $\Delta$  253.34) .

USP Metoprolol Related Compound B RS [( $\pm$ )1-chloro-2-hydroxy-3-[4-(2-methoxyethyl)phenoxy]-propane] (C<sub>12</sub>H<sub>17</sub>ClO<sub>3</sub>  $\Delta$  244.71) .

USP Metoprolol Related Compound C RS [( $\pm$ )4-[2-hydroxy-3-(1-methylethyl)aminopropoxy]benzaldehyde] (C<sub>13</sub>H<sub>19</sub>NO<sub>3</sub>  $\Delta$  237.29) .

USP Metoprolol Related Compound D RS [( $\pm$ ) N,N-bis[2-hydroxy-3-[4-(2-methoxyethyl)phenoxy]propyl](1-methylethyl)amine] (C<sub>27</sub>H<sub>41</sub>NO<sub>6</sub>  $\Delta$  475.62) .

USP Metoprolol Succinate RS

USP Metoprolol Tartrate RS .

USP Metrifonate RS [trichlorfon] .

USP Metronidazole RS .

USP Metronidazole Benzoate RS



USP Metyrapone RS

USP Metyrosine RS

USP Mexiletine Hydrochloride RS .

USP Mezlocillin Sodium RS

USP Mibolerone RS

USP Miconazole RS

USP Miconazole Nitrate RS .

USP Powdered Milk Thistle Extract RS .

USP Milrinone RS

USP Milrinone Related Compound A RS [1,6-dihydro-2-methyl-6-oxo-(3,4'-bipyridine)-5-carboxamide] (C<sub>12</sub>H<sub>11</sub>N<sub>3</sub>O<sub>2</sub> ♦ 229.23) .

USP Mineral Oil RS .

USP Minocycline Hydrochloride RS .

USP Minoxidil RS

USP Mirtazapine RS .

Add the following:

▲USP Mirtazapine Related Compound A RS [1,2,3,4,10,14b-hexahydropyrazino[2,1-a]pyrido[2,3-c][2]benzazepine (C<sub>16</sub>H<sub>17</sub>N<sub>3</sub> ♦ 251.33). ▲USP32

USP Mitomycin RS

USP Mitotane RS

USP Mitoxantrone Hydrochloride RS .

USP Mitoxantrone System Suitability Mixture RS [9,10-antracenedione, 8-amino-1,4-dihydroxy-5-[2-[(2-hydroxyethyl)amino]ethyl]amino]-, hydrochloride] (C<sub>18</sub>H<sub>19</sub>N<sub>3</sub>O<sub>5</sub>·HCl ♦ 393.83) .

USP Modafinil RS.

USP Molindone Hydrochloride RS .

USP Mometasone Furoate RS

USP Monensin Sodium RS.

USP Monobenzone RS

USP Monoethanolamine RS

USP Monoglycerides RS .

USP Monostearyl Maleate RS

USP Morantel Tartrate RS .

USP Moricizine Hydrochloride RS

USP Morphine Sulfate RS .

USP Mupirocin RS

USP Mupirocin Lithium RS

USP Mycophenolate Mofetil RS.

USP Mycophenolate Mofetil Related Compound A RS [2-morpholinoethyl (E)-6-(1,3-dihydro-4,6-dihydroxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-methyl-4-hexenoate] (C23H31NO7  $\Delta$  419.47).

USP Mycophenolate Mofetil Related Compound B RS [(RS)-7-hydroxy-5-methoxy-4-methyl-6-[2-(5-methyl-2-oxo-tetrahydrofuran-5-yl)ethyl]-3H-isobenzofuranyl-1-one] (C17H20O6  $\Delta$  320.34).

USP Nabumetone RS

USP Nabumetone Related Compound A RS [1-(6-methoxy-2-naphthyl)-but-1-en-3-one] (C15H14O2  $\Delta$  226.27).

USP Nadolol RS

USP Nafcillin Sodium RS .

USP Naftifine Hydrochloride RS .

USP Nalidixic Acid RS

USP Nalorphine Hydrochloride RS .

USP Naloxone RS .

USP Naltrexone RS .

USP Naltrexone Related Compound A RS [N-(3-butenyl)-noroxymorphone hydrochloride] (C20H23NO4·HCl  $\Delta$  377.87).

USP Nandrolone RS .

USP Nandrolone Decanoate RS

USP Nandrolone Phenpropionate RS .

USP Naphazoline Hydrochloride RS .

USP Naphthalene RS .

USP Naproxen RS

USP Naproxen Sodium RS .

USP Narasin RS.

USP Naratriptan Hydrochloride RS

USP Naratriptan Related Compound A RS [3-(1-methylpiperidin-4-yl)-1H-indole hydrochloride] (C14H18N2·HCl  $\Delta$  250.8).

USP Naratriptan Related Compound B RS [2-[3-(1-methyl-1,2,3,6-tetrahydropyridin-4-yl)-1H-indol-5-yl]ethanesulfonic acid methylamide oxalate] (C17H23N3O2S·C2H2O4  $\Delta$  423.5).

USP Naratriptan Resolution Mixture RS— A mixture of naratriptan hydrochloride with approximately 0.1% each of naratriptan related compound A [3-(1-methylpiperidin-4-yl)-1H-indole hydrochloride] and naratriptan related compound B [2-[3-(1-methyl-1,2,3,6-tetrahydropyridin-4-yl)-1H-indol-5-yl]ethanesulfonic acid methylamide oxalate].

USP Natamycin RS

USP Near-Infrared Calibrator RS.

USP Nefazodone Hydrochloride RS. 

USP Nefazodone Related Compound A RS  [1-(3-chloropropyl)-4-(chlorophenyl)piperazine] (C13H18Cl2N2  $\Delta$  273.20).

USP Nefazodone Related Compound B RS  [2-(3-(4-(chlorophenyl)-1-piperazinyl)propyl)-5-ethyl-2,4-dihydro-4-(2-phenoxyethyl)-3H-1,2,4-triazol-3-one] (C25H32ClN5O2  $\Delta$  470.01).

USP Neomycin Sulfate RS.

USP Neostigmine Bromide RS.

USP Neostigmine Methylsulfate RS.

USP Neotame RS.

USP Neotame Related Compound A RS  [N-[3,3-dimethylbutyl]-L-aspartyl]-L-phenylalanine].

USP Netilmicin Sulfate RS.

USP Nevirapine Anhydrous RS .

USP Nevirapine Hemihydrate RS .

USP Nevirapine Related Compound A RS  [5,11-dihydro-6H-11-ethyl-4-methyl-dipyrido[3,2-b:2',3'-e][1,4]diazepin-6-one] (C14H14N4O  $\Delta$  254.29).

USP Nevirapine Related Compound B RS  [5,11-dihydro-4-methyl-6H-dipyrido[3,2-b:2',3'-e][1,4]diazepin-6-one] (C12H10N4O  $\Delta$  226.23).

USP Niacin RS .

USP Niacinamide RS .

USP Nicotine Bitartrate Dihydrate RS .

USP Nifedipine RS .

USP Nifedipine Nitrophenylpyridine Analog RS  [dimethyl 4-(2-nitrophenyl)-2,6-dimethylpyridine-3,5-dicarboxylate] (C17H16N2O6  $\Delta$  344.33).

USP Nifedipine Nitrosophenylpyridine Analog RS  [dimethyl 4-(2-nitrosophenyl)-2,6-dimethylpyridine-3,5-dicarboxylate] (C17H16N2O5  $\Delta$  328.33).

USP Nimodipine RS .

USP Nimodipine Related Compound A RS  [(2-methoxyethyl-1-methylethyl)-2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate] (C21H24N2O7  $\Delta$  416.42).

USP Nitrofurantoin RS.

USP Nitrofurantoin Related Compound A RS  [N-(aminocarbonyl)-N-((5-nitro-2-furanyl)methylene)-amino]glycine].

USP Nitrofurazone RS .

USP Nitrofurazone Related Compound A RS  [5-nitro-2-furfuraldazine] (C10H6N4O6  $\Delta$  278.18).

USP Nitrofurfural Diacetate RS  (C9H9NO7  $\Delta$  243.17).

USP Nitrogen RS.

USP Diluted Nitroglycerin RS .

USP Nitrous Oxide RS.

USP Nizatidine RS .

USP Nonoxynol 9 RS .

USP Nordazepam RS  [7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one] (C<sub>15</sub>H<sub>11</sub>CIN<sub>2</sub>O  $\Delta$  270.72).

USP Norepinephrine Bitartrate RS .

USP Norethindrone RS .

USP Norethindrone Acetate RS .

USP Norethynodrel RS .

USP Norfloxacin RS .

USP Norgestimate RS .

USP Norgestimate Related Compound A RS. .

USP Norgestimate Oxime Mixture RS  [mixture of syn-17-deacetyl norgestimate and anti-17-deacetyl norgestimate].

USP Norgestrel RS .

USP Noroxymorphone Hydrochloride RS .

USP Norphenylephrine Hydrochloride RS. .

USP Nortriptyline Hydrochloride RS .

USP Noscapine RS .

USP Novobiocin RS .

USP Nystatin RS .

USP Octinoxate RS  [octyl methoxycinnamate].

USP Octisalate RS  [octyl salicylate].

USP Octocrylene RS .

USP Octoxynol 9 RS .

USP Octyldodecanol RS .

USP Ofloxacin RS .

USP Ofloxacin Related Compound A RS  [9-fluoro-3-methyl-7-oxo-10-(piperazin-1-yl)-2,3-dihydro-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid].

USP Oleic Acid RS. .

USP Oleoyl Polyoxylglycerides RS .

USP Oleyl Oleate RS. .

USP Omeprazole RS .

USP Omeprazole Magnesium RS. .



USP Omeprazole Related Compound A RS [(omeprazole sulfone, 5-methoxy-2-[[[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulfonyl]-1H-benzimidazole)] (C17H19N3O4S  $\diamond$  361.42  
 $\diamond$  CAS-88546-55-8).

USP Ondansetron Hydrochloride RS

USP Ondansetron Related Compound A RS [3[(dimethylamino)methyl]-1,2,3,9-tetrahydro-9-methyl-4H-carbazol-4-one].

USP Ondansetron Related Compound B RS [6,6'-methylene bis-[(1,2,3,9-tetrahydro-9-methyl-3-[(2-methyl-1H-imidazol-1-yl)-methyl]-4H-carbazol-4-one].

USP Ondansetron Related Compound C RS [1,2,3,9-tetrahydro-9-methyl-4H-carbazol-4-one].

USP Ondansetron Related Compound D RS [1,2,3,9-tetrahydro-9-methyl-3-methylene-4H-carbazol-4-one].

USP Ondansetron Resolution Mixture RS— Ondansetron hydrochloride having approximately 0.4% w/w of both ondansetron related compound A and 6,6'-methylene bis-[(1,2,3,9-tetrahydro-9-methyl-3-[(2-methyl-1H-imidazol-1-yl)-methyl]-4H-carbazol-4-one)].

USP Orphenadrine Citrate RS .

USP Oxacillin Sodium RS .

USP Oxandrolone RS

USP Oxandrolone Related Compound A RS [(7,8-didehydro-oxandrolone) or (17 $\beta$ -hydroxy-17 $\alpha$ -methyl-2-oxa-5 $\alpha$ -androst-7-en-3-one)].

USP Oxandrolone Related Compound B RS [(anhydro-oxandrolone) or (17 $\beta$ -17-dimethyl-2-oxa-18-nor-5 $\alpha$ -androstan-3-one)].

USP Oxandrolone Related Compound C RS [anhydro-oxandrolone (17,17-dimethyl-2-oxa-18-nor-5 $\alpha$ -androstan-3-one)].

USP Oxaprozin RS

USP Oxazepam RS

USP Oxfendazole RS

USP Oxprenolol Hydrochloride RS .

USP Oxtipryphline RS .

USP Oxybenzone RS

USP Oxybutynin Chloride RS

USP Oxybutynin Related Compound A RS [phenylcyclohexylglycolic acid] (C14H18O3  $\diamond$  234.30) .

USP Oxybutynin Related Compound B RS [methyl ester of phenylcyclohexylglycolic acid, or CHMME (cyclohexyl mandelic acid methyl ester)] .

USP Oxybutynin Related Compound C RS [methylethyl analog of oxybutynin chloride, or (4-(ethylmethylamino) but-2-ynyl ( $\pm$ ) 2-cyclohexyl-2-hydroxy-2-phenylacetate hydrochloride)] .

USP Oxycodone RS

USP Oxygen–Helium RS.

USP Oxymetazoline Hydrochloride RS

USP Oxymetholone RS

USP Oxymorphone RS

USP Oxyquinoline Sulfate RS .



USP Oxytetracycline RS .

USP Oxytocin RS .

USP Paclitaxel RS .

USP Paclitaxel Related Compound A RS  [cephalomannine] .

USP Paclitaxel Related Compound B RS  [10-deacetyl-7-epipaclitaxel] .

USP Paclitaxel Impurity Mixture RS— Mixture of paclitaxel and the following related compounds: propyl analog, cephalomannine, sec-butyl analog, n-butyl analog, benzyl analog, baccatin VI, pentyl analog, and 7-epipaclitaxel.

USP Padimate O RS .

USP Palmitic Acid RS .

USP Pamidronate Disodium RS. .

USP Pamoic Acid RS  (C<sub>23</sub>H<sub>16</sub>O<sub>6</sub>  $\Delta$  388.38) .

USP Pancreatin Amylase and Protease RS .

USP Pancreatin Lipase RS .

USP Pancuronium Bromide RS  [3 $\alpha$ ,17 $\beta$ -dihydroxy-2 $\beta$ ,16 $\beta$ -dipiperidinyl-5 $\alpha$ -androstane, 3,17-diacetate, dimethobromide] (C<sub>35</sub>H<sub>60</sub>Br<sub>2</sub>N<sub>2</sub>O<sub>4</sub>  $\Delta$  732.67).

USP Racemic Panthenol RS .

USP Pantolactone RS .

Add the following:

•USP Pantoprazole Sodium RS . • (RB 18-Jun-2008)

Add the following:

•USP Pantoprazole Related Compound A RS [5-(difluoromethoxy)-2-[(3,4-dimethoxy-2-pyridinyl)methyl]sulfonyl]-1H-benzimidazole] (C<sub>16</sub>H<sub>15</sub>F<sub>2</sub>N<sub>3</sub>O<sub>5</sub>S  $\Delta$  399.37). • (RB 18-Jun-2008)

Add the following:

•USP Pantoprazole Related Compound B RS [5-(difluoromethoxy)-2-[(3,4-dimethoxy-2-pyridinyl)methyl]thio]-1H-benzimidazole] (C<sub>16</sub>H<sub>15</sub>F<sub>2</sub>N<sub>3</sub>O<sub>3</sub>S  $\Delta$  367.37). • (RB 18-Jun-2008)

Add the following:

•USP Pantoprazole Related Compound C RS [5-(difluoromethoxy)-1H-benzimidazole-2-thiol] (C<sub>8</sub>H<sub>6</sub>F<sub>2</sub>N<sub>2</sub>O<sub>2</sub>S  $\Delta$  216.21). • (RB 18-Jun-2008)

Add the following:

•USP Pantoprazole Related Compound D and F Mixture RS— A mixture of 5-(difluoromethoxy)-2-[(RS)-[(3,4-dimethoxypyridin-2-yl)methyl]sulfinyl]-1-methyl-1H-benzimidazole and 6-(difluoromethoxy)-2-[(RS)-[(3,4-dimethoxypyridin-2-yl)methyl]sulfinyl]-1-methyl-1H-benzimidazole (C<sub>17</sub>H<sub>17</sub>F<sub>2</sub>N<sub>3</sub>O<sub>4</sub>S  $\Delta$  398.40). • (RB 18-Jun-2008)

Add the following:

•USP Pantoprazole Related Compound E RS— A mixture of the stereoisomers of 6,6'-bis(difluoromethoxy)-2,2'-bis[(3,4-dimethoxypyridin-2-yl)methyl]sulfinyl]-1H,1'H-5,5'-bibenzimidazolyl (C<sub>32</sub>H<sub>28</sub>F<sub>4</sub>N<sub>6</sub>O<sub>8</sub>S<sub>2</sub>  $\Delta$  764.74). • (RB 18-Jun-2008)

USP Papain RS .

USP Papaverine Hydrochloride RS .

USP Parachlorophenol RS. .

USP Paramethasone Acetate RS .

USP Paraffin RS.



USP Parbendazole RS

USP Paricalcitol RS.

USP Paricalcitol Solution RS.

USP Paromomycin Sulfate RS.

USP Paroxetine Hydrochloride RS .

USP Paroxetine Related Compound B RS [trans-4-phenyl-3-[(3,4-methylenedioxy)phenoxy]methylpiperidine hydrochloride].

USP Paroxetine Related Compound C RS [(+)-trans-paroxetine hydrochloride].

USP Paroxetine Related Compound E Mixture RS [paroxetine hydrochloride spiked with 1-methyl-4-(p-fluorophenyl)-1,2,3,6-tetrahydropyridine].

USP Paroxetine Related Compound F RS [trans-(−)-1-methyl-3-[1,3-benzodioxol-5-yloxy)methyl]-4-(fluorophenyl)piperidine] (C<sub>20</sub>H<sub>22</sub>FNO<sub>3</sub> 343.39).

USP Paroxetine Related Compound G RS [(±)trans-3-[(1,3-benzodioxol-5-yloxy)methyl]-4-(4'-fluorophenyl-4'-phenyl)piperidine hydrochloride] (C<sub>25</sub>H<sub>24</sub>FNO<sub>3</sub> 405.46).

USP Paroxetine System Suitability Mixture A RS —Mixture of approximately 1% paroxetine related compound A [piperidine, 3-[(1,3-benzodioxol-5-yloxy)methyl]-4-(4-methoxyphenyl)-hydrochloride (3 S-trans); and 1% of paroxetine related compound B [piperidine, 3-[(1,3-benzodioxol-5-yloxy)methyl]-4-phenyl-, hydrochloride (3 S-trans)]] in a matrix of paroxetine hydrochloride.

USP Parthenolide RS.

USP Particle Count RS (2 blanks and 2 suspensions).

USP Penbutolol Sulfate RS.

USP Penicillamine RS .

USP Penicillamine Disulfide RS (C<sub>10</sub>H<sub>20</sub>N<sub>2</sub>O<sub>4</sub>S<sub>2</sub>).

USP Penicillin G Benzathine RS.

USP Penicillin G Potassium RS.

USP Penicillin G Procaine RS.

USP Penicillin G Sodium RS.

USP Penicillin V RS .

USP Penicillin V Potassium RS.

USP Pentazocine RS .

USP Pentetic Acid RS .

USP Pentobarbital RS .

USP Pentoxyline RS .

USP Perflubron RS .

USP Pergolide Mesylate RS.

USP Pergolide Sulfoxide RS [(8 $\beta$ )-8-[(methylsulfinyl)methyl]-6-propyl-d-ergoline].

USP Perphenazine RS .



USP Perphenazine Sulfoxide RS

USP Phenacetin Melting Point RS

USP Phenazopyridine Hydrochloride RS .

USP Phendimetrazine Tartrate RS .

USP Phenelzine Sulfate RS .

USP Pheniramine Maleate RS .

USP Phenmetrazine Hydrochloride RS .

USP Phenobarbital RS

USP Phenol RS.

USP Phenoxybenzamine Hydrochloride RS .

USP Phenoxyethanol RS [2-phenoxyethanol] .

USP Phensuximide RS

USP Phentermine Hydrochloride RS .

USP Phentolamine Mesylate RS .

USP I-Phenylalanine RS

USP Phenylbenzimidazole Sulfonic Acid RS

USP Phenylbutazone RS

USP Phenylephrine Hydrochloride RS .

USP Phenylethyl Alcohol RS (C8H10O  $\Delta$  122.17).

USP 5-Phenylhydantoin RS

USP Phenylpropanediol RS [1-phenyl-1,2-propanediol] (C9H12O2  $\Delta$  152.19) .

USP Phenylpropanolamine Bitartrate RS .

USP Phenylpropanolamine Hydrochloride RS .

USP Phenyltoloxamine Citrate RS

USP Phenyltoloxamine Related Compound A RS [2-(2-benzylphenoxy)ethylmethylamine hydrochloride] (C16H19NO·HCl  $\Delta$  277.79) .

USP Phenytoin RS

USP Phenytoin Related Compound A RS [diphenylglycine] (C14H15NO2  $\Delta$  227.26) .

USP Phenytoin Related Compound B RS [diphenylhydantoic acid] (C15H14N2O3  $\Delta$  270.29) .

USP Phenytoin Sodium RS .

USP Cultured Rat Pheochromocytoma Reference Photomicrographs VRS.



USP Phosphated Riboflavin RS .

USP Physostigmine Salicylate RS .

USP Phytonadione RS .

USP Pilocarpine RS .

USP Pilocarpine Hydrochloride RS .

USP Pilocarpine Nitrate RS .

USP Pimozide RS .

USP Pindolol RS .

USP Piperacillin RS .

Add the following:

▲USP Piperazine Adipate RS. ▲USP32

Add the following:

▲USP Piperazine Dihydrochloride RS. ▲USP32

Add the following:

▲USP Piperazine Phosphate RS. ▲USP32

USP Piroxicam RS .

USP Plicamycin RS .

USP Polacrilex Resin RS .

USP Polacrilin Potassium RS .

USP Poloxamer Liquid RS. .

USP Poloxamer Solid RS. .

USP Poloxalene RS .

USP Polydimethylsiloxane RS .

USP High-Density Polyethylene RS .

Add the following:

▲USP Polyethylene Glycol 3350 RS. ▲USP32

USP Polyethylene Oxide RS .

USP Low-Density Polyethylene RS .

USP Polyethylene Terephthalate RS .

USP Polyethylene Terephthalate G RS .

USP Polyisobutylene RS.

USP Polymyxin B Sulfate RS .

USP Polyoxyl 20 Cetostearyl Ether RS.



USP Polyoxy 10 Oleyl Ether RS.

USP Polyoxy 10 Stearyl Ether RS.

USP Polyoxy 35 Castor Oil RS .

USP Polyoxy 40 Stearate RS

USP Polyoxy Lauryl Ether RS.

USP Polyoxy Oleate RS.

Add the following:

▲USP Polyvinyl Alcohol RS. ▲USP32

USP Posterior Pituitary RS .

USP Potassium Gluconate RS .

USP Potassium Guaiacolsulfonate RS

USP Potassium Perchlorate RS.

USP Potassium Sucrose Octasulfate RS [note—Sucrosofate Potassium is USAN] [ $\alpha$ -d-glucopyranoside, 1,3,4,6-tetra-O-sulfo- $\beta$ -d-fructofuranosyl, tetrakis (hydrogen sulfate), octapotassium salt, heptahydrate] (C12H14K8O3S8·7H<sub>2</sub>O  $\Delta$  1413.64  $\Delta$  CAS-76578-81-9). (anhydrous C12H14K8O3S8  $\Delta$  1287.53  $\Delta$  CAS-73264-44-5) .

USP Potassium Trichloroammineplatinate RS (Cl<sub>3</sub>H<sub>3</sub>KNPt  $\Delta$  357.58) .

USP Pralidoxime Chloride RS

USP Pramoxine Hydrochloride RS .

USP Pravastatin Sodium RS.

USP Pravastatin Related Compound A RS [3- $\alpha$ -hydroxyisocompactin] or [sodium (3R,5R)-3,5-dihydroxy-7-[(1S,2S,3S,8S,8aR)-3-hydroxy-2-methyl-8-[(2S)-2-methylbutanoyl]oxy]-1,2,3,7,8,8a-hexahydronephthalen-1-yl]heptanoate] (C<sub>23</sub>H<sub>35</sub>NaO<sub>7</sub>  $\Delta$  446.51) .

USP Pravastatin Related Compound B RS [6'-epi-pravastatin] or [sodium (3R,5R)-3,5-dihydroxy-7-[(1S,2S,6R,8S,8aR)-6-hydroxy-2-methyl-8-[(2S)-2-methylbutanoyl]oxy]-1,2,6,7,8,8a-hexahydronephthalen-1-yl]heptanoate] (C<sub>23</sub>H<sub>35</sub>NaO<sub>7</sub>  $\Delta$  446.51) .

USP Pravastatin 1,1,3,3-Tetramethylbutylamine RS.

USP Praziquantel RS

USP Praziquantel Related Compound A RS [2-benzoyl-1,2,3,6,7,11b-hexahydro-4H-pyrazino[2,1-a]isoquinolin-4-one] (C<sub>19</sub>H<sub>18</sub>N<sub>2</sub>O<sub>2</sub>  $\Delta$  306.37) .

USP Praziquantel Related Compound B RS [2-(cyclohexylcarbonyl)-2,3,6,7-tetrahydro-4H-pyrazino[2,1-a]isoquinolin-4-one] (C<sub>19</sub>H<sub>22</sub>N<sub>2</sub>O<sub>2</sub>  $\Delta$  310.40) .

USP Praziquantel Related Compound C RS [2-(N-formylhexahydrohippuroyl-1,2,3,4-tetrahydroisoquinolin-1-one] (C<sub>19</sub>H<sub>22</sub>N<sub>2</sub>O<sub>4</sub>  $\Delta$  342.39) .

USP Prazosin Hydrochloride RS .

USP Prednicarbate RS

USP Prednicarbate Related Compound A RS [1,2-dihydroprednicarbate] .

USP Prednicarbate Related Compound B RS [prednisolone-17-ethylcarbonate] .

USP Prednicarbate Related Compound C RS [prednisolone-21-propionate] .

USP Prednisolone RS



USP Prednisolone Acetate RS .

USP Prednisolone Hemisuccinate RS

USP Prednisolone Tebutate RS

USP Prednisone RS

USP Prednisone Tablets RS (Dissolution Calibrator, Disintegrating) .

USP Prilocaine Hydrochloride RS

USP Prilocaine RS.

USP Prilocaine Related Compound A RS [o-toluidine hydrochloride] (CH<sub>3</sub>C<sub>6</sub>H<sub>4</sub>NH<sub>2</sub>Cl  $\Delta$  143.62  $\Delta$  CAS-636-21-5) .

USP Prilocaine Related Compound B RS [(RS)-N-(4-methylphenyl)-2-(propylamino)propanamide] (C<sub>13</sub>H<sub>20</sub>N<sub>2</sub>O  $\Delta$  220.31) .

USP Primaquine Phosphate RS .

USP Primidone RS

USP Probenecid RS

USP Probucol RS

USP Probucol Related Compound A RS [2,2',6,6'-tetra-tert-butylidiphenoquinone] (C<sub>28</sub>H<sub>40</sub>O<sub>2</sub>  $\Delta$  408.63) .

USP Probucol Related Compound B RS [4,4'-(dithio)bis(2,6-di-tert-butylphenol)] (C<sub>28</sub>H<sub>42</sub>O<sub>2</sub>  $\Delta$  474.78) .

USP Probucol Related Compound C RS [4-[(3,5-di-tert-butyl-2-hydroxyphenylthio)isopropylidenethio]-2,6-di-tert-butylphenol] (C<sub>31</sub>H<sub>48</sub>O<sub>2</sub>S<sub>2</sub>  $\Delta$  516.86) .

USP Procainamide Hydrochloride RS .

USP Procaine Hydrochloride RS .

USP Procarbazine Hydrochloride RS .

USP Prochlorperazine Maleate RS .

USP Procyclidine Hydrochloride RS .

USP Progesterone RS

USP L-Proline RS .

USP Promazine Hydrochloride RS .

USP Promethazine Hydrochloride RS .

USP Propafenone Hydrochloride RS .

USP 2-Propanol RS.

USP Propantheline Bromide RS .

USP Propantheline Bromide Related Compound A RS [9-hydroxypropantheline bromide] (C<sub>23</sub>H<sub>30</sub>BrNO<sub>4</sub>  $\Delta$  464.39) .

USP Proparacaine Hydrochloride RS



USP Propofol RS

USP Propofol Related Compound A RS [3,3'-5,5'-tetraisopropylidiphenol].

USP Propofol Related Compound B RS [2,6-diisopropylbenzoquinone].

USP Propofol Related Compound C RS [2,6-diisopropylphenylisopropyl ether] (C14H22O  $\Delta$  206.32).

USP Propofol Resolution Mixture RS [propofol and 2-isopropyl-6-n-propylphenol].

USP Propoxycaaine Hydrochloride RS .

USP Propoxyphene Hydrochloride RS .

USP Propoxyphene Napsylate RS .

USP Propoxyphene Related Compound A RS [a-d-4-dimethylamino-1,2-diphenyl-3-methyl-2-butanol hydrochloride] (C19H25NO·HCl  $\Delta$  319.87).

USP Propoxyphene Related Compound B RS [ $\alpha$ -d-2-Acetoxy-4-dimethylamino-1,2-diphenyl-3-methylbutane] (C21H27NO2  $\Delta$  325.45).

USP Propranolol Hydrochloride RS .

USP Propylene Carbonate RS .

USP Propylene Glycol RS .

USP Propylene Glycol Dicaprylate/Dicaprate RS.

USP Propylene Glycol Dilaurate RS.

USP Propylene Glycol Monocaprylate Type I RS.

USP Propylene Glycol Monocaprylate Type II RS.

USP Propylene Glycol Monolaurate RS.

USP Propyl Gallate RS .

USP Propylparaben RS .

USP Propylthiouracil RS .

USP Prostaglandin A1 RS .

USP Prostaglandin B1 RS (C20H32O4  $\Delta$  336.47).

USP Protein A RS (C1995H3163N597O697S3  $\Delta$  46,760).

USP rProtein A RS (C1917H3039N565O658S3  $\Delta$  44,618).

USP rProtein A, B4, C-Cys RS (C1177H1854N326O384S1  $\Delta$  26,747.6).

USP rProtein A, C-Cys RS (C1478H2320N432O503S4  $\Delta$  34,317.5).

USP Protriptyline Hydrochloride RS .

USP Pseudoephedrine Hydrochloride RS .

USP Pseudoephedrine Sulfate RS .

USP Pyrantel Pamoate RS .



USP Pyrazinamide RS

USP Pyrethrum Extract RS.

USP Pyridostigmine Bromide RS.

USP Pyridoxine Hydrochloride RS.

USP Pyrilamine Maleate RS.

USP Pyrimethamine RS .

USP Pyrvinium Pamoate RS .

USP Quazepam RS .

USP Quazepam Related Compound A RS [7-chloro-1-(2,2,2 trifluoroethyl)-5-(2-Fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepine-2-one].

USP Quercetin RS.

USP Quinapril Hydrochloride RS.

USP Quinapril Related Compound A RS [ethyl [3S-((2R\*), 3a, 11ab)]-1,3,4,6,11,11a-hexahydro-3-methyl-1,4-dioxo-a-(2-phenylethyl)-2H-pyrazino[1,2-b]isoquinoline-2-acetate] (C25H27N2O4  $\Delta$  419.49).

USP Quinapril Related Compound B RS [3-isoquinolinecarboxylic acid, 2-[2-[(1-carboxy-3-phenylpropyl)amino]-1-oxopropyl]-1,2,3,4-tetrahydro-, [3S-[2R\*(R\*)],3R\*]] (C23H26N2O5  $\Delta$  410.47).

USP Quinic Acid RS.

USP Quinidine Gluconate RS.

USP Quinidine Sulfate RS.

USP Quinine Sulfate RS.

USP Quininone RS (C20H22N2O2  $\Delta$  322.40).

USP 3-Quinuclidinyl Benzilate RS (C21H23NO3  $\Delta$  337.42).

USP Raloxifene Hydrochloride RS.

USP Ramipril RS .

USP Ramipril Related Compound A RS [(2S,3aS,6aS)-1-[(S)2-[(S)1-(methoxycarbonyl)-3-phenylpropyl]amino]-1-oxopropyl]-octahydrocyclopenta[b]pyrrole-2-carboxylic acid] (C22H30N2O5  $\Delta$  402.48).

USP Ramipril Related Compound B RS [(2S,3aS,6aS)-1-[(S)2-[(S)1-(methylethoxy)carbonyl-3-phenylpropyl]amino]-1-oxopropyl]-octahydrocyclopenta[b]pyrrole-2-carboxylic acid] (C24H34N2O5  $\Delta$  430.54).

USP Ramipril Related Compound C RS [(2S,3aS,6aS)-1-[(S)2-[(S)1-ethoxycarbonyl-3-cyclohexyl propyl]amino]-1-oxopropyl]-octahydrocyclopenta[b]pyrrole-2-carboxylic acid] (C23H38N2O5  $\Delta$  422.56).

USP Ramipril Related Compound D RS [ethyl (2S)2-[(3S,5aS,8aS, 9aS)-3-methyl-1,4-dioxodecahydro-1H-cyclopenta[e]pyrrolo[1,2-a]pyrazin-2-yl]-4-phenyl-butanoate] Ramipril Diketopiperazine (C23H30N2O4  $\Delta$  398.50).

USP Ranitidine Hydrochloride RS .

USP Ranitidine Related Compound A RS [5-[(2-aminoethyl)thio]methyl]-N,N-dimethyl-2-furanmethanamine, hemifumarate salt].



USP Ranitidine Related Compound B RS [N,N'-bis[2-[[5-[(dimethylamino)methyl]-2-furanyl]methyl]thio]ethyl]-2-nitro-1,1-ethenediamine].

USP Ranitidine Related Compound C RS [N-[2-[[5-[(dimethylamino)methyl]-2-furanyl]methyl]sulfinyl]ethyl]-N-methyl-2-nitro-1,1-ethenediamine].

USP Ranitidine Resolution Mixture RS— It is a mixture of ranitidine hydrochloride and four related impurities: ranitidine-N-oxide, ranitidine complex nitroacetamide, ranitidine diamine hemifumarate, and ranitidine amino alcohol hemifumarate.

Ranitidine-N-oxide: N,N-dimethyl[5-[[2-[(1-methylamino)-2-nitroethenyl]amino]ethyl]sulphanyl]methyl]furan-2-yl]methanamine N-oxide.

Ranitidine complex nitroacetamide: N-[2-[[5-[(dimethylamino)methyl]furan-2-yl]methyl]sulphanyl]ethyl]-2-nitroacetamide.

Ranitidine diamine hemifumarate (related compound A): [5-[(2-aminoethyl)thio]methyl]-N,N-dimethyl-2-furanmethanamine, hemifumarate salt].

Ranitidine amino alcohol hemifumarate: [5-[(dimethylamino)methyl]furan-2-yl]methanol.

USP Rauwolfia Serpentina RS .

USP Repaglinide RS .

USP Repaglinide Related Compound A RS [(S)-3-methyl-1-[2-(1-piperidinyl)phenyl]butylamine, N-acetyl-L-glutamate salt] (C16H26N2·C7H11NO5  $\Delta$  435.6).

USP Repaglinide Related Compound B RS [3-ethoxy-4-ethoxycarbonylphenylacetic acid] (C13H16O5  $\Delta$  252.27).

USP Repaglinide Related Compound C RS [(S)-2-ethoxy-4-[2-[[2-phenyl-1-[2-(1-piperidinyl)phenyl]ethyl]amino]-2-oxoethyl]benzoic acid] (C30H34N2O4  $\Delta$  486.61).

USP Reserpine RS .

USP Resorcinol RS .

USP Ribavirin RS .

USP Riboflavin RS .

USP Rifabutin RS .

USP Rifampin RS .

USP Rifampin Quinone RS .

USP Rimexolone RS .

USP Risperidone RS [3-[2-[4-(6-fluoro-1,2-benzisoxazol-3-yl)piperidino]ethyl]-6,7,8,9-tetrahydro-2-methyl-4H-pyrido[1,2-a]pyrimidin-4-one] (410.48  $\Delta$  CAS-106266-06-2).

USP Risperidone System Suitability Mixture RS —Contains risperidone and about 0.2% of each of the following:

Z-oxime-3-[2-[4-[(Z)-(2,4-difluorophenyl)(hydroxymino)methyl]piperidin-1-yl]ethyl]-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one;

9-hydroxyrisperidone-(6RS)-3-[2-[4-(6-fluoro-1,2-benzisoxazol-3-yl)piperidin-1-yl]ethyl]-2,6-dimethyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one;

6-methylrisperidone-(6RS)-3-[2-[4-(6-fluoro-1,2-benzisoxazol-3-yl)piperidin-1-yl]ethyl]-2,6-dimethyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one.

USP Ritodrine Hydrochloride RS .

USP Ritonavir RS. .

USP Ritonavir Related Compounds Mixture RS.

USP Ropivacaine Hydrochloride RS. .

USP Ropivacaine Related Compound A RS [2,6-dimethylaniline hydrochloride] (C8H12ClN  $\Delta$  157.64.  $\Delta$  CAS-21436-98-6).

USP Ropivacaine Related Compound B RS [(R)-ropivacaine hydrochloride monohydrate; (R)-(-)-1-propylpiperidene-2-carboxylic acid (2,6-dimethylphenyl)-amide hydrochloride monohydrate] (C17H26N2O  $\Delta$  328.89).

USP Roxarsone RS .



USP Rutin RS

USP Saccharin RS

USP Saccharin Calcium RS .

USP Saccharin Sodium RS .

USP Salicylamide RS

USP Salicylic Acid RS

USP Salicylic Acid Related Compound A RS [4-hydroxybenzoic acid] (C7H6O3  $\Delta$  138.12  $\Delta$  CAS-99-96-7).

USP Salicylic Acid Related Compound B RS [4-hydroxyisophthalic acid] (C8H6O4  $\Delta$  182.13).

USP Salicylic Acid Tablets RS (Dissolution Calibrator, Non-disintegrating) .

USP Salsalate RS

USP Saquinavir Mesylate RS.

USP Saquinavir Related Compound A RS [N-tert-butyl-decahydro-2-[2(R)-hydroxy-4-phenyl-3(S)-[[N-(2-quinolylcarbonyl)-d-asparaginyl]amino]butyl]-4aS,8aS)-isoquinoline-3(S)-carboxamide] (C38H50N6O5  $\Delta$  670.84) .

USP Scopolamine Hydrobromide RS .

USP Scopoletin RS .

USP Secobarbital RS

USP Selegiline Hydrochloride RS .

USP Selenomethionine RS.

USP Sennosides RS .

USP I-Serine RS .

USP Sesame Oil Related Compound A RS.

USP Sesame Oil Related Compound B RS.

USP Sevoflurane RS

USP Sevoflurane Related Compound A RS [1,1,1,3,3-pentafluoroisopropenyl fluoromethyl ether] (C4H2F6O  $\Delta$  179.97) .

USP Sevoflurane Related Compound B RS [1,1,1,3,3,3-hexafluoro-2-methoxy-propane].

USP Sevoflurane Related Compound C RS [1,1,1,3,3,3-hexafluoro-2-propanol].

USP Sevomethyl Ether RS [1,1,1,3,3,3-hexafluoro-2-methoxy-propane].

USP Silver Sulfadiazine RS

USP Silybin RS .

USP Silydianin RS .

USP Simethicone RS



USP Simvastatin RS

USP Sincalide RS.

USP Sisomicin Sulfate RS .

USP  $\beta$ -Sitosterol RS .

USP Human Fibroblast-Derived Temporary Skin Substitute Reference Photomicrographs VRS.

USP Sodium Ascorbate RS .

USP Sodium Benzoate RS.

USP Sodium Bromide RS.

USP Sodium Butyrate RS .

USP Sodium Fluoride RS .

USP Sodium Lactate RS .

USP Sodium Nitroprusside RS .

USP Sodium Propionate RS .

USP Sodium Salicylate RS.

USP Sodium Starch Glycolate Type A RS .

USP Sodium Starch Glycolate Type B RS .

USP Sodium Stearyl Fumarate RS .

USP Somatropin RS .

USP 1,4-Sorbitan RS (C6H12O5  $\Delta$  164.16) .

USP Sorbitol RS .

USP Sotalol Hydrochloride RS .

USP Sotalol Related Compound A RS [N[(4-[(1-methylethyl)amino]acetyl]phenyl)methanesulfonamide monohydrochloride] (C12H18N2O3S·HCl  $\Delta$  306.81) .USP Sotalol Related Compound B RS [N-(4-formylphenyl)methanesulfonamide] (C8H9NO3S  $\Delta$  199.23) .USP Sotalol Related Compound C RS [N-[4-{2-[(1-methylethyl)amino]ethyl}phenyl)methanesulfonamide monohydrochloride] (C12H20N2O2S·HCl  $\Delta$  292.83) .

Add the following:

▲USP Defatted Powdered Soy RS ▲USP32

USP Spectinomycin Hydrochloride RS .

USP Spironolactone RS .

USP Squalane RS .

USP Stanozolol RS .

USP Stavudine RS .



USP Stavudine System Suitability Mixture RS —It is a mixture of stavudine and the following related compounds: thymidine, thymine, alpha-stavudine, and xylo-thymidine.

USP Stearic Acid RS .

USP Stearyl Alcohol RS .

USP Stearyl Polyoxylglycerides RS .

USP Powdered St. John's Wort Extract RS.

USP Streptomycin Sulfate RS .

USP Succinylcholine Chloride RS .

USP Succinylmonocholine Chloride RS .

USP Sucralose RS .

USP Sucrose RS .

USP Sufentanil Citrate RS .

USP Sulbactam RS .

USP Sulconazole Nitrate RS .

USP Sulfabenzamide RS .

USP Sulfacetamide RS .

USP Sulfacetamide Sodium RS .

USP Sulfachloropyridazine RS .

USP Sulfadiazine RS .

USP Sulfadimethoxine RS .

USP Sulfadoxine RS .

USP Sulfamerazine RS .

USP Sulfamethazine RS .

USP Sulfamethizole RS .

USP Sulfamethoxazole RS .

USP Sulfamethoxazole N4-Glucoside RS .

USP Sulfanilamide RS .

USP Sulfanilamide Melting Point RS .

USP Sulfanilic Acid RS (C6H7NO3S  $\Delta$  173.19).

USP Sulfapyridine RS .

USP Sulfapyridine Melting Point RS .



USP Sulfaquinoxaline RS.

USP Sulfaquinoxaline Related Compound A RS [N1-N2-diquinoxalin-2-ylsulfanilamide] (C22H16N6SO2  $\Delta$  428.50).

USP Sulfasalazine RS .

USP Sulfathiazole RS .

USP Sulfinpyrazone RS .

USP Sulfisoxazole RS .

USP Sulfisoxazole Acetyl RS .

USP Sulindac RS .

USP Sulisobenzene RS.

USP Sumatriptan RS .

USP Sumatriptan Succinate RS [1H-indole-5-methanesulfonamide, 3-[2-(dimethylamino)ethyl]-N-methyl-, butanedioate (1:1)] (C14H21N3O2S·C4H6O4  $\Delta$  413.49).USP Sumatriptan Succinate Related Compound A RS [[3-[2-(dimethylamino)ethyl]-2-[3-[2-(dimethylamino)ethyl]-1H-indol-5-yl]methyl]-1H-indol-5-yl]-N-methylmethanesulfonamide succinate salt] (C27H37N5O2S·C4H6O4  $\Delta$  613.77).USP Sumatriptan Succinate Related Compound C RS [[3-[2-(dimethylamino)ethyl]-1-(hydroxymethyl)-1H-indol-5-yl]-N-methylmethanesulfonamide succinate salt] (C15H23N3O3S·0.5 C4H6O4  $\Delta$  384.47).

USP Sumatriptan Succinate Related Impurities RS [Mixture of sumatriptan succinate, [3-[2-(methylamino)ethyl]-1H-indol-5-yl]-N-methylmethanesulfonamide maleate salt, sumatriptan succinate related compound C, [3-[2-(dimethylamino-N-oxide)ethyl]-1H-indol-5-yl]-N-methylmethanesulfonamide, and [3-[2-(aminoethyl)-1H-indol-5-yl]-N-methylmethanesulfonamide].

USP Suprofen RS .

USP Tacrine Hydrochloride RS.

USP Tagatose RS .

USP Tamoxifen Citrate RS.

Add the following:

▲USP Tamsulosin Hydrochloride RS. ▲USP32

Add the following:

▲USP Racemic Tamsulosin Hydrochloride RS. ▲USP32

USP Taurine RS .

USP Tazobactam RS.

USP Tazobactam Related Compound A RS [(2S,3S)-2-amino-3-methyl-3-sulfino-4-(1H-1,2,3-triazol-1-yl)butyric acid] (C7H12N4O4S  $\Delta$  248.26).

USP Temazepam RS .

USP Terazosin Hydrochloride RS.

USP Terazosin Related Compound A RS [1-(4-amino-6,7-dimethoxy-2-quinazolinyl)piperazine, dihydrochloride] (C14H19N5O2·2HCl  $\Delta$  362.25).USP Terazosin Related Compound B RS [1-(4-hydroxy-6,7-dimethoxy-2-quinazolinyl)-4-[(tetrahydro-2-furanyl)carbonyl]piperazine] (C19H24N4O5  $\Delta$  388.42).



USP Terazosin Related Compound C RS [1,4-bis(4-amino-6,7-dimethoxy-2-quinazolinyl)piperazine, dihydrochloride] (C<sub>24</sub>H<sub>28</sub>N<sub>8</sub>O<sub>4</sub>·2HCl ⚡ 565.45).

USP Terbinafine Hydrochloride RS.

USP Terbutaline Sulfate RS.

USP Terbutaline Related Compound A RS [3,5-dihydroxy- $\alpha$ -t-butylaminoacetophenone sulfate].

USP Terconazole RS.

USP Terpin Hydrate RS.

USP Testolactone RS.

USP Testosterone RS.

USP Testosterone Cypionate RS.

USP Testosterone Enanthate RS.

USP Testosterone Propionate RS.

USP Tetracaine Hydrochloride RS.

USP Tetracycline Hydrochloride RS.

USP  $\Delta$ 8-Tetrahydrocannabinol RS.

USP  $\Delta$ 9-Tetrahydrocannabinol RS.

USP exo-Tetrahydrocannabinol RS [(6aR, 10aR)-6,6-dimethyl-9-methylene-3-pentyl-6a,7,8,9,10,10a-hexahydro-6H-benzo[c]chromen-1-ol] (C<sub>21</sub>H<sub>30</sub>O<sub>2</sub> ⚡ 314.46).

USP Tetrahydrozoline Hydrochloride RS.

USP 3,3'-5,5'-Tetraisopropylidiphenol RS.

USP Thalidomide RS.

USP Theophylline RS.

USP Thiabendazole RS.

USP Thiacetarsamide RS.

USP Thiamine Hydrochloride RS.

USP Thiethylperazine Maleate RS.

USP Thimerosal RS.

USP Thioguanine RS.

USP Thiopental RS.

USP Thioridazine RS.

USP Thioridazine Hydrochloride RS.

USP Thiostrepton RS.



USP Thiotepa RS

USP Thiothixene RS .

USP (E)-Thiothixene RS

USP L-Threonine RS .

USP Thymol RS.

USP Tiagabine Hydrochloride RS

USP Tiagabine Related Compound A RS [(R)-ethyl 1-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]-3-piperidinecarboxylate] (C<sub>22</sub>H<sub>29</sub>NO<sub>2</sub>S<sub>2</sub>·HCl  $\Delta$  440.0) .USP Racemic Tiagabine Hydrochloride Mixture RS [(S)-(+), (R)-(-)-1-[4,4-bis(3-methyl-2-thienyl)-3-butene]nipeptic acid, hydrochloride] (C<sub>20</sub>H<sub>25</sub>NO<sub>2</sub>S<sub>2</sub>·HCl  $\Delta$  412.0) .

USP Tiamulin RS.

USP Tiamulin Fumarate RS .

USP Tiamulin Related Compound A RS [tosyl pleuromutilin] .

USP Ticarcillin Monosodium Monohydrate RS (C<sub>15</sub>H<sub>15</sub>N<sub>2</sub>Na O<sub>6</sub>S<sub>2</sub>·H<sub>2</sub>O  $\Delta$  424.43) .

USP Tiletamine Hydrochloride RS .

USP Tilmicosin RS .

USP Timolol Maleate RS .

USP Tinidazole RS .

USP Tinidazole Related Compound A RS [(2-methyl-5-nitroimidazole] (C<sub>4</sub>H<sub>5</sub>N<sub>3</sub>O<sub>2</sub>  $\Delta$  127.10) .USP Tinidazole Related Compound B RS [1-(2-ethyl-sulfonylethyl)-2-methyl-4-nitroimidazole] (C<sub>8</sub>H<sub>13</sub>N<sub>3</sub>O<sub>4</sub>S  $\Delta$  247.28) .

USP Tioconazole RS .

USP Tioconazole Related Compound A RS [1-[2,4-dichloro- $\beta$ -[(3-thenyl)-oxy]phenethyl]imidazole hydrochloride] (C<sub>16</sub>H<sub>14</sub>Cl<sub>2</sub> N<sub>2</sub>OS·HCl  $\Delta$  389.73) .USP Tioconazole Related Compound B RS [1-[2,4-dichloro- $\beta$ -[(2,5-dichloro-3-thenyl)oxy]phenethyl]imidazole hydrochloride] (C<sub>16</sub>H<sub>12</sub>Cl<sub>4</sub>N<sub>2</sub>OS·HCl  $\Delta$  458.62) .USP Tioconazole Related Compound C RS [1-[2,4-dichloro- $\beta$ -[(5-bromo-2-chloro-3-thenyl)-oxy]-phenethyl]imidazole hydrochloride] (C<sub>16</sub>H<sub>13</sub>BrCl<sub>2</sub>N<sub>2</sub>OS·HCl  $\Delta$  468.63) .

USP Tizanidine Hydrochloride RS.

USP Tizanidine Related Compound A RS [4-amino-5-chloro-2,1,3-benzothiadiazole] (C<sub>6</sub>H<sub>4</sub>CIN<sub>3</sub>S  $\Delta$  185.63) .USP Tizanidine Related Compound B RS [N-acetyl tizanidine] (C<sub>11</sub>H<sub>10</sub>CIN<sub>5</sub>OS  $\Delta$  295.75) .USP Tizanidine Related Compound C RS [1-acetylimidazolidine-2-thione] (C<sub>5</sub>H<sub>8</sub>N<sub>2</sub>OS  $\Delta$  144.20) .

USP Tobramycin RS .

USP Tocainide Hydrochloride RS .

USP Alpha Tocopherol RS .

USP Alpha Tocopheryl Acetate RS .



USP Alpha Tocopheryl Acid Succinate RS

USP Tolazamide RS

USP Tolazoline Hydrochloride RS .

USP Tolbutamide RS

USP Tolcapone RS

USP Tolcapone Related Compound A RS [4N-methyl-3,4-dihydroxybenzophenone] (C14H12O3  $\Delta$  228.24) .

USP Tolcapone Related Compound B RS [4-hydroxy-3-methoxy-4'-methyl-5-nitrobenzophenone] (C15H13NO5  $\Delta$  287.27) .

USP Tolmetin Sodium RS .

USP Tolnaftate RS

USP o-Toluenesulfonamide RS (C7H9NO2S  $\Delta$  171.22) .

USP p-Toluenesulfonamide RS (C7H9NO2S  $\Delta$  171.22) .

USP Tomato Extract Containing Lycopene RS .

USP Topiramate RS.

USP Topiramate Related Compound A RS [2,3 : 4,5-bis-O-(1-methylethylidene)- $\beta$ -d-fructopyranose] (C12H20O6  $\Delta$  260.28) .

USP Torsemide RS (Form 1) .

USP Torsemide Related Compound A RS [4-[(3-methylphenyl)amino]-3-pyridinesulfonamide] (C12H13N3O2S  $\Delta$  263.32) .

USP Torsemide Related Compound B RS [N-[(n-butylamino)carbonyl]-4-[(3-methylphenyl)amino]-3-pyridinesulfonamide] (C17H22N4O3S  $\Delta$  362.45) .

USP Torsemide Related Compound C RS [N-[(ethylamino)carbonyl]-4-[(3-methylphenyl)amino]-3-pyridinesulfonamide] (C15H18N4O3S  $\Delta$  334.39) .

USP Transplatin RS

USP Trazodone Hydrochloride RS .

USP Trenbolone RS.

USP Trenbolone Acetate RS .

USP Tretinoin RS

USP Triacetin RS

USP Triamcinolone RS

USP Triamcinolone Acetonide RS

USP Triamcinolone Diacetate RS .

USP Triamcinolone Hexacetonide RS

USP Triamterene RS

USP Triazolam RS



USP Tributyl Citrate RS

USP Trichlormethiazide RS

USP Triclosan RS

USP Triclosan Related Compound A RS [1,3,7-trichlorodibenzo-p-dioxin] (C12H5Cl3O2) ⚡ 287.53 .

USP Triclosan Related Compound B RS [2,8 dichlorodibenzo-p-dioxin] (C12H6Cl2O2) ⚡ 253.09 .

USP Triclosan Related Compounds Mixture A RS.

USP Trientine Hydrochloride RS .

USP Triethyl Citrate RS

USP Trifluoperazine Hydrochloride RS .

USP Triflupromazine Hydrochloride RS .

USP Trifluridine RS

USP Trifluridine Related Compound A RS [5-carboxy-2'-deoxyuridine] (C10H12N2O7) ⚡ 272.22 .

USP Trihexyphenidyl Hydrochloride RS .

USP Trimeprazine Tartrate RS .

USP Trimethobenzamide Hydrochloride RS .

USP Trimethoprim RS

USP Trimipramine Maleate RS.

USP Trimipramine Related Compound A RS [5-[3-(dimethylamino)-2-methylpropyl]-5H-dibenz[b,f]azepine] (C20H24N2) ⚡ 292.42.

USP Trioxsalen RS

USP Tripelennamine Hydrochloride RS .

USP Triprolidine Hydrochloride RS .

USP Triprolidine Hydrochloride Z-isomer RS

USP Trisalicylic Acid RS (C21H14O7) ⚡ 378.34 .

USP Trolamine RS

USP Troleandomycin RS

USP Tromethamine RS

USP Tropicamide RS

USP Trypsin Crystallized RS .

Add the following:

▲USP Tryptophan Related Compound A RS [3,3'-(ethylenebis(1H-indole-1,3-diy))bis[2S]-2-aminopropanoic] acid] (C24H26N4O4) ⚡ 432.49. ▲USP32

Add the following:

▲USP Tryptophan Related Compound B RS [2-acetamido-3-(1H-indol-3-yl)propanoic acid] (C13H14N2O3) ⚡ 246.3. ▲USP32



USP L-Tryptophan RS .

USP Tubocurarine Chloride RS .

USP Tylosin RS .

USP Tylosin Tartrate RS .

USP Tyloxapol RS .

USP L-Tyrosine RS .

USP Ubidecarenone RS .

USP Ubidecarenone Related Compound A RS [coenzyme Q9].

USP Ubidecarenone for System Suitability RS .

USP Undecylenic Acid RS .

USP Uracil Arabinoside RS .

USP Urea RS .

USP Ursodiol RS .

USP Valerenic Acid RS .

USP Powdered Valerian RS.

USP Valganciclovir Hydrochloride RS .

USP d-Valganciclovir RS .

USP L-Valine RS .

USP Valproic Acid RS .

USP Valproic Acid Related Compound A RS [diallylacetate] (C8H12O2 140.18) .

USP Valrubicin RS .

USP Valrubicin Related Compound A RS [N-trifluoroacetyl-14-bromodaunorubicin-13,13-dimethylketal] .

USP Valsartan RS .

USP Valsartan Related Compound A RS [(R-N-valeryl-N-([2'-(1H-tetrazole-5-yl)biphen-4-yl]methyl)valine] (C24H29N5O3 435.52) .

USP Valsartan Related Compound B RS [(S-N-butyryl-N-([2'-(1H-tetrazole-5-yl)biphen-4-yl]methyl)-valine] (C23H27N5O3 421.49) .

USP Valsartan Related Compound C RS [(S-N-valeryl-N-([2'-(1H-tetrazole-5-yl)biphen-4-yl]methyl)-valine benzyl ester] (C31H35N5O3 525.64) .

USP Vancomycin Hydrochloride RS .

USP Vancomycin B with Monodechlorovancomycin RS.

USP Vanillin RS .

USP Vanillin Melting Point RS .



USP Vasopressin RS.

USP Vecuronium Bromide RS

USP Vecuronium Bromide Related Compound A RS [3 $\alpha$ ,17 $\beta$ -diacetyl-oxy-2 $\beta$ ,16 $\beta$ -bispiperidinyl-5 $\alpha$ -androstan] (C<sub>33</sub>H<sub>54</sub>N<sub>2</sub>O<sub>4</sub> ♦ 542.79).USP Vecuronium Bromide Related Compound B RS [piperidinium, 1-[(2 $\beta$ ,3 $\alpha$ ,5 $\alpha$ ,16 $\beta$ ,17 $\beta$ )-3-acetyloxy-17-hydroxy-2-(1-piperidinyl)androstan-16-yl]-1-methyl bromide] (C<sub>32</sub>H<sub>55</sub>BrN<sub>2</sub>O<sub>3</sub> ♦ 595.69).USP Vecuronium Bromide Related Compound C RS [piperidinium, 1-[(2 $\beta$ ,3 $\alpha$ ,5 $\alpha$ ,16 $\beta$ ,17 $\beta$ )-3,17-dihydroxy-2-(1-piperidinyl)androstan-16-yl]-1-methyl bromide] (C<sub>30</sub>H<sub>53</sub>BrN<sub>2</sub>O<sub>2</sub> ♦ 553.66).USP Vecuronium Bromide Related Compound D RS (C<sub>26</sub>H<sub>41</sub>NO<sub>3</sub> ♦ 415.61).USP Vecuronium Bromide Related Compound F RS [piperidinium, 1-[(2 $\beta$ ,3 $\alpha$ ,5 $\alpha$ ,16 $\beta$ ,17 $\beta$ )-17-acetyloxy-3-hydroxy-2-(1-piperidinyl)androstan-16-yl]-1-methyl bromide] (C<sub>32</sub>H<sub>55</sub>BrN<sub>2</sub>O<sub>3</sub> ♦ 595.69).

USP Verapamil Hydrochloride RS

USP Verapamil Related Compound A RS [3,4-dimethoxy- $\alpha$ -[3-(methylamino)propyl]- $\alpha$ -(1-methylethyl)-benzeneacetonitrile monohydrochloride] (C<sub>17</sub>H<sub>26</sub>N<sub>2</sub>O<sub>2</sub>·HCl ♦ 326.87).USP Verapamil Related Compound B RS [benzeneacetonitrile,  $\alpha$ -[2-[[2-(3,4-dimethoxyphenyl)-ethyl]methylamino]ethyl]-3,4-dimethoxy- $\alpha$ -(1-methylethyl)-, monohydrochloride] (C<sub>26</sub>H<sub>36</sub>N<sub>2</sub>O<sub>4</sub>·HCl ♦ 477.05).

USP Verapamil Related Compound D RS

USP Verapamil Related Compound E RS [3,4-dimethoxybenzaldehyde].

USP Verapamil Related Compound F RS [(3,4-dimethoxyphenyl)methanol].

USP Verteporfin RS (C<sub>41</sub>H<sub>42</sub>N<sub>4</sub>O<sub>8</sub> ♦ 718.79).USP Verteporfin Related Compound A RS [trans-( $\pm$ )-18-ethenyl-4,4a-dihydro-3,4-bis(methoxycarbonyl)-4a,8,14,19-tetramethyl-23H,25H-benzo[b]porphine-9,13-dipropionic acid] (C<sub>40</sub>H<sub>40</sub>N<sub>4</sub>O<sub>8</sub> ♦ 704.77).

USP Vidarabine RS .

USP Vinblastine Sulfate RS .

USP Vincristine Sulfate RS .

USP Vinorelbine Related Compound A RS [4-O-deacetylvinorelbine] (C<sub>43</sub>H<sub>52</sub>N<sub>4</sub>O<sub>7</sub>·2C<sub>4</sub>H<sub>6</sub>O<sub>6</sub> ♦ 1037.07).

USP Vinorelbine Tartrate RS

USP Vitamin A RS

USP Vitamin D Assay System Suitability RS .

USP Vitexin RS .

USP Warfarin RS .

USP Warfarin Related Compound A RS [3-(o-hydroxyphenyl)-5-phenyl-2-cyclohexen-1-one] (C<sub>18</sub>H<sub>16</sub>O<sub>2</sub> ♦ 264.33).USP Xanthanoic Acid RS (C<sub>14</sub>H<sub>10</sub>O<sub>3</sub> ♦ 226.23).USP Xanthone RS (C<sub>13</sub>H<sub>8</sub>O<sub>2</sub> ♦ 196.21).

USP Xylazine RS

USP Xylazine Hydrochloride RS .

USP Xylitol RS

USP Xylometazoline Hydrochloride RS .

USP Xylose RS .

USP Yohimbine Hydrochloride RS

USP Zalcitabine RS

USP Zalcitabine Related Compound A RS [2',3'-didehydro-2',3'-dideoxycytidine] .

USP Zidovudine RS

USP Zidovudine Related Compound B RS [3'-chloro-3'-deoxythymidine] (C10H13ClN2O4  $\Delta$  260.68) .

USP Zidovudine Related Compound C RS [thymine] (C5H6N2O2  $\Delta$  126.12) .

USP Zileuton RS

USP Zileuton Related Compound A RS [N-(1-benzo-[b]thien-2-ylethyl)urea] (C11H12N2O  $\Delta$  220.30) .

USP Zileuton Related Compound B RS [2-(benzo[b]thien-2-oyl)benzo[b]thiopene] (C17H10OS2  $\Delta$  294.40) .

USP Zileuton Related Compound C RS [1-benzo-[b]thien-2-ylethanone] (C10H8OS  $\Delta$  176.24) .

USP Zolazepam Hydrochloride RS .

#### ◀ 16 ▶ AUTOMATED METHODS OF ANALYSIS

Where a sufficiently large number of similar units are to be subjected routinely to the same type of examination, automated methods of analysis may be far more efficient and precise than manual methods. Such automated methods have been found especially useful in testing the content uniformity of tablets and capsules and in facilitating methods requiring precisely controlled experimental conditions. Many manufacturing establishments, as well as the laboratories of regulatory agencies, have found it convenient to utilize automated methods as alternatives to Pharmacopeial methods (see Procedures under Tests and Assays in the General Notices and Requirements). In addition, the detection system and calculation of results for automated methods are often computerized.

Before an automated method for testing an article is adopted as an alternative, it is advisable to ascertain that the results obtained by the automated method are equivalent in accuracy and precision to those obtained by the prescribed Pharmacopeial method, bearing in mind the further principle stated in the General Notices and Requirements that "where a difference appears, or in the event of dispute, only the result obtained by the procedure given in this Pharmacopeia is conclusive."

It is necessary to monitor the performance of the automated analytical system continually by assaying standard preparations of known composition frequently interspersed among the test preparations. Where immiscible solvents are employed in the automated apparatus for rapid extractions, they are often separated for analysis before complete extraction is attained, and the chemical reactions utilized in automated methods rarely are stoichiometric. Both the accuracy and the precision of the determinations depend upon precise adjustment of the equipment, so maintained that all standard and test preparations are exposed to identical physical and chemical manipulations for identical time intervals. Excessive variability in the response of the standard preparations indicates that the analytical system is malfunctioning and that the test results are therefore invalid. However, where automated systems are shown to operate reliably, the precision of the automated method may surpass that of the manual procedure employing the same basic chemistry.

Many of the manual methods given in this Pharmacopeia can be adapted for use in automated equipment incorporating either discrete analyzers or continuous flow systems and operating under a variety of conditions. On the other hand, an analytical scheme devised for a particular automated system may not be readily transposable for use either in a manual procedure or in other types of automated equipment.

The apparatus required for manual methods is, in general, less complicated than the apparatus of automated systems, even those systems used for the direct automated measurement of a single analyte (i.e., the substance being determined or analyzed for) in a binary mixture. However, because of their versatility, automated systems designed for the rapid determination of a specified substance often can be readily modified by the addition of suitable modules and accessories to permit the determination of one or more additional substances in a dosage form. Such extended systems have been utilized, for example, in the automated analysis of articles containing both estrogens and progestogens.

The accompanying pertinent diagrams represent examples of automated methods. Diagrams for official methods are reproduced here rather than in the individual monographs. The descriptions of the procedural details in these methods exemplify the general approach in automated analysis applicable to dosage forms. It should be noted that the diagrams, with many minutiae, are an indispensable part of the directions for conducting the analysis.

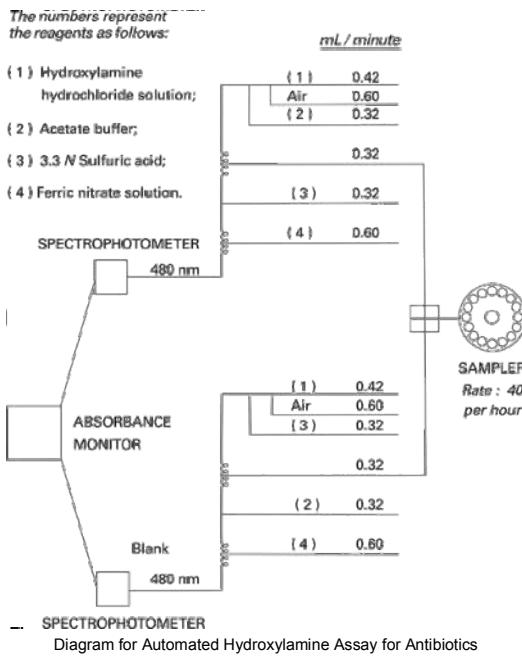
#### DIAGRAMS

The diagrams shown below are arranged in alphabetic order by the name of the drug first mentioned, where the diagram is for a procedure for a specific article. Where there is no procedure in this chapter for a particular diagram, reference is to be made to the named monograph.

#### ANTIBIOTICS—HYDROXYLAMINE ASSAY

The following procedure is applicable for the assay of those Pharmacopeial antibiotics, such as cephalosporins and penicillins, that possess the beta-lactam structure.

Apparatus— Automatic analyzer consisting of (1) a liquid sampler, (2) a proportioning pump, (3) suitable spectrophotometers equipped with matched flow cells and analysis capability at 480 nm, (4) a means of recording spectrophotometric readings, and/or computer for data retrieval and calculation, and (5) a manifold consisting of the components illustrated in the accompanying pertinent [diagram](#).



## Diagram for Automated Hydroxylamine Assay for Antibiotics

## Reagents—

Hydroxylamine Hydrochloride Solution— Dissolve 20 g of hydroxylamine hydrochloride in 5 mL of polyoxyethylene (23) lauryl ether solution (1 in 1000), and add water to make 1000 mL.

Acetate Buffer— Dissolve 173 g of sodium hydroxide and 20.6 g of sodium acetate in water to make 1000 mL. Dilute 75 mL of this solution with water to 500 mL, and mix.

Ferric Nitrate Solution— Suspend 233 g of ferric nitrate in about 600 mL of water, add 2.8 mL of sulfuric acid, stir until the ferric nitrate is dissolved, add 1 mL of polyoxyethylene (23) lauryl ether, dilute with water to 1000 mL, and mix.

USP Reference Standards (11)—Use the USP Reference Standard as directed in the individual monograph.

Standard Preparation— Unless otherwise directed in the individual monograph, dissolve an accurately weighed quantity of the USP Reference Standard in water, and quantitatively dilute with water to obtain a solution having a known concentration of about 1 mg per mL.

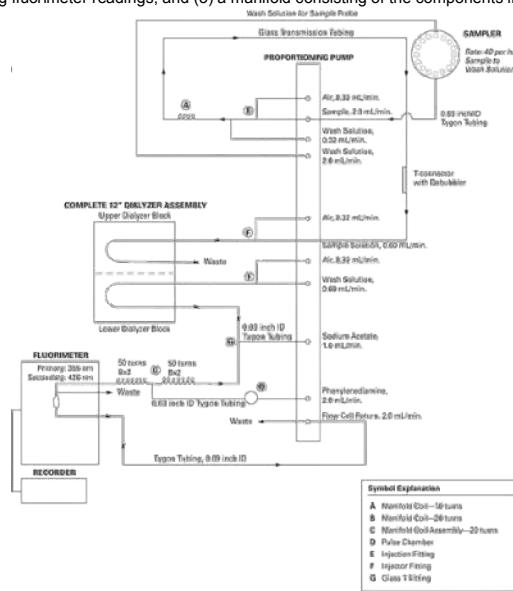
**Assay Preparation**— Unless otherwise directed in the individual monograph, using the specimen under test, prepare as directed under Standard Preparation.

Procedure— With the sample line pumping water, the other lines pumping their respective reagents, and the spectrophotometer set at 480 nm, standardize the system until a steady absorbance baseline has been established. Transfer portions of the Standard Preparation and the Assay Preparation to sampler cups, and place in the sampler. Start the sampler, and conduct determinations of the Standard Preparation and the Assay Preparation typically at the rate of 40 per hour, using a ratio of about 2:1 for sample and wash time. Calculate the potency by the formula given in the individual monograph, in which C is the concentration, in mg per mL, of USP Reference Standard in the Standard Preparation; P is the potency, in  $\mu$ g per mg, of the USP Reference Standard; and AU and AS are the absorbances, corrected for the absorbances of the respective blanks, of the solutions from the Assay Preparation and the Standard Preparation, respectively.

#### ASSAY FOR ASCORBIC ACID

The following procedure is applicable for the assay of ascorbic acid in Pharmacopeial multivitamin-minerals combination products (solid and liquid dosage forms) that contain components that interfere in other methods of assay.

Apparatus— Automatic analyzer consisting of (1) a liquid sampler; (2) a proportioning pump; (3) a suitable fluorimeter equipped with a flow cell and filters: primary—335 nm, and secondary—426 nm; (4) a means of recording fluorimeter readings; and (5) a manifold consisting of the components illustrated in the accompanying pertinent diagram.



### Diagram for Automated Ascorbic Acid

## Reagents—



Extracting Solution— Dissolve 600 g of metaphosphoric acid in 1200 mL of water. Add 400 mL of glacial acetic acid, dilute with water to 2000 mL, and mix.

Dilute Extracting Solution— Dissolve 60 g of metaphosphoric acid in 1200 mL of water. Add 160 mL of glacial acetic acid, dilute with water to 2000 mL, and mix.

**Surfactant Solution**— Prepare a 30% solution of polyoxyethylene (23) lauryl ether by melting 150 g in a container on a steam bath and slowly adding approximately 250 mL of water with continuous stirring. Cool and dilute with water to make 500 mL.

Wash Solution— Add 1 mL of Surfactant Solution to 3000 mL of Dilute Extracting Solution, and mix.

Carbon Extraction Solution— Dissolve 60 g of metaphosphoric acid in 1200 mL of water. Add 160 mL of glacial acetic acid, and mix. Add 33 g of activated charcoal powder, mix, and dilute with water to 2000 mL. Continually mix the solution at a rate that maintains homogeneity.

**Sodium Acetate Solution**— Dissolve 500 g of sodium acetate trihydrate in water to make 1000 mL, mix, and filter.

Phenylenediamine Solution— Dissolve 200 mg of o-phenylenediamine dihydrochloride in water to make 1000 mL, and mix. Prepare fresh daily.

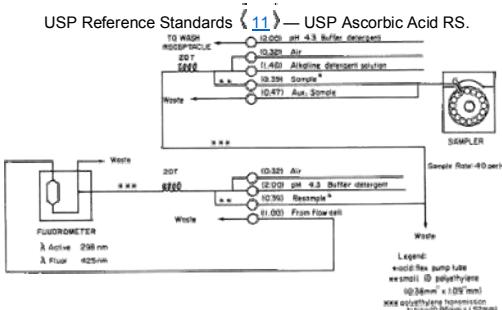


Diagram for Automated Aspirin Determinative Step of the Dissolution Test for Aspirin, Alumina, and Magnesium Oxide Tablets

Standard Stock Solution— Dissolve an accurately weighed quantity of USP Ascorbic Acid RS in Dilute Extracting Solution to obtain a solution having a known concentration of about 0.1 mg per ml.

Standard Preparations— Transfer 10.0, 20.0, 30.0, 40.0, and 50.0 mL of Standard Stock Solution to separate 100-mL volumetric flasks, dilute the contents of each flask with Carbon Extracting Solution to volume, mix, and filter to obtain Standard Preparations A, B, C, D, and E having known concentrations of 10 µg, 20 µg, 30 µg, 40 µg, and 50 µg of USP Ascorbic Acid RS per mL, respectively.

### Assay Preparation—

For Liquid Preparations— Transfer an accurately measured volume of the liquid preparation, equivalent to 150 mg of ascorbic acid, to a 100-mL volumetric flask. Add 10 mL of Extracting Solution and 6 mL of glacial acetic acid. Dilute with water to volume, and mix. Transfer 2.0 mL of this solution to a 100-mL volumetric flask, dilute with Carbon Extracting Solution to volume, mix, and filter.

For Tablet Preparations— Weigh and finely powder not fewer than 20 Tablets. Transfer an accurately weighed quantity of the powder, equivalent to about 250 mg of ascorbic acid, to a 250-mL volumetric flask. Add 25 mL of Extracting Solution, 15 mL of glacial acetic acid, and about 100 mL of water, and swirl to mix. Heat for 15 minutes in a 70° water bath, swirling after about 7 minutes. Cool, and dilute with water to volume. Transfer 2.0 mL of this solution to a 100-mL volumetric flask, dilute with Carbon Extracting Solution to volume, mix, and filter.

For Capsule Preparations— Empty the contents, if necessary by cutting open with a sharp blade, of not fewer than 20 Capsules in a suitable container, and mix thoroughly. Transfer a portion of the capsule contents, equivalent to about 250 mg of ascorbic acid, to a 250-mL volumetric flask, and proceed as directed for Tablets above, beginning with "Add 25 mL of ."

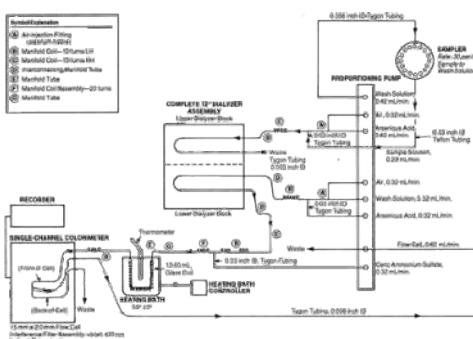
Procedure— With the sample line pumping the Wash Solution, the other lines pumping their respective reagents, and the fluorimeter equipped with proper filters, standardize the system by pumping until a steady baseline has been established. Transfer portions of the Standard Preparations and the Assay Preparation to sample cups, and place in the sampler. Start the sampler, and conduct determinations of each Standard Preparation and the Assay Preparation at the rate of 40 per hour, using a ratio of about 2:1 for sample and wash time. Derive a standard response line by plotting the respective Standard Preparation concentration (10.0, 20.0, 30.0, 40.0, and 50.0  $\mu\text{g}$  per mL) versus transmittance. From the measured transmittance and the standard response line, determine the ascorbic acid concentration,  $C$ , in  $\mu\text{g}$  per mL, of the Assay Preparation. Calculate the quantity, in mg, of  $\text{C}_6\text{H}_8\text{O}_6$  in the portion of liquids, tablets, or capsule contents taken by the appropriate formula:

For Liquids: 5C<sub>1</sub>V in which V is the volume, in mL, of liquid preparation taken to prepare the Assay Preparation.

For Tablets or Capsules: 12.5G

#### ASSAY FOR IODIDE

Apparatus— Automatic analyzer consisting of (1) a liquid sampler, (2) a proportioning pump, (3) a heating bath, (4) a suitable colorimeter equipped with a 2.0- × 50-mm flow cell and analysis capability at 420 nm, (5) a means of recording colorimetric readings, and (6) a manifold consisting of the components illustrated in the accompanying pertinent *diagram*.



### Diagram for Automated Iodide Assay

### Reagents—

Acetic Acid Carrier Solution— Transfer 3.0 mL of glacial acetic acid to a 2000-mL volumetric flask containing about 800 mL of water. Add 2 mL of polyoxyethylene (23) lauryl ether, and dilute with water to volume.

**Surfactant Solution**— Prepare a 30% solution of polyoxyethylene (23) lauryl ether by melting 150 g in a container on a steam bath and slowly adding approximately 250 mL of water with continuous stirring. Cool, and dilute with water to make 500 mL.

Arsenious Acid Solution— Transfer 19.6 g of arsenic trioxide and 14.0 g of sodium hydroxide to a 2000-mL volumetric flask. Add about 150 mL of water, and dissolve with stirring. Dilute with water to a volume of about 800 mL, and add 66 mL of sulfuric acid. Cool to room temperature. Transfer 50.0 g of sodium chloride to the solution, and mix to dissolve. Add 2 mL of Surfactant Solution, dilute with water to volume, mix, and filter.

Ceric Ammonium Sulfate Solution— Transfer 12.65 g of ceric ammonium sulfate to a 1000-mL volumetric flask. Add about 700 mL of water followed by 100 mL of sulfuric acid, swirling to mix. Heat to dissolve, and cool to room temperature. Add 1 mL of Surfactant Solution, dilute with water to volume, mix, and filter.

3% Acetic Acid Solution— Transfer 30 mL of glacial acetic acid to a 1000-mL volumetric flask containing about 300 mL of water. Dilute with water to volume, and mix.

#### Standard Preparations—

Standard Stock Solution— Transfer an accurately weighed quantity of 1.3080 g of potassium iodide, previously dried for 24 hours at 105°, to a 1000-mL volumetric flask. Dilute with water to volume, and mix to obtain a solution having an iodide concentration of 1000 µg per mL.

Intermediate Standard Solution— Quantitatively dilute a suitable volume of Standard Stock Solution with water to obtain a solution having an iodide concentration of 1 µg per mL.

Working Standard Preparations— Transfer 2.0, 4.0, 6.0, 8.0, and 10.0 mL of Intermediate Standard Solution to separate 100-mL volumetric flasks. Add 5 mL of 3% Acetic Acid Solution. Dilute the contents of each flask with water to volume, and mix to obtain Standard Preparations A, B, C, D, and E having known iodide concentrations of about 0.02 µg per mL, 0.04 µg per mL, 0.06 µg per mL, 0.08 µg per mL, and 0.1 µg per mL, respectively.

#### Assay Preparation—

For Liquid Preparations— Transfer an accurately measured volume of the liquid preparation, equivalent to 16 µg of iodide, to a 200-mL volumetric flask. Add 10 mL of 3% Acetic Acid Solution to dissolve, dilute with deionized water to volume, mix, and filter. Transfer 10.0 mL of this solution to a 100-mL volumetric flask, add 5.0 mL of 3% Acetic Acid Solution, dilute with deionized water to volume, mix, and filter to obtain a solution having an iodide concentration of about 0.08 µg per mL.

For Tablet Preparations— Weigh and finely powder not fewer than 20 Tablets. Transfer an accurately weighed quantity of the powder, equivalent to about 250 µg of iodide, to a 250-mL volumetric flask. Add 100 mL of 1 N hydrochloric acid, and mix with the aid of sonication for 30 minutes. Dilute with water to volume, mix, and filter. Transfer 8.0 mL of the filtered solution to a 100-mL volumetric flask, add 5 mL of 3% Acetic Acid Solution, dilute with water to volume, and mix to obtain a solution having an iodide concentration of about 0.08 µg per mL.

For Capsule Preparations— Empty the contents, if necessary by cutting open with a sharp blade, of not fewer than 20 Capsules into a suitable container, and mix thoroughly. Transfer a portion of the capsule contents, equivalent to about 250 µg of iodide, to a 250-mL volumetric flask and proceed as directed for Tablets above, beginning with "Add 100 mL of ."

Procedure— With the sample line pumping the Acetic Acid Carrier Solution, the other lines pumping their respective reagents, and the colorimeter equipped with 420-nm filters, standardize the system until a steady baseline has been established. Transfer portions of the Standard Preparations and the Assay Preparation to the sampler cups, and place in the sampler. Start the sampler, and conduct determinations of each Standard Preparation and the Assay Preparation at the rate of 30 per hour, using a ratio of about 1:4 for sample and wash time. Derive a standard response line by plotting the respective Standard Preparation concentration (0.02, 0.04, 0.06, 0.08, and 0.10 µg per mL) versus absorbance. [note—This is an indirect absorbance relationship: the greater the iodide amount, the less the absorbance.] From the measured transmittance and the standard response line, determine the iodide concentration, C, in µg per mL, of the Assay Preparation. Calculate the quantity, in µg, of iodide in the portion of liquids, tablets, or capsules contents taken by the formula:

For Liquids:  $2000C/V$  in which V is the volume, in mL, of the liquid preparation taken to prepare the Assay Preparation.

For Tablets and Capsules: 3125C.

#### CONTENT UNIFORMITY OF NITROGLYCERIN TABLETS

This is not to be considered as the official method. It is detailed here for further illustration of descriptions of automated methods.

Apparatus— Automatic analyzer consisting of (1) a liquid sampler, (2) a proportioning pump, (3) a heating bath, (4) a suitable spectrophotometer equipped with a 5-mm flow cell and analysis capability at 545 nm, (5) a means of recording spectrophotometric readings, and (6) a manifold consisting of the components illustrated in the accompanying pertinent diagram.

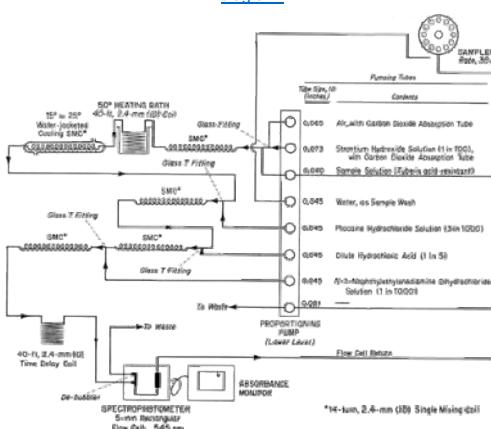


Diagram for Automated Assay for Nitroglycerin Tablets

#### Reagents—

1 Percent Strontium Hydroxide Solution— Dissolve 20.0 g of strontium hydroxide [Sr(OH)<sub>2</sub>·8H<sub>2</sub>O] in 1800 mL of carbon dioxide-free water, heating if necessary. Cool to room temperature, dilute with carbon dioxide-free water to 2000 mL, and mix. Allow to stand overnight, and filter. Store the clear solution in tightly closed containers, protected from carbon dioxide.

0.3 Percent Procaine Hydrochloride Solution— Dissolve 3.0 g of procaine hydrochloride in water to make 1000 mL.

0.1 Percent N-(1-Naphthyl)ethylenediamine Dihydrochloride Solution— Dissolve 1.0 g of N-(1-naphthyl)ethylenediamine dihydrochloride in water to make 1000 mL. Prepare fresh each week.

Standard Preparation— Dissolve an accurately weighed portion of 10% nitroglycerin-beta lactose absorbate, previously standardized, in water, and dilute quantitatively and stepwise with water to obtain a solution having a known concentration of about 30 µg per mL.

Test Preparation— Dissolve 1 Nitroglycerin Tablet in water to obtain a solution having a concentration of about 30 µg of nitroglycerin per mL.

Procedure— With the sample line pumping water, the other lines pumping their respective reagents, and the spectrophotometer set at 545 nm, standardize the system by pumping until a steady absorbance baseline has been established. Transfer portions of the Standard Preparation and the Test Preparation to sampler cups, and place in the sampler. Start the sampler, and conduct determinations of the Standard Preparation and the Test Preparation at a rate of 30 per hour, using a ratio of 1:1 for sample and wash time. First, run two standards, discarding the first value, then continue the run using one standard after each five samples, recording the absorbance values. Calculate the quantity, in mg, of C<sub>3</sub>H<sub>5</sub>N<sub>3</sub>O<sub>9</sub> in the Tablet taken by the formula:



in which T is the labeled quantity, in mg, of nitroglycerin in the Tablet; D is the concentration, in  $\mu\text{g}$  per mL, of nitroglycerin in the solution from the Tablet, based on the labeled quantity per Tablet and the extent of dilution; C is the concentration, in  $\mu\text{g}$  per mL, of nitroglycerin in the Standard Preparation; AU is the absorbance of the Test Preparation; and AS is the average of the absorbances of the two Standard Preparations that bracket the Test Preparation.

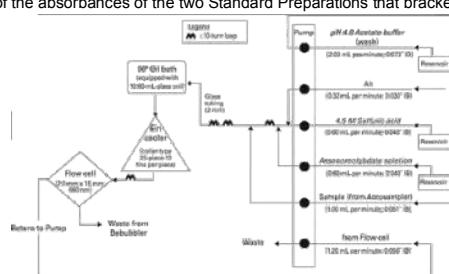


Diagram of Dissolution Test Method for Erythromycin Ethylsuccinate Tablets Labeled as Chewable

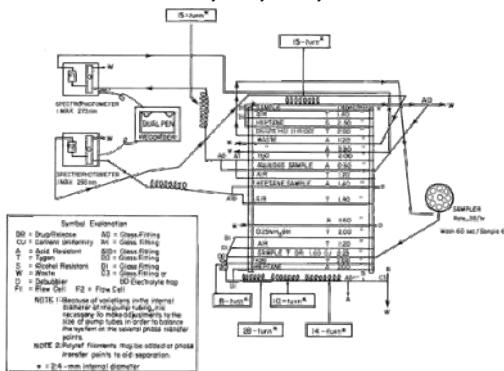


Diagram for Automated Drug Release and Content Uniformity Test for Propranolol Hydrochloride and Hydrochlorothiazide Extended-Release Capsules

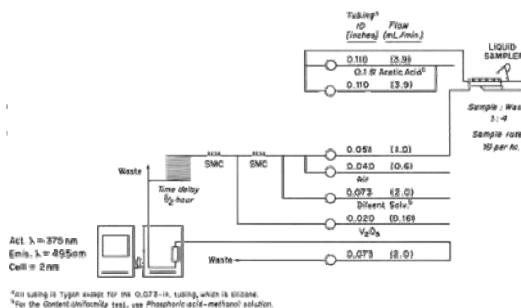


Diagram for Automated Dissolution and Content Uniformity Test for Reserpine Tablets

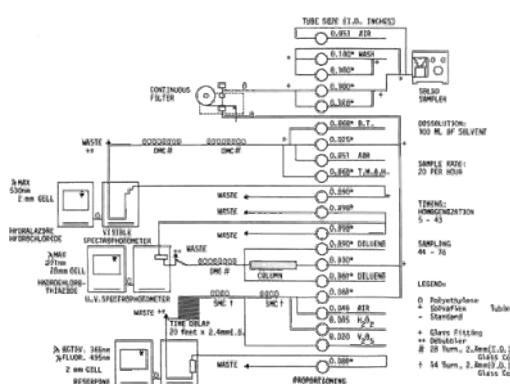


Diagram for Automated Content Uniformity Test for Reserpine, Hydralazine Hydrochloride, and Hydrochlorothiazide Tablets

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USEPA.

Auxiliary Information—Please <a href="#">check for your question in the FAQs</a> before contacting UCI.		
Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Gary E. Ritchie, M.Sc.</a> Scientific Fellow 1-301-816-8353	(GC05) General Chapters 05
Reference Standards	Lili Wang, Technical Services Scientist 1-301-816-8129 <a href="mailto:RSTech@usp.org">RSTech@usp.org</a>	



Temperature reading devices suitable for Pharmacopeial tests conform to specifications that are traceable to a NIST standard. Temperature reading devices may be of the liquid-in-glass type or an analog or digital temperature indicator type, such as a resistance temperature device, thermistor, or thermocouple.

An analog or digital temperature indicator consists of a temperature probe, which houses a sensor. The probe is attached to a meter capable of translating a signal in ohms or millivolts into a temperature reading. The temperature probe portion of the analog or digital temperature indicator that is submerged in the medium whose temperature is being measured must be made of inert material. Standardization of analog and digital temperature indicator devices is performed on an established testing frequency with a temperature standard traceable to NIST. In the selection of a temperature reading device, careful consideration of the condition under which it is to be used is essential.

Liquid-in-glass thermometers may be standardized for total immersion, partial immersion, or full immersion. Insofar as practicable, each thermometer should be employed according to the condition of immersion under which it was standardized. Standardization of thermometers is performed on an established testing frequency with a temperature standard traceable to NIST. Refer to the current issue of ASTM standards E1. Standardization of liquid-in-glass thermometers for total immersion involves immersion of the thermometer to the top of the liquid column, with the remainder of the stem and the upper expansion chamber exposed to ambient temperature. Standardization for partial immersion involves immersion of the thermometer to the indicated immersion line etched on the front of the thermometer, with the remainder of the stem exposed to ambient temperature. Standardization for full immersion involves immersion of the entire thermometer, with no portion of the stem exposed to ambient temperature. For use under other conditions of immersion, an emergent stem correction is necessary to obtain correct temperature readings.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Antonio Hernandez-Cardoso, B.S.</a> Scientist, Latin American Specialist 1-301-816-8308	(GC05) General Chapters 05

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### 31 VOLUMETRIC APPARATUS

Most of the volumetric apparatus available in the United States is calibrated at 20°, although the temperatures generally prevailing in laboratories more nearly approach 25°. To minimize volumetric error, the temperature should be the same for the volumetric apparatus, the material being prepared, the solvents being used to prepare the volumetric solutions, the area in which they are prepared, and the final volume adjustment.

**Use**— To attain the degree of precision required in many Pharmacopeial assays involving volumetric measurements and directing that a quantity be "accurately measured," the apparatus must be chosen and used with care. A buret should be of such size that the titrant volume represents not less than 30% of the nominal volume. Where less than 10 mL of titrant is to be measured, a 10-mL buret or a microburet generally is required.

The design of volumetric apparatus is an important factor in assuring accuracy. For example, the length of the graduated portions of graduated cylinders should be not less than five times the inside diameter, and the tips of burets and pipets should restrict the outflow rate to not more than 500 µL per second.

**Standards of Accuracy**— The capacity tolerances for volumetric flasks, transfer pipets, and burets are those accepted by the National Institute of Standards and Technology (Class A),<sup>1</sup> as indicated in the accompanying tables. Use Class A volumetric apparatus unless otherwise specified in the individual monograph. For plastic volumetric apparatus the accepted capacity tolerances are Class B.<sup>2</sup>

The capacity tolerances for measuring (i.e., "graduated") pipets of up to and including 10-mL capacity are somewhat larger than those for the corresponding sizes of transfer pipets, namely, 10, 20, and 30 µL for the 2-, 5-, and 10-mL sizes, respectively.

Transfer and measuring pipets calibrated "to deliver" should be drained in a vertical position and then touched against the wall of the receiving vessel to drain the tips. Volume readings on burets should be estimated to the nearest 0.01 mL for 25- and 50-mL burets, and to the nearest 0.005 mL for 5- and 10-mL burets. Pipets calibrated "to contain" are called for in special cases, generally for measuring viscous fluids like syrups; however, a volumetric flask may be substituted for a "to contain" pipet. In such cases, the pipet or flask should be washed clean, after draining, and the washings added to the measured portion.

#### Volumetric Flasks

Designated volume, mL	10	25	50	100	250	500	1000
Limit of error, mL	0.02	0.03	0.05	0.08	0.12	0.20	0.30
Limit of error, %	0.20	0.12	0.10	0.08	0.05	0.04	0.03

#### Transfer Pipets

Designated volume, mL	1	2	5	10	25	50	100
Limit of error, mL	0.006	0.006	0.01	0.02	0.03	0.05	0.08
Limit of error, %	0.60	0.30	0.20	0.20	0.12	0.10	0.08

#### Burets

Designated volume, mL	10 ("micro" type)			25	50
Subdivisions, mL	0.02			0.1	0.1
Limit of error, mL	0.02			0.03	0.05

1 See ASTM 288-06, ASTM E287-02, ASTM E1189-00, and ASTM E969-02.

2 See ASTM E 288, Fed. Spec. NNN-F-289, and ISO Standard 384.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Horacio N. Pappa, Ph.D.</a> Senior Scientist and Latin American Liaison 1-301-816-8319	(GC05) General Chapters 05

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### 41 WEIGHTS AND BALANCES

The intent of this section is to bring the requirements for weights into conformity with American National Standard ANSI/ASTM E617, "Laboratory Weights and Precision Mass Standards." This standard is incorporated by reference and should be consulted for full descriptions and information on the tolerances and construction of weights.<sup>1</sup>

Pharmacopeial tests and assays require balances that vary in capacity, sensitivity, and reproducibility. Unless otherwise specified, when substances are to be "accurately weighed" for

say, the weighing is to be performed with a weighing device whose measurement uncertainty (random plus systematic error) does not exceed 0.1% of the reading. Measurement uncertainty is satisfactory if three times the standard deviation of not less than ten replicate weighings divided by the amount weighed, does not exceed 0.001. Unless otherwise specified, for titrimetric limits tests, the weighing shall be performed to provide the number of significant figures in the weight of the analyte that corresponds to the number of significant figures in the concentration of the titrant.

The class designations below are in order of increasing tolerances.

Class 1.1 weights are used for calibration of low-capacity, high-sensitivity balances. They are available in various denominations from 1 to 500 mg. The tolerance for any denomination in this class is 5 µg. They are recommended for calibration of balances using optical or electrical methods for accurately weighing quantities below 20 mg.

Class 1 weights are designated as high-precision standards for calibration. They may be used for weighing accurately quantities below 20 mg. (For weights of 10 g or less, the requirements of class 1 are met by USP XXI class M.)

Class 2 weights are used as working standards for calibration, built-in weights for analytical balances, and laboratory weights for routine analytical work. (The requirements of class 2 are met by USP XXI class S.)<sup>2</sup>

Class 3 and class 4 weights are used with moderate-precision laboratory balances. (Class 3 requirements are met by USP XXI class S-1; class 4 requirements are met by USP XXI class P.)<sup>2</sup>

A weight class is chosen so that the tolerance of the weights used does not exceed 0.1% of the amount weighed. Generally, class 2 may be used for quantities greater than 20 mg, class 3 for quantities of greater than 50 mg, and class 4 for quantities of greater than 100 mg. Weights should be calibrated periodically, preferably against an absolute standard weight.

1 Copies of ASTM Standard E 617-81 (Reapproved 1985) may be obtained from the American Society for Testing and Materials, 1916 Race Street, Philadelphia, PA 19103.

2 Note that the designations S and P no longer designate weight classes but rather weight grades, that is, design limitations such as range of density of materials, surface area, surface finish, corrosion resistance, and hardness.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Horacio N. Pappa, Ph.D.</a> Senior Scientist and Latin American Liaison 1-301-816-8319	(GC05) General Chapters 05

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#### 51 ANTIMICROBIAL EFFECTIVENESS TESTING

Antimicrobial preservatives are substances added to nonsterile dosage forms to protect them from microbiological growth or from microorganisms that are introduced inadvertently during or subsequent to the manufacturing process. In the case of sterile articles packaged in multiple-dose containers, antimicrobial preservatives are added to inhibit the growth of microorganisms that may be introduced from repeatedly withdrawing individual doses.

Antimicrobial preservatives should not be used as a substitute for good manufacturing practices or solely to reduce the viable microbial population of a nonsterile product or control the presterilization bioburden of multidose formulations during manufacturing. Antimicrobial preservatives in compendial dosage forms meet the requirements for Added Substances under Ingredients and Processes in the General Notices.

All useful antimicrobial agents are toxic substances. For maximum protection of patients, the concentration of the preservative shown to be effective in the final packaged product should be below a level that may be toxic to human beings.

The concentration of an added antimicrobial preservative can be kept at a minimum if the active ingredients of the formulation possess an intrinsic antimicrobial activity. Antimicrobial effectiveness, whether inherent in the product or whether produced because of the addition of an antimicrobial preservative, must be demonstrated for all injections packaged in multiple-dose containers or for other products containing antimicrobial preservatives. Antimicrobial effectiveness must be demonstrated for multiple-dose topical and oral dosage forms and for other dosage forms such as ophthalmic, otic, nasal, irrigation, and dialysis fluids (see [Pharmaceutical Dosage Forms](#) 1151).

This chapter provides tests to demonstrate the effectiveness of antimicrobial protection. Added antimicrobial preservatives must be declared on the label. The tests and criteria for effectiveness apply to a product in the original, unopened container in which it was distributed by the manufacturer.

#### PRODUCT CATEGORIES

For the purpose of testing, compendial articles have been divided into four categories (see [Table 1](#)). The criteria of antimicrobial effectiveness for these products are a function of the route of administration.

Table 1. Compendial Product Categories

Category	Product Description
1	Injections, other parenterals including emulsions, otic products, sterile nasal products, and ophthalmic products made with aqueous bases or vehicles.
2	Topically used products made with aqueous bases or vehicles, nonsterile nasal products, and emulsions, including those applied to mucous membranes.
3	Oral products other than antacids, made with aqueous bases or vehicles.
4	Antacids made with an aqueous base.

#### TEST ORGANISMS

Use cultures of the following microorganisms<sup>1</sup>: *Candida albicans* (ATCC No. 10231), *Aspergillus niger* (ATCC No. 16404), *Escherichia coli* (ATCC No. 8739), *Pseudomonas aeruginosa* (ATCC No. 9027), and *Staphylococcus aureus* (ATCC No. 6538). The viable microorganisms used in the test must not be more than five passages removed from the original ATCC culture. For purposes of the test, one passage is defined as the transfer of organisms from an established culture to fresh medium. All transfers are counted. In the case of organisms maintained by seed-lot techniques, each cycle of freezing, thawing, and revival in fresh medium is taken as one transfer. A seed-stock technique should be used for long-term storage of cultures. Cultures received from the ATCC should be resuscitated according to directions. If grown in broth, the cells are pelleted by centrifugation. Resuspend in 1/20th the volume of fresh maintenance broth, and add an equal volume of 20% (v/v in water) sterile glycerol. Cells grown on agar may be scraped from the surface into the 10% glycerol broth. Dispense small aliquots of the suspension into sterile vials. Store the vials in liquid nitrogen or in a mechanical freezer at no more than  $-50^{\circ}\text{C}$ . When a fresh seed-stock

material is required, it may be removed and used to inoculate a series of working cultures. These working cultures may then be used periodically (each day in the case of bacteria and yeast) to start the inoculum culture.

#### MEDIA

All media used in the test must be tested for growth promotion. Use the microorganisms indicated above under Test Organisms.

#### PREPARATION OF INOCULUM

Preparatory to the test, inoculate the surface of a suitable volume of solid agar medium from a recently revived stock culture of each of the specified microorganisms. The culture conditions for the inoculum culture are described in [Table 2](#) in which the suitable media are Soybean-Casein Digest or Sabouraud Dextrose Agar Medium (see [Microbial Enumeration Tests](#) [61](#) and [Tests for Specified Microorganisms](#) [62](#)).

To harvest the bacterial and *C. albicans* cultures, use sterile saline TS, washing the surface growth, collecting it in a suitable vessel, and adding sufficient sterile saline TS to obtain a microbial count of about  $1 \times 10^8$  colony-forming units (cfu) per mL. To harvest the cells of *A. niger*, use sterile saline TS containing 0.05% of polysorbate 80, and add sufficient sterile saline TS to obtain a count of about  $1 \times 10^8$  cfu per mL.

Alternatively, the stock culture organisms may be grown in a suitable liquid medium (i.e., Soybean-Casein Digest Broth or Sabouraud Dextrose Broth) and the cells harvested by centrifugation, then washed and resuspended in sterile saline TS to obtain a microbial count of about  $1 \times 10^8$  cfu per mL. [note—The estimate of inoculum concentration may be performed by turbidimetric measurements for the challenge microorganisms. Refrigerate the suspension if it is not used within 2 hours.]

Determine the number of cfu per mL in each suspension, using the conditions of media and microbial recovery incubation times listed in Table 2 to confirm the initial cfu per mL estimate. This value serves to calibrate the size of inoculum used in the test. The bacterial and yeast suspensions are to be used within 24 hours of harvest, but the fungal preparation may be stored under refrigeration for up to 7 days.

#### PROCEDURE

The test can be conducted either in five original containers if sufficient volume of product is available in each container and the product container can be entered aseptically (i.e., needle and syringe through an elastomeric rubber stopper), or in five sterile, capped bacteriological containers of suitable size into which a sufficient volume of product has been transferred. Inoculate each container with one of the prepared and standardized inoculum, and mix. The volume of the suspension inoculum used is between 0.5% and 1.0% of the volume of the product. The concentration of test microorganisms that is added to the product (Categories 1, 2, and 3) are such that the final concentration of the test preparation after inoculation is between  $1 \times 10^5$  and  $1 \times 10^6$  cfu per mL of the product. For Category 4 products (antacids) the final concentration of the test preparation after inoculation is between  $1 \times 10^3$  and  $1 \times 10^4$  cfu per mL of the product.

The initial concentration of viable microorganisms in each test preparation is estimated based on the concentration of microorganisms in each of the standardized inoculum as determined by the plate-count method.

Incubate the inoculated containers at  $22.5 \pm 2.5^\circ$ . Sample each container at the appropriate intervals specified in [Table 3](#). Record any changes observed in appearance at these intervals. Determine by the plate-count procedure the number of cfu present in each test preparation for the applicable intervals (see Procedure under [Microbial Enumeration Tests](#) [61](#) and [Tests for Specified Microorganisms](#) [62](#)). Incorporate an inactivator (neutralizer) of the specific antimicrobial in the plate count or in the appropriate dilution prepared for plating. These conditions are determined in the validation study for that sample based upon the conditions of media and microbial recovery incubation times listed in [Table 2](#). Using the calculated concentrations of cfu per mL present at the start of the test, calculate the change in  $\log_{10}$  values of the concentration of cfu per mL for each microorganism at the applicable test intervals, and express the changes in terms of log reductions.

Table 2. Culture Conditions for Inoculum Preparation

Organism	Suitable Medium	Incubation Temperature	Inoculum Incubation Time	Microbial Recovery Incubation Time
Escherichia coli (ATCC No. 8739)	Soybean-Casein Digest Broth; Soybean-Casein Digest Agar	$32.5 \pm 2.5^\circ$	18 to 24 hours	3 to 5 days
Pseudomonas aeruginosa (ATCC No. 9027)	Soybean-Casein Digest Broth; Soybean-Casein Digest Agar	$32.5 \pm 2.5^\circ$	18 to 24 hours	3 to 5 days
Staphylococcus aureus (ATCC No. 6538)	Soybean-Casein Digest Broth; Soybean-Casein Digest Agar	$32.5 \pm 2.5^\circ$	18 to 24 hours	3 to 5 days
Candida albicans (ATCC No. 10231)	Sabouraud Dextrose Agar; Sabouraud Dextrose Broth	$22.5 \pm 2.5^\circ$	44 to 52 hours	3 to 5 days
Aspergillus niger (ATCC No. 16404)	Sabouraud Dextrose Agar; Sabouraud Dextrose Broth	$22.5 \pm 2.5^\circ$	6 to 10 days	3 to 7 days

#### CRITERIA FOR ANTIMICROBIAL EFFECTIVENESS

The requirements for antimicrobial effectiveness are met if the criteria specified under [Table 3](#) are met (see Significant Figures and Tolerances under General Notices). No increase is defined as not more than 0.5  $\log_{10}$  unit higher than the previous value measured.

Table 3. Criteria for Tested Microorganisms

For Category 1 Products	
Bacteria:	Not less than 1.0 log reduction from the initial calculated count at 7 days, not less than 3.0 log reduction from the initial count at 14 days, and no increase from the 14 days' count at 28 days.
Yeast and Molds:	No increase from the initial calculated count at 7, 14, and 28 days.
For Category 2 Products	
Bacteria:	Not less than 2.0 log reduction from the initial count at 14 days, and no increase from the 14 days' count at 28 days.
Yeast and Molds:	No increase from the initial calculated count at 14 and 28 days.
For Category 3 Products	
Bacteria:	Not less than 1.0 log reduction from the initial count at 14 days, and no increase from the 14 days' count at 28 days.
Yeast and Molds:	No increase from the initial calculated count at 14 and 28 days.
For Category 4 Products	
Bacteria, Yeast, Molds:	No increase from the initial calculated count at 14 and 28 days.



and Molds:

1 Available from American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209 (<http://www.atcc.org>).Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Radhakrishna S Tirumalai, Ph.D.</a> Senior Scientist 1-301-816-8339	(MSA05) Microbiology and Sterility Assurance

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## 55 BIOLOGICAL INDICATORS—RESISTANCE PERFORMANCE TESTS

### TOTAL Viable SPORE COUNT

For paper carrier biological indicators, remove three specimens of the relevant biological indicators from their original individual containers. Disperse the paper into component fibers by placing the test specimens in a sterile 250-mL cup of a suitable blender containing 100 mL of chilled, sterilized [Purified Water](#) and blending for a time known to be adequate to achieve a homogeneous suspension. It is not unusual for blending times of 15 minutes or more to be required for optimal recovery. Transfer a 10-mL aliquot of the suspension to a sterile, screw-capped 16- × 125-mm tube. For [Biological Indicator for Steam Sterilization, Paper Carrier](#), heat the tube containing the suspension in a water bath at 95° to 100° for 15 minutes (heat shock), starting the timing when the temperature reaches 95°. For [Biological Indicator for Dry-Heat Sterilization, Paper Carrier](#), and for [Biological Indicator for Ethylene Oxide Sterilization, Paper Carrier](#), heat the tube containing the suspension in a water bath at 80° to 85° for 10 minutes, starting the timing when the temperature of the spore suspension reaches 80°. Cool rapidly in an ice-water bath at 0° to 4°. Transfer two 1-mL aliquots to suitable tubes, and make appropriate serial dilutions in sterilized [Purified Water](#); the dilutions being selected as calculated to yield preferably 30 to 300 colonies, but not less than 6, on each of a pair of plates when treated as described below. Where the biological indicator has a low spore concentration, it may be necessary to modify the dilution series and to use more plates at each dilution. Prepare a separate series of plates for each aliquot. Place 1.0 mL of each selected dilution in each of two 15- × 100-mm Petri dishes. Within 20 minutes, add to each plate 20 mL of Soybean–Casein Digest Agar Medium that has been melted and cooled to 45° to 50°. Swirl to attain a homogeneous suspension, and allow it to solidify. Incubate the plates in an inverted position at 55° to 60° for [Biological Indicator for Steam Sterilization, Paper Carrier](#), and at 30° to 35° for [Biological Indicator for Ethylene Oxide Sterilization, Paper Carrier](#) and for [Biological Indicator for Dry-Heat Sterilization, Paper Carrier](#) or at the optimal recovery temperature specified by the manufacturer. Examine the plates after 24 and 48 hours, recording for each plate the number of colonies; and use the number of colonies observed after 48 hours to calculate the results. Calculate the average number of spores per specimen from the results, using the appropriate dilution factor. The test is valid if the log number of spores per carrier at 48 hours is equal to or greater than the log number after 24 hours in each case. For [Biological Indicator for Steam Sterilization, Self-Contained](#), aseptically remove the three carriers from the container, and proceed as directed for [Biological Indicator for Steam Sterilization, Paper Carrier](#).

For [Biological Indicators for Moist Heat, Dry Heat, and Gaseous Modes of Sterilization, Nonpaper Carriers](#), aseptically remove the three carriers from their original packaging or container. Place each carrier in a suitable sterile container containing 100 mL of chilled [Purified Water](#), and sonicate or shake on a reciprocal shaker for an appropriate time. Fifteen minutes or more may be required for optimal recovery. A previous study should be conducted that ensures that the recovery method results in at least 50% to 300% recovery of the labeled spore viable count. Transfer a 10-mL aliquot of the suspension to a sterile, screw-capped 16- × 125-mm tube. Heat the tubes containing suspensions of *Bacillus atrophaeus*, *Bacillus subtilis*, and *Bacillus coagulans* at 80° to 85° for 10 minutes. Heat the tubes containing a suspension of *Geobacillus stearothermophilus* at 95° to 100° for 15 minutes. Start the timing when the lowest temperature of the stated temperature ranges is reached. Cool rapidly in an ice-water bath at 0° to 4°. Transfer two 1-mL aliquots to suitable tubes, and make appropriate serial dilutions in [Purified Water](#). The selected dilutions should be those that will preferably yield 30 to 300 colonies but not fewer than 6 on each pair of plates when treated as described below. When the biological indicator has a low spore concentration, it may be necessary to modify the dilution series and to use more plates at each dilution. Prepare a separate series of plates for each aliquot. Place 1.0 mL of each selected dilution in each of two 15- × 100-mm Petri dishes. Within 20 minutes add the aliquot to each plate containing 20 mL of agar that has been melted and cooled to between 45° and 50°. Swirl to attain a homogeneous suspension.

For *G. stearothermophilus*, *B. atrophaeus*, *B. subtilis*, and *B. coagulans*, use Soybean–Casein Digest Agar Medium and incubate the plates in an inverted position aerobically at the following respective temperatures for each microorganism: 55° to 60°, 30° to 35°, and 48° to 52°, or at the optimum temperature specified by the biological indicator manufacturer. Examine the plates after 24 and 48 hours. Record the number of colonies observed on each plate. Calculate the average number of spores per carrier from the results, using the appropriate dilution factor. The test is valid if the log number of spores per carrier at 48 hours is equal to or greater than the log number after 24 hours in each case.

For [Biological Indicators for Moist Heat, Dry Heat, and Gaseous Modes of Sterilization, Liquid Spore Suspensions](#), using *G. stearothermophilus*, *B. atrophaeus*, *B. subtilis*, and *B. coagulans* as biological indicators, prepare an appropriate serial dilution of the original spore suspension in chilled [Purified Water](#) contained in a sterile, screw-capped 16- × 125-mm tube, and proceed with the viable spore count procedures specified under [Biological Indicators for Moist Heat, Dry Heat, and Gaseous Modes of Sterilization, Nonpaper Carriers](#).

### D-VALUE DETERMINATION

Conduct all the tests described in this section under aseptic conditions, using sterilized equipment for nonthermophilic microorganisms. D-value determination for *G. stearothermophilus* and *B. coagulans* can be performed in a controlled but unclassified environment.

#### Apparatus

The test equipment for the determination of microbial resistance is described in substantial detail in ISO 18472, Sterilization of Health Care Products—Biological and Chemical Indicators—Test Equipment.<sup>1</sup> The details of individual Biological Indicator Evaluation Resistometers (BIERs) vary with the specifics of their design and the particular sterilization process in conjunction with which they are used. Provided that the performance of the BIER vessel meets the requirements of the ISO standard for exposure of the biological indicator, design differences are acceptable.

#### Procedure

Carry out the tests for D value at each of the applicable sets of sterilization conditions for which the packaged biological indicator under test is labeled for use. Take a sufficient number of groups of specimens of biological indicators in their original individual containers, each group consisting of not less than 5 specimens. The number of groups provides a range of observations from not less than one labeled D value below the labeled survival time through not less than one labeled D value above the labeled kill time. Place each group on a separate suitable specimen holder that permits each specimen to be exposed to the prescribed sterilizing condition at a specific location in the sterilizing chamber of the BIER. Check the BIER apparatus for operating parameters using specimen holders without specimens. Select a series of sterilizing times in increments from the shortest time for the specimens to be tested. The differences in sterilizing times over the series are as constant as feasible, and the difference between adjacent times is no greater than 75% of the labeled D value.

Test procedures for the use of BIER vessels for the evaluation of microbial resistance are defined in a series of ISO standards under the 11138 series.<sup>2,3,4,5</sup> The appropriate standard should be followed for the biological indicator. The test methods and carriers used with the BIER may be adapted to the specifics of the biological indicator. The method and apparatus used for paper carriers may differ from those for other carriers and will be substantially different from those used for suspensions of biological indicators.

The D-value exposure conditions for alternative material carriers are the same as the conditions used to determine the D value for paper carriers. If the manufacturer's label permits usage of the biological indicator carrier with multiple sterilization methods, then data on D value, survival time, and kill time will need to be provided by the manufacturer for each sterilization method. It is possible that biological indicators inoculated onto carriers other than paper will be used for gaseous or vapor sterilization/decontamination methods such as vapor phase hydrogen peroxide and chlorine dioxide.



Standard physical conditions for the evaluation of biological indicators for use with vapor phase hydrogen peroxide or chlorine dioxide have not been defined. In the case of chlorine dioxide, concentration of the gas, relative humidity, and temperature are critical process control conditions that can be accurately measured. The manufacturer of biological indicators marketed for use with chlorine dioxide should state the conditions under which the D-value determination was conducted so that the user can at least discern the resistance of a lot of biological indicators as compared to their own anticipated use conditions. The situation with vapor phase hydrogen peroxide is a more complex one. Various equipment manufacturers

have proposed different decontamination or sterilization conditions. Thus, there is no standard process for the conduct of vapor phase hydrogen decontamination or surface sterilization. It follows, then, that there are no industry standard biological indicator evaluation methods for vapor hydrogen peroxide, and it has been reported that there may not be a direct correlation between vapor concentration and rate or even effectiveness of biological indicator inactivation. Additionally, it is difficult to accurately assess relative humidity, which is often defined as a critical process parameter, in the presence of vapor hydrogen peroxide. For these reasons it is more reasonable to consider resistance of biological indicators to be a relative or comparative measure from the manufacturer rather than a true D value. It follows that, depending upon equipment and processes employed, it may be impossible for an end user to duplicate the biological indicator resistance tests performed by the manufacturer.

For [Biological Indicators for Moist Heat, Dry Heat, and Gaseous Modes of Sterilization, Liquid Spore Suspensions](#), conduct D-value determinations for each of the microorganisms that are provided as a liquid spore crop suspension. The test is conducted using appropriate serial dilutions predicated upon the stated spore titer of the suspension in [Purified Water](#) in a sterile tube.

Where the suspension is placed on or in a substrate such as an elastomeric closure or formulated product, its resistance may differ from that determined in [Purified Water](#). That difference may be significant to the usage of the biological indicators and appropriate measurements made prior to use in sterilization validation activities.

#### Recovery

After completion of the sterilizing procedure for [Biological Indicator for Dry-Heat Sterilization, Paper Carrier](#); [Biological Indicator for Ethylene Oxide Sterilization, Paper Carrier](#); or [Biological Indicator for Steam Sterilization, Paper Carrier](#), whichever is applicable, and within a noted time not more than 4 hours, aseptically remove and add each strip to a suitable medium (see Media under [Sterility Tests](#) 71) to submerge the biological indicator completely in a suitable tube. For each [Biological Indicator for Steam Sterilization, Self-Contained](#) specimen, the paper strip is immersed in the self-contained medium according to manufacturers' instructions, within a noted time not more than 4 hours. Incubate each tube at the optimal recovery temperature specified by the manufacturer. Observe each inoculated medium-containing tube at appropriate intervals for a total of 7 days after inoculation. (Where growth is observed at any particular observation time, further incubation of the specimen(s) concerned may be omitted.) Note the number of specimens showing no evidence of growth at any time.

For [Biological Indicators for Moist Heat, Dry Heat, and Gaseous Modes of Sterilization, Nonpaper Carriers](#), recovery of spores from the biological indicator carriers will follow recovery procedures described in the procedures under Total Viable Spore Count. D-value determination methods for paper carrier biological indicators may be used to calculate the D value for nonpaper carriers. Incubation conditions for the microorganisms that may be used for nonpaper biological indicators are described in the Total Viable Spore Count section.

For [Biological Indicators for Moist Heat, Dry Heat, and Gaseous Modes of Sterilization, Liquid Spore Suspensions](#), the method of recovery following sterilization exposure conditions are those methods described in the Total Viable Spore Count section for liquid suspensions, and when a dry heat D-value determination is made from *B. atrophaeus* suspensions, the same recovery procedures as described under [Biological Indicator for Steam Sterilization, Paper Carrier](#) are followed.

Where *C. sporogenes* is used as a biological indicator, methods for preparation, inoculation, and recovery methods and media must be adapted to accommodate the use of this anaerobic sporeformer.

#### Calculation

The determination of D values of biological indicators can be performed using the Limited Spearman-Karber, Survival Curve Method or Stumbo-Murphy-Cochran procedures 6,7,8. It is preferable to use the same method as that defined by the biological indicator manufacturer to determine D values. The use of a different method can result in differences that are more an artifact of the method than a variation in the performance of the biological indicator.

#### Survival Time and Kill Time

Take two groups, each consisting of 10 specimens of the relevant biological indicator, in their original, individual containers. Place the specimens of a group in suitable specimen holders that permit each specimen to be exposed to the sterilizing conditions at a specific location in the BIER chamber.

Expose the specimens for the required survival time, enter the chamber, and remove the holder(s) containing the 10 specimens. Repeat the above procedure immediately, or preheat if a substantial interval has elapsed, so as to subject the second holder(s) containing 10 specimens similarly to the first conditions, but for the required kill time.

The Survival time and kill time for all monographed biological indicators is described in the official monograph under the heading for each.

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- 1 ANSI/ AAMI/ ISO 18472:2006, Sterilization of Health Care Products—Biological and Chemical Indicators—Test Equipment. Association for the Advancement of Medical Instrumentation (AAMI), 1110 N. Glebe Road, Suite 220, Arlington, VA 22201-4795
- 2 ANSI/AAMI/ISO 11138-1:2006, Sterilization of health care products—Biological indicators—Part 1: General requirements, 2nd ed. Association for the Advancement of Medical Instrumentation (AAMI), 1110 N. Glebe Road, Suite 220, Arlington, VA.
- 3 ANSI/AAMI/ISO 11138-2:2006, Sterilization of health care products—Biological indicators—Part 2: Biological indicators for ethylene oxide sterilization processes, 3rd ed. Association for the Advancement of Medical Instrumentation (AAMI), 1110 N. Glebe Road, Suite 220, Arlington, VA.
- 4 ANSI/AAMI/ISO 11138-3:2006, Sterilization of health care products—Biological indicators—Part 3: Biological indicators for moist heat sterilization processes. Association for the Advancement of Medical Instrumentation (AAMI), 1110 N. Glebe Road, Suite 220, Arlington, VA.
- 5 ANSI/AAMI/ISO 11138-4:2006, Sterilization of health care products—Biological indicators—Part 4: Biological indicators for dry heat sterilization processes. Association for the Advancement of Medical Instrumentation (AAMI), 1110 N. Glebe Road, Suite 220, Arlington, VA.
- 6 Pflug, I.J. Syllabus for an Introductory Course in the Microbiology and Engineering of Sterilization Processes, 4th ed. St. Paul, MN: Environmental Sterilization Services, 1980.
- 7 Pflug, I.J., and G.M. Smith. The Use of Biological Indicators for Monitoring Wet-Heat Sterilization Processes, in Sterilization of Medical Products, ed. E.R.L. Gaughan and K. Kereluk. New Brunswick, NJ: Johnson and Johnson, 1977, 193–230.
- 8 Holcomb, R.G., and I.J. Pflug. The Spearman-Karber Method of Analyzing Quantal Assay Microbial Destruction Data, in Microbiology and Engineering Sterilization Processes, ed. I.J. Pflug. St. Paul, MN: Environmental Sterilization Services, 1979.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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## 61 MICROBIOLOGICAL EXAMINATION OF NONSTERILE PRODUCTS: MICROBIAL ENUMERATION TESTS

### INTRODUCTION

The tests described hereafter will allow quantitative enumeration of mesophilic bacteria and fungi that may grow under aerobic conditions.



The tests are designed primarily to determine whether a substance or preparation complies with an established specification for microbiological quality. When used for such purposes follow the instructions given below, including the number of samples to be taken, and interpret the results as stated below.

The methods are not applicable to products containing viable microorganisms as active ingredients.

Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence to the Pharmacopeial method has been demonstrated.

#### GENERAL PROCEDURES

Carry out the determination under conditions designed to avoid extrinsic microbial contamination of the product to be examined. The precautions taken to avoid contamination must be such that they do not affect any microorganisms that are to be revealed in the test.

If the product to be examined has antimicrobial activity, this is, insofar as possible, removed or neutralized. If inactivators are used for this purpose, their efficacy and their absence of toxicity for microorganisms must be demonstrated.

If surface-active substances are used for sample preparation, their absence of toxicity for microorganisms and their compatibility with any inactivators used must be demonstrated.

#### ENUMERATION METHODS

Use the Membrane Filtration method or one of the Plate-Count Methods, as directed. The Most-Probable-Number (MPN) Method is generally the least accurate method for microbial counts; however, for certain product groups with very low bioburden, it may be the most appropriate method.

The choice of a method is based on factors such as the nature of the product and the required limit of microorganisms. The method chosen must allow testing of a sufficient sample size to judge compliance with the specification. The suitability of the chosen method must be established.

#### GROWTH PROMOTION TEST AND SUITABILITY OF THE COUNTING METHOD

##### General Considerations

The ability of the test to detect microorganisms in the presence of product to be tested must be established.

Suitability must be confirmed if a change in testing performance or a change in the product that may affect the outcome of the test, is introduced.

##### Preparation of Test Strains

Use standardized stable suspensions of test strains or prepare as stated below. Seed-lot culture maintenance techniques (seed-lot systems) are used so that the viable microorganisms used for inoculation are not more than 5 passages removed from the original master seed-lot. Grow each of the bacterial and fungal test strains separately as described in [Table 1](#).

Table 1. Preparation and Use of Test Microorganisms

Microorganism	Preparation of Test Strain	Growth Promotion		Suitability of Counting Method in the Presence of Product	
		Total Aerobic Microbial Count	Total Yeasts and Molds Count	Total Aerobic Microbial Count	Total Yeasts and Molds Count
Staphylococcus aureus such as ATCC 6538, NCIMB 9518, CIP 4.83, or NBRC 13276	Soybean-Casein Digest Agar or Soybean-Casein Digest Broth 30°–35° 18–24 hours	Soybean-Casein Digest Agar and Soybean-Casein Digest Broth ≤ 100 cfu 30°–35° ≤ 3 days		Soybean-Casein Digest Agar/MPN Soybean-Casein Digest Broth ≤ 100 cfu 30°–35° ≤ 3 days	
Pseudomonas aeruginosa such as ATCC 9027, NCIMB 8626, CIP 82.118, or NBRC 13275	Soybean-Casein Digest Agar or Soybean-Casein Digest Broth 30°–35° 18–24 hours	Soybean-Casein Digest Agar and Soybean-Casein Digest Broth ≤ 100 cfu 30°–35° ≤ 3 days		Soybean-Casein Digest Agar/MPN Soybean-Casein Digest Broth ≤ 100 cfu 30°–35° ≤ 3 days	
Bacillus subtilis such as ATCC 6633, NCIMB 8054, CIP 52.62, or NBRC 3134	Soybean-Casein Digest Agar or Soybean-Casein Digest Broth 30°–35° 18–24 hours	Soybean-Casein Digest Agar and Soybean-Casein Digest Broth ≤ 100 cfu 30°–35° ≤ 3 days		Soybean-Casein Digest Agar/MPN Soybean-Casein Digest Broth ≤ 100 cfu 30°–35° ≤ 3 days	
Candida albicans such as ATCC 10231, NCPF 3179, IP 48.72, or NBRC 1594	Sabouraud Dextrose Agar or Sabouraud Dextrose Broth 20°–25° 2–3 days	Soybean-Casein Digest Agar ≤ 100 cfu 30°–35° ≤ 5 days	Sabouraud Dextrose Agar ≤ 100 cfu 20°–25° ≤ 5 days	Soybean-Casein Digest Agar ≤ 100 cfu 30°–35° ≤ 5 days MPN: not applicable	Sabouraud Dextrose Agar ≤ 100 cfu 20°–25° ≤ 5 days
Aspergillus niger such as ATCC 16404, IMI 149007, IP 1431.83, or NBRC 9455	Sabouraud Dextrose Agar or Potato-Dextrose Agar 20°–25° 5–7 days, or until good sporulation is achieved	Soybean-Casein Digest Agar ≤ 100 cfu 30°–35° ≤ 5 days	Sabouraud Dextrose Agar ≤ 100 cfu 20°–25° ≤ 5 days	Soybean-Casein Digest Agar ≤ 100 cfu 30°–35° ≤ 5 days MPN: not applicable	Sabouraud Dextrose Agar ≤ 100 cfu 20°–25° ≤ 5 days

Use Buffered Sodium Chloride-Peptone Solution pH 7.0 or Phosphate Buffer Solution pH 7.2 to make test suspensions; to suspend *A. niger* spores, 0.05% of polysorbate 80 may be added to the buffer. Use the suspensions within 2 hours, or within 24 hours if stored between 2° and 8°. As an alternative to preparing and then diluting a fresh suspension of vegetative cells of *A. niger* or *B. subtilis*, a stable spore suspension is prepared and then an appropriate volume of the spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2° to 8° for a validated period of time.



#### Negative Control

To verify testing conditions, a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of microorganisms.

#### Growth Promotion of the Media

Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from the ingredients described.

Inoculate portions/plates of Soybean-Casein Digest Broth and Soybean-Casein Digest Agar with a small number (not more than 100 cfu) of the microorganisms indicated in [Table 1](#), using a separate portion/plate of medium for each. Inoculate plates of Sabouraud Dextrose Agar with a small number (not more than 100 cfu) of the microorganisms indicated in [Table 1](#), using a separate plate of medium for each. Incubate according to the conditions described in [Table 1](#).

For solid media, growth obtained must not differ by a factor greater than 2 from the calculated value for a standardized inoculum. For a freshly prepared inoculum, growth of the microorganisms comparable to that previously obtained with a previously tested and approved batch of medium occurs. Liquid media are suitable if clearly visible growth of the microorganisms comparable to that previously obtained with a previously tested and approved batch of medium occurs.

#### Suitability of the Counting Method in the Presence of Product Preparation of the Sample

The method for sample preparation depends on the physical characteristics of the product to be tested. If none of the procedures described below can be demonstrated to be satisfactory, a suitable alternative procedure must be developed.

**Water-Soluble Products**— Dissolve or dilute (usually a 1 in 10 dilution is prepared) the product to be examined in Buffered Sodium Chloride-Peptone Solution pH 7.0, Phosphate Buffer Solution pH 7.2, or Soybean-Casein Digest Broth. If necessary, adjust to a pH of 6 to 8. Further dilutions, where necessary, are prepared with the same diluent.

**Nonfatty Products Insoluble in Water**— Suspend the product to be examined (usually a 1 in 10 dilution is prepared) in Buffered Sodium Chloride-Peptone Solution pH 7.0, Phosphate Buffer Solution pH 7.2, or Soybean-Casein Digest Broth. A surface-active agent such as 1 g per L of polysorbate 80 may be added to assist the suspension of poorly wettable substances. If necessary, adjust to a pH of 6 to 8. Further dilutions, where necessary, are prepared with the same diluent.

**Fatty Products**— Dissolve in isopropyl myristate sterilized by filtration, or mix the product to be examined with the minimum necessary quantity of sterile polysorbate 80 or another noninhibitory sterile surface-active reagent heated, if necessary, to not more than 40° or, in exceptional cases, to not more than 45°. Mix carefully and if necessary maintain the temperature in a water bath. Add a sufficient quantity of the prewarmed chosen diluent to make a 1 in 10 dilution of the original product. Mix carefully, while maintaining the temperature for the shortest time necessary for the formation of an emulsion. Further serial 10-fold dilutions may be prepared using the chosen diluent containing a suitable concentration of sterile polysorbate 80 or another noninhibitory sterile surface-active reagent.

**Fluids or Solids in Aerosol Form**— Aseptically transfer the product into a membrane filter apparatus or a sterile container for further sampling. Use either the total contents or a defined number of metered doses from each of the containers tested.

**Transdermal Patches**— Remove the protective cover sheets ("release liners") of the transdermal patches and place them, adhesive side upwards, on sterile glass or plastic trays. Cover the adhesive surface with a suitable sterile porous material (e.g., sterile gauze) to prevent the patches from sticking together, and transfer the patches to a suitable volume of the chosen diluent containing inactivators such as polysorbate 80 and/or lecithin. Shake the preparation vigorously for at least 30 minutes.

#### inoculation and dilution

Add to the sample prepared as directed above and to a control (with no test material included) a sufficient volume of the microbial suspension to obtain an inoculum of not more than 100 cfu. The volume of the suspension of the inoculum should not exceed 1% of the volume of diluted product.

To demonstrate acceptable microbial recovery from the product, the lowest possible dilution factor of the prepared sample must be used for the test. Where this is not possible due to antimicrobial activity or poor solubility, further appropriate protocols must be developed. If inhibition of growth by the sample cannot otherwise be avoided, the aliquot of the microbial suspension may be added after neutralization, dilution, or filtration.

#### neutralization/removal of antimicrobial activity

The number of microorganisms recovered from the prepared sample diluted as described in Inoculation and Dilution and incubated following the procedure described in Recovery of Microorganisms in the Presence of Product, is compared to the number of microorganisms recovered from the control preparation.

If growth is inhibited (reduction by a factor greater than 2), then modify the procedure for the particular enumeration test to ensure the validity of the results. Modification of the procedure may include, for example,

1. An increase in the volume of the diluent or culture medium;
2. Incorporation of a specific or general neutralizing agents into the diluent;
3. Membrane filtration; or
4. A combination of the above measures.

**Neutralizing Agents**— Neutralizing agents may be used to neutralize the activity of antimicrobial agents (see [Table 2](#)). They may be added to the chosen diluent or the medium preferably before sterilization. If used, their efficacy and their absence of toxicity for microorganisms must be demonstrated by carrying out a blank with neutralizer and without product.

Table 2. Common Neutralizing Agents/Methods for Interfering Substances

Interfering Substance	Potential Neutralizing Agents/Method
Glutaraldehyde, mercurials	Sodium hydrogen sulfite (Sodium bisulfite)
Phenolics, alcohol, aldehydes, sorbate	Dilution
Aldehydes	Glycine
Quaternary ammonium compounds (QACs), parahydroxybenzoates (parabens), bis-biguaniides	Lecithin
QACs, iodine, parabens	Polysorbate
Mercurials	Thioglycollate
Mercurials, halogens, aldehydes	Thiosulfate
EDTA (edetate)	Mg or Ca ions

If no suitable neutralizing method can be found, it can be assumed that the failure to isolate the inoculated organism is attributable to the microbicidal activity of the product. This information serves to indicate that the article is not likely to be contaminated with the given species of the microorganism. However, it is possible that the product inhibits only some of the microorganisms specified herein, but does not inhibit others not included among the test strains or those for which the latter are not representative. Then, perform the test with the highest dilution factor compatible with microbial growth and the specific acceptance criterion.

#### recovery of microorganisms in the presence of product

For each of the microorganisms listed, separate tests are performed. Only microorganisms of the added test strain are counted.

**Membrane Filtration**— Use membrane filters having a nominal pore size not greater than 0.45 µm. The type of filter material is chosen in such a way that the bacteria-retaining efficiency is not affected by the components of the sample to be investigated. For each of the microorganisms listed, one membrane filter is used.



Transfer a suitable quantity of the sample prepared as described under Preparation of the Sample, Inoculation and Dilution, and Neutralization/Removal of Antimicrobial Activity (preferably representing 1 g of the product, or less if large numbers of cfu are expected) to the membrane filter, filter immediately, and rinse the membrane filter with an appropriate volume of diluent.

For the determination of total aerobic microbial count (TAMC), transfer the membrane filter to the surface of the Soybean-Casein Digest Agar. For the determination of total combined yeasts and molds count (TYMC), transfer the membrane to the surface of the Sabouraud Dextrose Agar. Incubate the plates as indicated in [Table 1](#). Perform the counting.

Plate-Count Methods— Perform plate-count methods at least in duplicate for each medium, and use the mean count of the result.

Pour-Plate Method— For Petri dishes 9 cm in diameter, add to the dish 1 mL of the sample prepared as described under Preparation of the Sample, Inoculation and Dilution, and Neutralization/Removal of Antimicrobial Activity and 15 to 20 mL of Soybean-Casein Digest Agar or Sabouraud Dextrose Agar, both media maintained at not more than 45°. If larger Petri dishes are used, the amount of agar medium is increased accordingly. For each of the microorganisms listed in [Table 1](#), at least two Petri dishes are used.

Incubate the plates as indicated in [Table 1](#). Take the arithmetic mean of the counts per medium, and calculate the number of cfu in the original inoculum.

Surface-Spread Method— For Petri dishes 9 cm in diameter, add 15 to 20 mL of Soybean-Casein Digest Agar or Sabouraud Dextrose Agar at about 45° to each Petri dish, and allow to solidify. If larger Petri dishes are used, the volume of the agar is increased accordingly. Dry the plates, for example, in a laminar-airflow cabinet or in an incubator. For each of the microorganisms listed in [Table 1](#), at least two Petri dishes are used. Spread a measured volume of not less than 0.1 mL of the sample, prepared as directed under Preparation of the Sample, Inoculation and Dilution, and Neutralization/Removal of Antimicrobial Activity over the surface of the medium. Incubate and count as directed for Pour-Plate Method.

Most-Probable-Number (MPN) Method— The precision and accuracy of the MPN Method is less than that of the Membrane Filtration method or the Plate-Count Method. Unreliable results are obtained particularly for the enumeration of molds. For these reasons, the MPN Method is reserved for the enumeration of TAMC in situations where no other method is available. If the use of the method is justified, proceed as follows.

Prepare a series of at least three serial 10-fold dilutions of the product as described for Preparation of the Sample, Inoculation and Dilution, and Neutralization/Removal of Antimicrobial Activity. From each level of dilution, three aliquots of 1 g or 1 mL are used to inoculate three tubes with 9 to 10 mL of Soybean-Casein Digest Broth. If necessary a surface-active agent such as polysorbate 80, or an inactivator of antimicrobial agents may be added to the medium. Thus, if three levels of dilution are prepared, nine tubes are inoculated.

Incubate all tubes at 30° to 35° for not more than 3 days. If reading of the results is difficult or uncertain owing to the nature of the product to be examined, subculture in the same broth or in Soybean-Casein Digest Agar for 1 to 2 days at the same temperature, and use these results. From [Table 3](#), determine the most probable number of microorganisms per g or mL of the product to be examined.

Table 3. Most-Probable-Number Values of Microorganisms

Observed Combinations of Numbers of Tubes Showing Growth in Each Set			MPN per g or per mL of Product	95% Confidence Limits
Number of g or mL of Product per Tube	0.1	0.01		
0	0	0	<3	0-9.4
0	0	1	3	0.1-9.5
0	1	0	3	0.1-10
0	1	1	6.1	1.2-17
0	2	0	6.2	1.2-17
0	3	0	9.4	3.5-35
1	0	0	3.6	0.2-17
1	0	1	7.2	1.2-17
1	0	2	11	4-35
1	1	0	7.4	1.3-20
1	1	1	11	4-35
1	2	0	11	4-35
1	2	1	15	5-38
1	3	0	16	5-38
2	0	0	9.2	1.5-35
2	0	1	14	4-35
2	0	2	20	5-38
2	1	0	15	4-38
2	1	1	20	5-38
2	1	2	27	9-94
2	2	0	21	5-40
2	2	1	28	9-94
2	2	2	35	9-94
2	3	0	29	9-94
2	3	1	36	9-94
3	0	0	23	5-94
3	0	1	38	9-104
3	0	2	64	16-181
3	1	0	43	9-181
3	1	1	75	17-199
3	1	2	120	30-360
3	1	3	160	30-380
3	2	0	93	18-360
3	2	1	150	30-380
3	2	2	210	30-400



3	2	3	290	90-990
3	3	0	240	40-990
3	3	1	460	90-1980
3	3	2	1100	200-4000
3	3	3	>1100	

#### results and interpretation

When verifying the suitability of the Membrane Filtration method or the Plate-Count Method, a mean count of any of the test organisms not differing by a factor greater than 2 from the value of the control defined in Inoculation and Dilution in the absence of product must be obtained. When verifying the suitability of the MPN Method, the calculated value from the inoculum must be within 95% confidence limits of the results obtained with the control.

If the above criteria cannot be met for one of more of the organisms tested with any of the described methods, the method and test conditions that come closest to the criteria are used to test the product.

#### TESTING OF PRODUCTS

##### Amount Used for the Test

Unless otherwise directed, use 10 g or 10 mL of the product to be examined taken with the precautions referred to above. For fluids or solids in aerosol form, sample 10 containers. For transdermal patches, sample 10 patches.

The amount to be tested may be reduced for active substances that will be formulated in the following conditions: the amount per dosage unit (e.g., tablet, capsule, injection) is less than or equal to 1 mg, or the amount per g or mL (for preparations not presented in dose units) is less than 1 mg. In these cases, the amount of sample to be tested is not less than the amount present in 10 dosage units or 10 g or 10 mL of the product.

For materials used as active substances where the sample quantity is limited or batch size is extremely small (i.e., less than 1000 mL or 1000 g), the amount tested shall be 1% of the batch unless a lesser amount is prescribed or justified and authorized.

For products where the total number of entities in a batch is less than 200 (e.g., samples used in clinical trials), the sample size may be reduced to two units, or one unit if the size is less than 100.

Select the sample(s) at random from the bulk material or from the available containers of the preparation. To obtain the required quantity, mix the contents of a sufficient number of containers to provide the sample.

##### Examination of the Product

###### membrane filtration

Use a filtration apparatus designed to allow the transfer of the filter to the medium. Prepare the sample using a method that has been shown to be suitable as described in Growth Promotion Test and Suitability of the Counting Method, transfer the appropriate amount to each of two membrane filters, and filter immediately. Wash each filter following the procedure shown to be suitable.

For the determination of TAMC, transfer one of the membrane filters to the surface of Soybean-Casein Digest Agar. For the determination of TYMC, transfer the other membrane to the surface of Sabouraud Dextrose Agar. Incubate the plate of Soybean-Casein Digest Agar at 30° to 35° for 3 to 5 days and the plate of Sabouraud Dextrose Agar at 20° to 25° for 7 days. Calculate the number of cfu per g or per mL of product.

When examining transdermal patches, separately filter 10% of the volume of the preparation described for Preparation of the Sample through each of two sterile filter membranes. Transfer one membrane to Soybean-Casein Digest Agar for TAMC and the other membrane to Sabouraud Dextrose Agar for TYMC.

###### plate-count methods

Pour-Plate Method— Prepare the sample using a method that has been shown to be suitable as described in Growth Promotion Test and Suitability of the Counting Method. Prepare for each medium at least two Petri dishes for each level of dilution. Incubate the plates of Soybean-Casein Digest Agar at 30° to 35° for 3 to 5 days and the plates of Sabouraud Dextrose Agar at 20° to 25° for 5 to 7 days. Select the plates corresponding to a given dilution and showing the highest number of colonies less than 250 for TAMC and 50 for TYMC. Take the arithmetic mean per culture medium of the counts, and calculate the number of cfu per g or per mL of product.

Surface-Spread Method— Prepare the sample using a method that has been shown to be suitable as described in Growth Promotion Test and Suitability of the Counting Method. Prepare at least two Petri dishes for each medium and each level of dilution. For incubation and calculation of the number of cfu, proceed as directed for the Pour-Plate Method.

###### most-probable-number method

Prepare and dilute the sample using a method that has been shown to be suitable as described in Growth Promotion Test and Suitability of the Counting Method. Incubate all tubes for 3 to 5 days at 30° to 35°. Subculture if necessary, using the procedure shown to be suitable. Record for each level of dilution the number of tubes showing microbial growth. Determine the most probable number of microorganisms per g or mL of the product to be examined from [Table 3](#).

#### Interpretation of the Results

The total aerobic microbial count (TAMC) is considered to be equal to the number of cfu found using Soybean-Casein Digest Agar; if colonies of fungi are detected on this medium, they are counted as part of TAMC. The total combined yeasts and molds count (TYMC) is considered to be equal to the number of cfu found using Sabouraud Dextrose Agar; if colonies of bacteria are detected on this medium, they are counted as part of TYMC. When the TYMC is expected to exceed the acceptance criterion due to the bacterial growth, Sabouraud Dextrose Agar containing antibiotics may be used. If the count is carried out by the MPN Method, the calculated value is TAMC.

When an acceptance criterion for microbiological quality is prescribed, it is interpreted as follows:

- 101 cfu: maximum acceptable count = 20;
- 102 cfu: maximum acceptable count = 200;
- 103 cfu: maximum acceptable count = 2000;

and so forth.

The recommended solutions and media are described in [Tests for Specified Microorganisms](#) (62)

(Official May 1, 2009)

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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## INTRODUCTION

The tests described hereafter will allow determination of the absence of, or limited occurrence of, specified microorganisms that may be detected under the conditions described.

The tests are designed primarily to determine whether a substance or preparation complies with an established specification for microbiological quality. When used for such purposes, follow the instructions given below, including the number of samples to be taken, and interpret the results as stated below.

Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence to the Pharmacopeial method has been demonstrated.

## GENERAL PROCEDURES

The preparation of samples is carried out as described in [Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests](#) 61.

If the product to be examined has antimicrobial activity, this is insofar as possible removed or neutralized as described in [Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests](#) 61.

If surface-active substances are used for sample preparation, their absence of toxicity for microorganisms and their compatibility with any inactivators used must be demonstrated as described in [Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests](#) 61.

## GROWTH-PROMOTING AND INHIBITORY PROPERTIES OF THE MEDIA AND SUITABILITY OF THE TEST

The ability of the test to detect microorganisms in the presence of the product to be tested must be established. Suitability must be confirmed if a change in testing performance or a change in the product that may affect the outcome of the test is introduced.

## Preparation of Test Strains

Use standardized stable suspensions of test strains as stated below. Seed-lot culture maintenance techniques (seed-lot systems) are used so that the viable microorganisms used for inoculation are not more than five passages removed from the original master seed-lot.

## aerobic microorganisms

Grow each of the bacterial test strains separately in containers containing Soybean-Casein Digest Broth or on Soybean-Casein Digest Agar at 30° to 35° for 18 to 24 hours. Grow the test strain for *Candida albicans* separately on Sabouraud Dextrose Agar or in Sabouraud Dextrose Broth at 20° to 25° for 2 to 3 days.

<i>Staphylococcus aureus</i>	such as ATCC 6538, NCIMB 9518, CIP 4.83, or NBRC 13276
<i>Pseudomonas aeruginosa</i>	such as ATCC 9027, NCIMB 8626, CIP 82.118, or NBRC 13275
<i>Escherichia coli</i>	such as ATCC 8739, NCIMB 8545, CIP 53.126, or NBRC 3972
<i>Salmonella enterica</i> ssp. <i>enterica</i> serotype <i>typhimurium</i> or, as an alternative,	such as ATCC 14028
<i>Salmonella enterica</i> ssp. <i>enterica</i> serotype <i>abony</i>	such as NBRC 100797, NCTC 6017, or CIP 80.39
<i>Candida albicans</i>	such as ATCC 10231, NCPF 3179, IP 48.72, or NBRC 1594

Use Buffered Sodium Chloride-Peptone Solution pH 7.0 or Phosphate Buffer Solution pH 7.2 to make test suspensions. Use the suspensions within 2 hours or within 24 hours if stored at 2° to 8°.

## clostridia

Use Clostridium sporogenes such as ATCC 11437 (NBRC 14293, NCIMB 12343, CIP 100651) or ATCC 19404 (NCTC 532 or CIP 79.3). Grow the clostridial test strain under anaerobic conditions in Reinforced Medium for Clostridia at 30° to 35° for 24 to 48 hours. As an alternative to preparing and then diluting down a fresh suspension of vegetative cells of *C. sporogenes*, a stable spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2° to 8° for a validated period.

## Negative Control

To verify testing conditions, a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of microorganisms.

## Growth Promotion and Inhibitory Properties of the Media

Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from ingredients. Verify suitable properties of relevant media as described in [Table 1](#).

Table 1. Growth Promoting, Inhibitory, and Indicative Properties of Media

Test/Medium	Property	Test Strains
<b>Test for bile-tolerant Gram-negative bacteria</b>		
Enterobacteria Enrichment Broth Mossel	Growth promoting	<i>E. coli</i>
		<i>P. aeruginosa</i>
	Inhibitory	<i>S. aureus</i>
Violet Red Bile Glucose Agar	Growth promoting + Indicative	<i>E. coli</i>
		<i>P. aeruginosa</i>
<b>Test for <i>Escherichia coli</i></b>		
MacConkey Broth	Growth promoting	<i>E. coli</i>
	Inhibitory	<i>S. aureus</i>
MacConkey Agar	Growth promoting + Indicative	<i>E. coli</i>
<b>Test for <i>Salmonella</i></b>		
Rappaport Vassiliadis <i>Salmonella</i> Enrichment Broth	Growth promoting	<i>Salmonella enterica</i> ssp. <i>enterica</i> serotype <i>typhimurium</i> or
		<i>Salmonella enterica</i> ssp. <i>enterica</i> serotype <i>abony</i>
	Inhibitory	<i>S. aureus</i>
Xylose Lysine Deoxycholate Agar	Growth promoting + Indicative	<i>Salmonella enterica</i> ssp. <i>enterica</i> serotype <i>typhimurium</i> or
		<i>Salmonella enterica</i> ssp. <i>enterica</i> serotype <i>abony</i>

	Indicative	E. coli
<b>Test for Pseudomonas aeruginosa</b>		
Cetrimide Agar	Growth promoting	P. aeruginosa
	Inhibitory	E. coli
<b>Test for Staphylococcus aureus</b>		
Mannitol Salt Agar	Growth promoting + Indicative	S. aureus
	Inhibitory	E. coli
<b>Test for Clostridia</b>		
Reinforced Medium for Clostridia	Growth promoting	Cl. sporogenes
Columbia Agar	Growth promoting	Cl. sporogenes
<b>Test for Candida albicans</b>		
Sabouraud Dextrose Broth	Growth promoting	C. albicans
Sabouraud Dextrose Agar	Growth promoting + Indicative	C. albicans

Test for Growth-Promoting Properties, Liquid Media— Inoculate a portion of the appropriate medium with a small number (not more than 100 cfu) of the appropriate microorganism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Clearly visible growth of the microorganism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

Test for Growth-Promoting Properties, Solid Media— Perform Surface-Spread Method (see Plate-Count Methods under [Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests \(61\)](#)), inoculating each plate with a small number (not more than 100 cfu) of the appropriate microorganism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Growth of the microorganism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

Test for Inhibitory Properties, Liquid or Solid Media— Inoculate the appropriate medium with at least 100 cfu of the appropriate microorganism. Incubate at the specified temperature for not less than the longest period of time specified in the test. No growth of the test microorganism occurs.

Test for Indicative Properties— Perform Surface-Spread Method (see Plate-Count Methods under [Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests \(61\)](#)), inoculating each plate with a small number (not more than 100 cfu) of the appropriate microorganism. Incubate at the specified temperature for a period of time within the range specified in the test. Colonies are comparable in appearance and indication reactions to those previously obtained with a previously tested and approved batch of medium.

#### Suitability of the Test Method

For each new product to be tested perform sample preparation as described in the relevant paragraph under Testing of Products. At the time of mixing, add each test strain in the prescribed growth medium. Inoculate the test strains individually. Use a number of microorganisms equivalent to not more than 100 cfu in the inoculated test preparation.

Perform the test as described in the relevant paragraph under Testing of Products using the shortest incubation period prescribed.

The specified microorganisms must be detected with the indication reactions as described under Testing of Products.

Any antimicrobial activity of the product necessitates a modification of the test procedure (see Neutralization/Removal of Antimicrobial Activity under [Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests \(61\)](#)).

For a given product, if the antimicrobial activity with respect to a microorganism for which testing is prescribed cannot be neutralized, then it is to be assumed that the inhibited microorganism will not be present in the product.

### TESTING OF PRODUCTS

#### Bile-Tolerant Gram-Negative Bacteria

Sample Preparation and Pre-Incubation— Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in [Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests \(61\)](#), but using Soybean-Casein Digest Broth as the chosen diluent, mix, and incubate at 20° to 25° for a time sufficient to resuscitate the bacteria but not sufficient to encourage multiplication of the organisms (usually 2 hours but not more than 5 hours).

Test for Absence— Unless otherwise prescribed, use the volume corresponding to 1 g of the product, as prepared in Sample Preparation and Pre-Incubation, to inoculate Enterobacteria Enrichment Broth Mossel. Incubate at 30° to 35° for 24 to 48 hours. Subculture on plates of Violet Red Bile Glucose Agar. Incubate at 30° to 35° for 18 to 24 hours.

The product complies with the test if there is no growth of colonies.

#### Quantitative Test—

Selection and Subculture— Inoculate suitable quantities of Enterobacteria Enrichment Broth Mossel with the preparation as directed under Sample Preparation and Pre-Incubation and/or dilutions of it containing respectively 0.1 g, 0.01 g, and 0.001 g (or 0.1 mL, 0.01 mL, and 0.001 mL) of the product to be examined. Incubate at 30° to 35° for 24 to 48 hours.

Subculture each of the cultures on a plate of Violet Red Bile Glucose Agar. Incubate at 30° to 35° for 18 to 24 hours.

Interpretation— Growth of colonies constitutes a positive result. Note the smallest quantity of the product that gives a positive result and the largest quantity that gives a negative result. Determine from [Table 2](#) the probable number of bacteria.

Table 2. Interpretation of Results

Results for Each Quantity of Product			Probable Number of Bacteria per g or mL of Product
0.1 g or 0.1 mL	0.01 g or 0.01 mL	0.001 g or 0.001 mL	
+	+	+	more than 103
+	+	-	less than 103 and more than 102
+	-	-	less than 102 and more than 10
-	-	-	less than 10

#### Escherichia coli

Sample Preparation and Pre-Incubation— Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in [Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests \(61\)](#), and use 10 mL or the quantity corresponding to 1 g or 1 mL, to inoculate a suitable amount (determined as described under Suitability of the Test Method) of Soybean-Casein Digest Broth, mix, and incubate at 30° to 35° for 18 to 24 hours.

Selection and Subculture— Shake the container, transfer 1 mL of Soybean-Casein Digest Broth to 100 mL of MacConkey Broth, and incubate at 42° to 44° for 24 to 48 hours.



Subculture on a plate of MacConkey Agar at 30° to 35° for 18 to 72 hours.

Interpretation— Growth of colonies indicates the possible presence of *E. coli*. This is confirmed by identification tests.

The product complies with the test if no colonies are present or if the identification tests are negative.

#### Salmonella

Sample Preparation and Pre-Incubation— Prepare the product to be examined as described in [Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests](#) (61), and use the quantity corresponding to not less than 10 g or 10 mL to inoculate a suitable amount (determined as described under Suitability of the Test Method) of Soybean–Casein Digest Broth, mix, and incubate at 30° to 35° for 18 to 24 hours.

Selection and Subculture— Transfer 0.1 mL of Soybean–Casein Digest Broth to 10 mL of Rappaport Vassiliadis Salmonella Enrichment Broth, and incubate at 30° to 35° for 18 to 24 hours. Subculture on plates of Xylose Lysine Deoxycholate Agar. Incubate at 30° to 35° for 18 to 48 hours.

Interpretation— The possible presence of *Salmonella* is indicated by the growth of well-developed, red colonies, with or without black centers. This is confirmed by identification tests.

The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

#### Pseudomonas aeruginosa

Sample Preparation and Pre-Incubation— Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in [Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests](#) (61), and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under Suitability of the Test Method) of Soybean–Casein Digest Broth, and mix. When testing transdermal patches, filter the volume of sample corresponding to one patch of the preparation (see Transdermal Patches under Preparation of the Sample in [Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests](#) (61)) through a sterile filter membrane, and place in 100 mL of Soybean–Casein Digest Broth. Incubate at 30° to 35° for 18 to 24 hours.

Selection and Subculture— Subculture on a plate of Cetrimide Agar, and incubate at 30° to 35° for 18 to 72 hours.

Interpretation— Growth of colonies indicates the possible presence of *P. aeruginosa*. This is confirmed by identification tests.

The product complies with the test if colonies are not present or if the confirmatory identification tests are negative.

#### Staphylococcus aureus

Sample Preparation and Pre-Incubation— Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in [Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests](#) (61), and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under Suitability of the Test Method) of Soybean–Casein Digest Broth, and homogenize. When testing transdermal patches, filter the volume of sample corresponding to one patch of the preparation (see Transdermal Patches under Preparation of the Sample in [Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests](#) (61)) through a sterile filter membrane, and place in 100 mL of Soybean–Casein Digest Broth. Incubate at 30° to 35° for 18 to 24 hours.

Selection and Subculture— Subculture on a plate of Mannitol Salt Agar, and incubate at 30° to 35° for 18 to 72 hours.

Interpretation— The possible presence of *S. aureus* is indicated by the growth of yellow or white colonies surrounded by a yellow zone. This is confirmed by identification tests.

The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

#### Clostridia

Sample Preparation and Heat Treatment— Prepare the product to be examined as described in [Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests](#) (61). Take two equal portions corresponding to not less than 1 g or 1 mL of the product to be examined. Heat one portion at 80° for 10 minutes, and cool rapidly. Do not heat the other portion.

Selection and Subculture— Transfer 10 mL of each of the mixed portions to two containers (38 mm × 200 mm) or other containers containing 100 mL of Reinforced Medium for Clostridia. Incubate under anaerobic conditions at 30° to 35° for 48 hours. After incubation, make subcultures from each tube on Columbia Agar, and incubate under anaerobic conditions at 30° to 35° for 48 hours.

Interpretation— The occurrence of anaerobic growth of rods (with or without endospores) giving a negative catalase reaction indicates the presence of Clostridia.

If no anaerobic growth of microorganisms is detected on Columbia Agar or the catalase test is positive, the product complies with the test.

#### Candida albicans

Sample Preparation and Pre-Incubation— Prepare the product to be examined as described in [Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests](#) (61), and use 10 mL or the quantity corresponding to not less than 1 g or 1 mL, to inoculate 100 mL of Sabouraud Dextrose Broth, and mix. Incubate at 30° to 35° for 3 to 5 days.

Selection and Subculture— Subculture on a plate of Sabouraud Dextrose Agar, and incubate at 30° to 35° for 24 to 48 hours.

Interpretation— Growth of white colonies may indicate the presence of *C. albicans*. This is confirmed by identification tests.

The product complies with the test if such colonies are not present or if the confirmatory identification tests are negative.

#### RECOMMENDED SOLUTIONS AND CULTURE MEDIA

[note—This section is given for information.]

The following solutions and culture media have been found satisfactory for the purposes for which they are prescribed in the test for microbial contamination in the Pharmacopeia.

Other media may be used if they have similar growth-promoting and inhibitory properties.

Stock Buffer Solution— Transfer 34 g of potassium dihydrogen phosphate to a 1000-mL volumetric flask, dissolve in 500 mL of [Purified Water](#), adjust with sodium hydroxide to a pH of 7.2 ± 0.2, add [Purified Water](#) to volume, and mix. Dispense in containers, and sterilize. Store at a temperature of 2° to 8°.

Phosphate Buffer Solution pH 7.2— Prepare a mixture of [Purified Water](#) and Stock Buffer Solution (800:1 v/v), and sterilize.

Buffered Sodium Chloride–Peptone Solution pH 7.0	
Potassium Dihydrogen Phosphate	3.6 g
Disodium Hydrogen Phosphate Dihydrate	7.2 g (equivalent to 0.067 M phosphate)
Sodium Chloride	4.3 g
Peptone (meat or casein)	1.0 g
Purified Water	1000 mL

Sterilize in an autoclave using a validated cycle.



Soybean-Casein Digest Broth	
Pancreatic Digest of Casein	17.0 g
Papaic Digest of Soybean	3.0 g
Sodium Chloride	5.0 g
Dibasic Hydrogen Phosphate	2.5 g
Glucose Monohydrate	2.5 g
Purified Water	1000 mL

Adjust the pH so that after sterilization it is  $7.3 \pm 0.2$  at  $25^{\circ}\text{C}$ . Sterilize in an autoclave using a validated cycle.

Soybean-Casein Digest Agar	
Pancreatic Digest of Casein	15.0 g
Papaic Digest of Soybean	5.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Purified Water	1000 mL

Adjust the pH so that after sterilization it is  $7.3 \pm 0.2$  at  $25^{\circ}\text{C}$ . Sterilize in an autoclave using a validated cycle.

Sabouraud Dextrose Agar	
Dextrose	40.0 g
Mixture of Peptic Digest of Animal Tissue and Pancreatic Digest of Casein (1:1)	10.0 g
Agar	15.0 g
Purified Water	1000 mL

Adjust the pH so that after sterilization it is  $5.6 \pm 0.2$  at  $25^{\circ}\text{C}$ . Sterilize in an autoclave using a validated cycle.

Potato Dextrose Agar	
Infusion from potatoes	200 g
Dextrose	20.0 g
Agar	15.0 g
Purified Water	1000 mL

Adjust the pH so that after sterilization it is  $5.6 \pm 0.2$  at  $25^{\circ}\text{C}$ . Sterilize in an autoclave using a validated cycle.

Sabouraud Dextrose Broth	
Dextrose	20.0 g
Mixture of Peptic Digest of Animal Tissue and Pancreatic Digest of Casein (1:1)	10.0 g
Purified Water	1000 mL

Adjust the pH so that after sterilization it is  $5.6 \pm 0.2$  at  $25^{\circ}\text{C}$ . Sterilize in an autoclave using a validated cycle.

Enterobacteria Enrichment Broth Mossel	
Pancreatic Digest of Gelatin	10.0 g
Glucose Monohydrate	5.0 g
Dehydrated Ox Bile	20.0 g
Potassium Dihydrogen Phosphate	2.0 g
Disodium Hydrogen Phosphate Dihydrate	8.0 g
Brilliant Green	15 mg
Purified Water	1000 mL

Adjust the pH so that after heating it is  $7.2 \pm 0.2$  at  $25^{\circ}\text{C}$ . Heat at  $100^{\circ}\text{C}$  for 30 minutes, and cool immediately.

Violet Red Bile Glucose Agar	
Yeast Extract	3.0 g
Pancreatic Digest of Gelatin	7.0 g
Bile Salts	1.5 g
Sodium Chloride	5.0 g
Glucose Monohydrate	10.0 g
Agar	15.0 g
Neutral Red	30 mg
Crystal Violet	2 mg
Purified Water	1000 mL

Adjust the pH so that after heating it is  $7.4 \pm 0.2$  at  $25^{\circ}\text{C}$ . Heat to boiling; do not heat in an autoclave.

MacConkey Broth	



Pancreatic Digest of Gelatin	20.0 g
Lactose Monohydrate	10.0 g
Dehydrated Ox Bile	5.0 g
Bromocresol Purple	10 mg
Purified Water	1000 mL

Adjust the pH so that after sterilization it is  $7.3 \pm 0.2$  at  $25^{\circ}$ . Sterilize in an autoclave using a validated cycle.

MacConkey Agar	
Pancreatic Digest of Gelatin	17.0 g
Peptones (meat and casein)	3.0 g
Lactose Monohydrate	10.0 g
Sodium Chloride	5.0 g
Bile Salts	1.5 g
Agar	13.5 g
Neutral Red	30.0 mg
Crystal Violet	1 mg
Purified Water	1000 mL

Adjust the pH so that after sterilization it is  $7.1 \pm 0.2$  at  $25^{\circ}$ . Boil for 1 minute with constant shaking, then sterilize in an autoclave using a validated cycle.

Rappaport Vassiliadis Salmonella Enrichment Broth	
Soya Peptone	4.5 g
Magnesium Chloride Hexahydrate	29.0 g
Sodium Chloride	8.0 g
Dipotassium Phosphate	0.4 g
Potassium Dihydrogen Phosphate	0.6 g
Malachite Green	0.036 g
Purified Water	1000 mL

Dissolve, warming slightly. Sterilize in an autoclave using a validated cycle, at a temperature not exceeding  $115^{\circ}$ . The pH is to be  $5.2 \pm 0.2$  at  $25^{\circ}$  after heating and autoclaving.

Xylose Lysine Deoxycholate Agar	
Xylose	3.5 g
L-Lysine	5.0 g
Lactose Monohydrate	7.5 g
Sucrose	7.5 g
Sodium Chloride	5.0 g
Yeast Extract	3.0 g
Phenol Red	80 mg
Agar	13.5 g
Sodium Deoxycholate	2.5 g
Sodium Thiosulfate	6.8 g
Ferric Ammonium Citrate	0.8 g
Purified Water	1000 mL

Adjust the pH so that after heating it is  $7.4 \pm 0.2$  at  $25^{\circ}$ . Heat to boiling, cool to  $50^{\circ}$ , and pour into Petri dishes. Do not heat in an autoclave.

Cetrimide Agar	
Pancreatic Digest of Gelatin	20.0 g
Magnesium Chloride	1.4 g
Dipotassium Sulfate	10.0 g
Cetrimide	0.3 g
Agar	13.6 g
Purified Water	1000 mL
Glycerol	10.0 mL

Heat to boiling for 1 minute with shaking. Adjust the pH so that after sterilization it is  $7.2 \pm 0.2$  at  $25^{\circ}$ . Sterilize in an autoclave using a validated cycle.

Mannitol Salt Agar	
Pancreatic Digest of Casein	5.0 g
Peptic Digest of Animal Tissue	5.0 g
Beef Extract	1.0 g
d-Mannitol	10.0 g
Sodium Chloride	75.0 g
Agar	15.0 g
Phenol Red	0.025 g
Purified Water	1000 mL



Heat to boiling for 1 minute with shaking. Adjust the pH so that after sterilization it is  $7.4 \pm 0.2$  at  $25^\circ$ . Sterilize in an autoclave using a validated cycle.

Reinforced Medium for Clostridia	
Beef Extract	10.0 g
Peptone	10.0 g
Yeast Extract	3.0 g
Soluble Starch	1.0 g
Glucose Monohydrate	5.0 g
Cysteine Hydrochloride	0.5 g
Sodium Chloride	5.0 g
Sodium Acetate	3.0 g
Agar	0.5 g
Purified Water	1000 mL

Hydrate the agar, and dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilization it is about  $6.8 \pm 0.2$  at  $25^\circ$ . Sterilize in an autoclave using a validated cycle.

Columbia Agar	
Pancreatic Digest of Casein	10.0 g
Meat Peptic Digest	5.0 g
Heart Pancreatic Digest	3.0 g
Yeast Extract	5.0 g
Maize Starch	1.0 g
Sodium Chloride	5.0 g
Agar, according to gelling power	10.0–15.0 g
Purified Water	1000 mL

Hydrate the agar, and dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilization it is  $7.3 \pm 0.2$  at  $25^\circ$ . Sterilize in an autoclave using a validated cycle. Allow to cool to  $45^\circ$  to  $50^\circ$ ; add, where necessary, gentamicin sulfate corresponding to 20 mg of gentamicin base, and pour into Petri dishes.

(Official May 1, 2009)

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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## 71 STERILITY TESTS

◆ Portions of this general chapter have been harmonized with the corresponding texts of the European Pharmacopeia and/or the Japanese Pharmacopeia. Those portions that are not harmonized are marked with symbols (◆◆) to specify this fact.◆

The following procedures are applicable for determining whether a Pharmacopeial article purporting to be sterile complies with the requirements set forth in the individual monograph with respect to the test for sterility. Pharmacopeial articles are to be tested by the Membrane Filtration method under Test for Sterility of the Product to be Examined where the nature of the product permits. If the membrane filtration technique is unsuitable, use the Direct Inoculation of the Culture Medium method under Test for Sterility of the Product to be Examined. All devices, with the exception of Devices with Pathways Labeled Sterile, are tested using the Direct Inoculation of the Culture Medium method. Provisions for retesting are included under Observation and Interpretation of Results.

Because sterility testing is a very exacting procedure, where asepsis of the procedure must be ensured for a correct interpretation of results, it is important that personnel be properly trained and qualified. The test for sterility is carried out under aseptic conditions. In order to achieve such conditions, the test environment has to be adapted to the way in which the sterility test is performed. The precautions taken to avoid contamination are such that they do not affect any microorganisms that are to be revealed in the test. The working conditions in which the tests are performed are monitored regularly by appropriate sampling of the working area and by carrying out appropriate controls.

These Pharmacopeial procedures are not by themselves designed to ensure that a batch of product is sterile or has been sterilized. This is accomplished primarily by validation of the sterilization process or of the aseptic processing procedures.

When evidence of microbial contamination in the article is obtained by the appropriate Pharmacopeial method, the result so obtained is conclusive evidence of failure of the article to meet the requirements of the test for sterility, even if a different result is obtained by an alternative procedure. ◆ For additional information on sterility testing, see [Sterilization and Sterility Assurance of Compendial Articles](#) (1211).◆

## MEDIA

Prepare media for the tests as described below, or dehydrated formulations may be used provided that, when reconstituted as directed by the manufacturer or distributor, they meet the requirements of the Growth Promotion Test of Aerobes, Anaerobes, and Fungi. Media are sterilized using a validated process.

The following culture media have been found to be suitable for the test for sterility. Fluid Thioglycollate Medium is primarily intended for the culture of anaerobic bacteria. However, it will also detect aerobic bacteria. Soybean–Casein Digest Medium is suitable for the culture of both fungi and aerobic bacteria.

Fluid Thioglycollate Medium	
I-Cystine	0.5 g
Sodium Chloride	2.5 g
Dextrose (C <sub>6</sub> H <sub>12</sub> O <sub>6</sub> ·H <sub>2</sub> O)	5.5/5.0 g
Agar, granulated (moisture content not exceeding 15%)	0.75 g

Yeast Extract (water-soluble)	5.0 g
Pancreatic Digest of Casein	15.0 g
Sodium Thioglycollate	0.5 g
or Thioglycolic Acid	0.3 mL
Resazurin Sodium Solution (1 in 1000), freshly prepared	1.0 mL
Purified Water	1000 mL

Mix the L-cystine, sodium chloride, dextrose, yeast extract, and pancreatic digest of casein with the purified water, and heat until solution is effected. Dissolve the sodium thioglycollate or thioglycolic acid in the solution and, if necessary, add 1 N sodium hydroxide so that, after sterilization, the solution will have a pH of  $7.1 \pm 0.2$ . If filtration is necessary, heat the solution again without boiling, and filter while hot through moistened filter paper. Add the resazurin sodium solution, mix, and place the medium in suitable vessels that provide a ratio of surface to depth of medium such that not more than the upper half of the medium has undergone a color change indicative of oxygen uptake at the end of the incubation period. Sterilize using a validated process. If the medium is stored, store at a temperature between  $2^{\circ}$  and  $25^{\circ}$  in a sterile, airtight container. If more than the upper one-third of the medium has acquired a pink color, the medium may be restored once by heating the containers in a water-bath or in free-flowing steam until the pink color disappears and by cooling quickly, taking care to prevent the introduction of nonsterile air into the container.

Fluid Thioglycollate Medium is to be incubated at  $32.5 \pm 2.5^{\circ}$ .

◆ Alternative Thioglycollate Medium

Prepare a mixture having the same composition as that of the Fluid Thioglycollate Medium, but omitting the agar and the resazurin sodium solution, sterilize as directed above, and allow to cool prior to use. The pH after sterilization is  $7.1 \pm 0.2$ . Incubate under anaerobic conditions for the duration of the incubation period.

Alternative Fluid Thioglycollate Medium is to be incubated at  $32.5 \pm 2.5^{\circ}$ .

Soybean-Casein Digest Medium	
Pancreatic Digest of Casein	17.0 g
Papaic Digest of Soybean Meal	3.0 g
Sodium Chloride	5.0 g
Dibasic Potassium Phosphate	2.5 g
Dextrose (C <sub>6</sub> H <sub>12</sub> O <sub>6</sub> H <sub>2</sub> O)	2.5/2.3 g
Purified Water	1000 mL

Dissolve the solids in the Purified Water, heating slightly to effect a solution. Cool the solution to room temperature, and adjust the pH with 1 N sodium hydroxide so that, after sterilization, it will have a pH of  $7.3 \pm 0.2$ . Filter, if necessary to clarify, dispense into suitable containers, and sterilize using a validated procedure. Store at a temperature between  $2^{\circ}$  and  $25^{\circ}$  in a sterile well-closed container, unless it is intended for immediate use.

Soybean-Casein Digest Medium is to be incubated at  $22.5 \pm 2.5^{\circ}$ .

◆ Media for Penicillins or Cephalosporins

Where sterility test media are to be used in the Direct Inoculation of the Culture Medium method under Test for Sterility of the Product to be Examined, modify the preparation of Fluid Thioglycollate Medium and the Soybean-Casein Digest Medium as follows. To the containers of each medium, transfer aseptically a quantity of  $\beta$ -lactamase sufficient to inactivate the amount of antibiotic in the specimen under test. Determine the quantity of  $\beta$ -lactamase required to inactivate the antibiotic by using a  $\beta$ -lactamase preparation that has been assayed previously for its penicillin- or cephalosporin-inactivating power. [note—Supplemented  $\beta$ -lactamase media can also be used in the membrane filtration test.]

Alternatively (in an area completely separate from that used for sterility testing), confirm that an appropriate amount of  $\beta$ -lactamase is incorporated into the medium, following either method under Validation Test, using less than 100 colony-forming units (cfu) of *Staphylococcus aureus* (see Table 1) as the challenge. Typical microbial growth of the inoculated

culture must be observed as a confirmation that the  $\beta$ -lactamase concentration is appropriate.

Table 1. Strains of the Test Microorganisms Suitable for Use in the Growth Promotion Test and the Validation Test

Aerobic bacteria	
<i>Staphylococcus aureus</i> <sup>◆1◆</sup>	ATCC 6538, CIP 4.83, NCTC 10788, NCIMB 9518
<i>Bacillus subtilis</i>	ATCC 6633, CIP 52.62, NCIMB 8054
<i>Pseudomonas aeruginosa</i> <sup>◆2◆</sup>	ATCC 9027, NCIMB 8626, CIP 82.118
Anaerobic bacterium	
<i>Clostridium sporogenes</i> <sup>◆3◆</sup>	ATCC 19404, CIP 79.3, NCTC 532 or ATCC 11437
Fungi	
<i>Candida albicans</i>	ATCC 10231, IP 48.72, NCPF 3179
<i>Aspergillus niger</i>	ATCC 16404, IP 1431.83, IMI 149007

◆1 An alternative to *Staphylococcus aureus* is *Bacillus subtilis* (ATCC 6633).

◆2 An alternative microorganism is *Micrococcus luteus* (*Kocuria rhizophila*), ATCC 9341.

◆3 An alternative to *Clostridium sporogenes*, when a nonspore-forming microorganism is desired, is *Bacillus vulgaris* (ATCC 8482). [note—Seed-lot culture maintenance techniques (seed-lot systems) are used so that the viable microorganisms used for inoculation are not more than five passages removed from the original master seed lot.]

Suitability Tests

The media used comply with the following tests, carried out before, or in parallel, with the test on the product to be examined.

sterility

Confirm the sterility of each sterilized batch of medium by incubating a portion of the media at the specified incubation temperature for 14 days. No growth of microorganisms occurs.

growth promotion test of aerobes, anaerobes, and fungi

Test each lot of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from ingredients <sup>◆1◆</sup>. Suitable strains of microorganisms are indicated in Table 1.

Inoculate portions of Fluid Thioglycollate Medium with a small number (not more than 100 cfu) of the following microorganisms, using a separate portion of medium for each of the



ollowing species of microorganism: Clostridium sporogenes, Pseudomonas aeruginosa, and Staphylococcus aureus. ♦Inoculate portions of Alternative Fluid Thioglycollate Medium with a small number (not more than 100 cfu) of Clostridium sporogenes.♦ Inoculate portions of Soybean–Casein Digest Medium with a small number (not more than 100 cfu) of the following microorganisms, using a separate portion of medium for each of the following species of microorganism: Aspergillus niger, Bacillus subtilis, and Candida albicans. Incubate for not more than 3 days in the case of bacteria and not more than 5 days in the case of fungi.

The media are suitable if a clearly visible growth of the microorganisms occurs.

♦storage

If prepared media are stored in unsealed containers, they can be used for 1 month, provided that they are tested for growth promotion within 2 weeks of the time of use and that color indicator requirements are met. If stored in tight containers, the media can be used for 1 year, provided that they are tested for growth promotion within 3 months of the time of use and that the color indicator requirements are met.♦

♦DILUTING AND RINSING FLUIDS FOR MEMBRANE FILTRATION

Fluid A

preparation

Dissolve 1 g of peptic digest of animal tissue in water to make 1 L, filter or centrifuge to clarify, if necessary, and adjust to a pH of  $7.1 \pm 0.2$ . Dispense into containers, and sterilize using a validated process.

preparation for penicillins or cephalosporins

Aseptically add to the above Preparation, if necessary, a quantity of sterile  $\beta$ -lactamase sufficient to inactivate any residual antibiotic activity on the membranes after the solution of the test specimen has been filtered (see Media for Penicillins or Cephalosporins).

Fluid D

To each L of Fluid A add 1 mL of polysorbate 80, adjust to a pH of  $7.1 \pm 0.2$ , dispense into containers, and sterilize using a validated process. Use this fluid for articles containing lecithin or oil, or for devices labeled as "sterile pathway."

Fluid K

Dissolve 5.0 g of peptic digest of animal tissue, 3.0 g of beef extract, and 10.0 g of polysorbate 80 in water to make 1 L. Adjust the pH to obtain, after sterilization, a pH of  $6.9 \pm 0.2$ .

Dispense into containers, and sterilize using a validated process.♦

VALIDATION TEST

Carry out a test as described below under Test for Sterility of the Product to be Examined using exactly the same methods, except for the following modifications.

Membrane Filtration

After transferring the content of the container or containers to be tested to the membrane, add an inoculum of a small number of viable microorganisms (not more than 100 cfu) to the final portion of sterile diluent used to rinse the filter.

Direct Inoculation

After transferring the contents of the container or containers to be tested (for catgut and other surgical sutures for veterinary use: strands) to the culture medium, add an inoculum of a small number of viable microorganisms (not more than 100 cfu) to the medium.

In both cases use the same microorganisms as those described above under Growth Promotion Test of Aerobes, Anaerobes, and Fungi. Perform a growth promotion test as a positive control. Incubate all the containers containing medium for not more than 5 days.

If clearly visible growth of microorganisms is obtained after the incubation, visually comparable to that in the control vessel without product, either the product possesses no antimicrobial activity under the conditions of the test or such activity has been satisfactorily eliminated. The test for sterility may then be carried out without further modification.

If clearly visible growth is not obtained in the presence of the product to be tested, visually comparable to that in the control vessels without product, the product possesses antimicrobial activity that has not been satisfactorily eliminated under the conditions of the test. Modify the conditions in order to eliminate the antimicrobial activity, and repeat the validation test.

This validation is performed (a) when the test for sterility has to be carried out on a new product; and (b) whenever there is a change in the experimental conditions of the test. The validation may be performed simultaneously with the Test for Sterility of the Product to be Examined.

TEST FOR STERILITY OF THE PRODUCT TO BE EXAMINED

♦Number of Articles to Be Tested

Unless otherwise specified elsewhere in this chapter or in the individual monograph, test the number of articles specified in [Table 3](#). If the contents of each article are of sufficient quantity (see [Table 2](#)), they may be divided so that equal appropriate portions are added to each of the specified media. [note—Perform sterility testing employing two or more of the specified media.] If each article does not contain sufficient quantities for each medium, use twice the number of articles indicated in [Table 3](#).♦

Table 2. Minimum Quantity to be Used for Each Medium

Quantity per Container	Minimum Quantity to be Used (unless otherwise justified and authorized)
Liquids (other than antibiotics)	
Less than 1 mL	The whole contents of each container
1–40 mL	Half the contents of each container, but not less than 1 mL
Greater than 40 mL, and not greater than 100 mL	20 mL
Greater than 100 mL	10% of the contents of the container, but not less than 20 mL
Antibiotic liquids	1 mL
Other preparations soluble in water or in isopropyl myristate	The whole contents of each container to provide not less than 200 mg
Insoluble preparations, creams, and ointments to be suspended or emulsified	Use the contents of each container to provide not less than 200 mg
Solids	
Less than 50 mg	The whole contents of each container
50 mg or more, but less than 300 mg	Half the contents of each container, but not less than 50 mg
300 mg–5 g	150 mg

greater than 5 g	500 mg
Devices	
Catgut and other surgical sutures for veterinary use	3 sections of a strand (each 30-cm long)
♦Surgical dressing/cotton/gauze (in packages)	100 mg per package
Sutures and other individually packaged single-use material	The whole device
Other medical devices	The whole device, cut into pieces or disassembled♦

Table 3. Minimum Number of Articles to be Tested in Relation to the Number of Articles in the Batch

Number of Items in the Batch	Minimum Number of Items to be Tested for Each Medium (unless otherwise justified and authorized)*
Parenteral preparations	
Not more than 100 containers	10% or 4 containers, whichever is the greater
More than 100 but not more than 500 containers	10 containers
More than 500 containers	2% or 20 containers, whichever is less
♦For large-volume parenterals	2% or 10 containers, whichever is less
Antibiotic solids	
Pharmacy bulk packages (<5 g)	20 containers
Pharmacy bulk packages ( $\geq$ 5 g)	6 containers
Bulks and blends	See Bulk solid products♦
Ophthalmic and other noninjectable preparations	
Not more than 200 containers	5% or 2 containers, whichever is the greater
More than 200 containers	10 containers
If the product is presented in the form of single-dose containers, apply the scheme shown above for preparations for parenteral use.	
Devices	
Catgut and other surgical sutures for veterinary use	2% or 5 packages, whichever is the greater, up to a maximum total of 20 packages
♦Not more than 100 articles	10% or 4 articles, whichever is greater
More than 100, but not more than 500 articles	10 articles
More than 500 articles	2% or 20 articles, whichever is less♦
Bulk solid products	
Up to 4 containers	Each container
More than 4 containers, but not more than 50 containers	20% or 4 containers, whichever is greater
More than 50 containers	2% or 10 containers, whichever is greater

\* If the contents of one container are enough to inoculate the two media, this column gives the number of containers needed for both the media together.

The test may be carried out using the technique of Membrane Filtration or by Direct Inoculation of the Culture Medium with the product to be examined. Appropriate negative controls are included. The technique of membrane filtration is used whenever the nature of the product permits; that is, for filterable aqueous preparations, for alcoholic or oily preparations, and for preparations miscible with, or soluble in, aqueous or oily solvents, provided these solvents do not have an antimicrobial effect in the conditions of the test.

#### Membrane Filtration

Use membrane filters having a nominal pore size not greater than 0.45  $\mu$ m whose effectiveness to retain microorganisms has been established. Cellulose nitrate filters, for example, are used for aqueous, oily, and weakly alcoholic solutions; and cellulose acetate filters, for example, are used for strongly alcoholic solutions. Specially adapted filters may be needed for certain products (e.g., for antibiotics).

The technique described below assumes that membranes about 50 mm in diameter will be used. If filters of a different diameter are used, the volumes of the dilutions and the washings should be adjusted accordingly. The filtration apparatus and membrane are sterilized by appropriate means. The apparatus is designed so that the solution to be examined can be introduced and filtered under aseptic conditions: it permits the aseptic removal of the membrane for transfer to the medium, or it is suitable for carrying out the incubation after adding the medium to the apparatus itself.

#### aqueous solutions

If appropriate, transfer a small quantity of a suitable, sterile diluent such as ♦Fluid A (see Diluting and Rinsing Fluids for Membrane Filtration)♦ onto the membrane in the apparatus and filter. The diluent may contain suitable neutralizing substances and/or appropriate inactivating substances, for example, in the case of antibiotics.

Transfer the contents of the container or containers to be tested to the membrane or membranes, if necessary, after diluting to the volume used in the Validation Test with the chosen sterile diluent, but using not less than the quantities of the product to be examined prescribed in Tables 2 and 3. Filter immediately. If the product has antimicrobial properties, wash the membrane not less than three times by filtering through it each time the volume of the chosen sterile diluent used in the Validation Test. Do not exceed a washing cycle of 5 times 200 mL, even if during validation it has been demonstrated that such a cycle does not fully eliminate the antimicrobial activity. Transfer the whole membrane to the culture medium or cut it aseptically into two equal parts, and transfer one half to each of two suitable media. Use the same volume of each medium as in the Validation Test. Alternatively, transfer the medium onto the membrane in the apparatus. Incubate the media for not less than 14 days.

#### soluble solids (other than antibiotics)

Use for each medium not less than the quantity prescribed in Tables 2 and 3 of the product dissolved in a suitable solvent, such as ♦Fluid A (Diluting and Rinsing Fluids for Membrane Filtration),♦ and proceed with the test as described above for Aqueous Solutions using a membrane appropriate to the chosen solvent.

#### oils and oily solutions

Use for each medium not less than the quantity of the product prescribed in Tables 2 and 3. Oils and oily solutions of sufficiently low viscosity may be filtered without dilution through a dry membrane. Viscous oils may be diluted as necessary with a suitable sterile diluent such as isopropyl myristate shown not to have antimicrobial activity in the conditions of the test.

Allow the oil to penetrate the membrane by its own weight, and then filter, applying the pressure or suction gradually. Wash the membrane at least three times by filtering through it



each time about 100 mL of a suitable sterile solution such as ♦Fluid A (see Diluting and Rinsing Fluids for Membrane Filtration)♦ containing a suitable emulsifying agent at a concentration shown to be appropriate in the validation of the test, for example polysorbate 80 at a concentration of 10 g per L ♦(Fluid K)♦. Transfer the membrane or membranes to the culture medium or media, or vice versa, as described above for Aqueous Solutions, and incubate at the same temperatures and for the same times.

#### ointments and creams

Use for each medium not less than the quantities of the product prescribed in [Tables 2](#) and [3](#). Ointments in a fatty base and emulsions of the water-in-oil type may be diluted to 1% in isopropyl myristate as described above, by heating, if necessary, to not more than 40°. In exceptional cases it may be necessary to heat to not more than 44°. Filter as rapidly as possible, and proceed as described above for Oils and Oily Solutions.

#### ♦prefilled syringes

For prefilled syringes without attached sterile needles, expel the contents of each syringe into one or two separate membrane filter funnels or into separate pooling vessels prior to transfer. If a separate sterile needle is attached, directly expel the syringe contents as indicated above, and proceed as directed for Aqueous Solutions. Test the sterility of the needle, using Direct Inoculation under Validation Test.

#### solids for injection other than antibiotics

Constitute the test articles as directed on the label, and proceed as directed for Aqueous Solutions or Oils and Oily Solutions, whichever applies. [note—If necessary, excess diluent can be added to aid in the constitution and filtration of the constituted test article.]

#### antibiotic solids for injection

Pharmacy Bulk Packages, < 5 g— From each of 20 containers, aseptically transfer about 300 mg of solids, into a sterile 500-mL conical flask, dissolve in about 200 mL of Fluid A (see Diluting and Rinsing Fluids for Membrane Filtration), and mix; or constitute, as directed in the labeling, each of 20 containers and transfer a quantity of liquid or suspension, equivalent to about 300 mg of solids, into a sterile 500-mL conical flask, dissolve in about 200 mL of Fluid A, and mix. Proceed as directed for Aqueous Solutions or Oils and Oily Solutions, whichever applies.

Pharmacy Bulk Packages, ≥ 5 g— From each of 6 containers, aseptically transfer about 1 g of solids into a sterile 500-mL conical flask, dissolve in about 200 mL of Fluid A, and mix; or constitute, as directed in the labeling, each of 6 containers and transfer a quantity of liquid, equivalent to about 1 g of solids, into a sterile 500-mL conical flask, dissolve in about 200 mL of Fluid A, and mix. Proceed as directed for Aqueous Solutions.

#### antibiotic solids, bulks, and blends

Aseptically remove a sufficient quantity of solids from the appropriate amount of containers (see [Table 2](#)), mix to obtain a composite, equivalent to about 6 g of solids, and transfer to a sterile 500-mL conical flask. Dissolve in about 200 mL of Fluid A, and mix. Proceed as directed for Aqueous Solutions.

#### sterile aerosol products

For fluid products in pressurized aerosol form, freeze the containers in an alcohol-dry ice mixture at least at -20° for about 1 hour. If feasible, allow the propellant to escape before aseptically opening the container, and transfer the contents to a sterile pooling vessel. Add 100 mL of Fluid D to the pooling vessel, and mix gently. Proceed as directed for Aqueous Solutions or Oils and Oily Solutions, whichever applies.

#### devices with pathways labeled sterile

Aseptically pass not less than 10 pathway volumes of Fluid D through each device tested. Collect the fluids in an appropriate sterile vessel, and proceed as directed for Aqueous Solutions or Oils and Oily Solutions, whichever applies.

In the case of sterile, empty syringes, draw sterile diluent into the barrel through the sterile needle, if attached, or through a sterile needle attached for the purpose of the test, and express the contents into a sterile pooling vessel. Proceed as directed above.♦

#### Direct Inoculation of the Culture Medium

Transfer the quantity of the preparation to be examined prescribed in [Tables 2](#) and [3](#) directly into the culture medium so that the volume of the product is not more than 10% of the volume of the medium, unless otherwise prescribed.

If the product to be examined has antimicrobial activity, carry out the test after neutralizing this with a suitable neutralizing substance or by dilution in a sufficient quantity of culture medium. When it is necessary to use a large volume of the product, it may be preferable to use a concentrated culture medium prepared in such a way that it takes into account the subsequent dilution. Where appropriate, the concentrated medium may be added directly to the product in its container.

#### oily liquids

Use media to which have been added a suitable emulsifying agent at a concentration shown to be appropriate in the validation of the test, for example polysorbate 80 at a concentration of 10 g per L.

#### ointments and creams

Prepare by diluting to about 1 in 10 by emulsifying with the chosen emulsifying agent in a suitable sterile diluent such as ♦Fluid A (see Diluting and Rinsing Fluids for Membrane Filtration).♦ Transfer the diluted product to a medium not containing an emulsifying agent.

Incubate the inoculated media for not less than 14 days. Observe the cultures several times during the incubation period. Shake cultures containing oily products gently each day. However, when thioglycollate medium or other similar medium is used for the detection of anaerobic microorganisms, keep shaking or mixing to a minimum in order to maintain anaerobic conditions.

#### catgut and other surgical sutures for veterinarian use

Use for each medium not less than the quantities of the product prescribed in [Tables 2](#) and [3](#). Open the sealed package using aseptic precautions, and remove three sections of the strand for each culture medium. Carry out the test on three sections, each 30-cm long, which have been cut off from the beginning, the center, and the end of the strand. Use whole strands from freshly opened cassette packs. Transfer each section of the strand to the selected medium. Use sufficient medium to cover adequately the material to be tested (20 mL to 150 mL).

#### ♦solids

Transfer a quantity of the product in the form of a dry solid (or prepare a suspension of the product by adding sterile diluent to the immediate container), corresponding to not less than the quantity indicated in [Tables 2](#) and [3](#). Transfer the material so obtained to 200 mL of Fluid Thioglycollate Medium, and mix. Similarly, transfer the same quantity to 200 mL of Soybean-Casein Digest Medium, and mix. Proceed as directed above.

#### purified cotton, gauze, surgical dressings, and related articles

From each package of cotton, rolled gauze bandage, or large surgical dressings being tested, aseptically remove two or more portions of 100- to 500-mg each from the innermost part of the sample. From individually packaged, single-use materials, aseptically remove the entire article. Immerse the portions or article in each medium, and proceed as directed above.

#### sterile devices

Articles can be immersed intact or disassembled. To ensure that device pathways are also in contact with the media, immerse the appropriate number of units per medium in a volume of medium sufficient to immerse the device completely, and proceed as directed above. For extremely large devices, immerse those portions of the device that are to come into contact



with the patient in a volume of medium sufficient to achieve complete immersion of those portions.

For catheters where the inside lumen and outside are required to be sterile, either cut them into pieces such that the medium is in contact with the entire lumen or fill the lumen with medium, and then immerse the intact unit.♦

#### OBSERVATION AND INTERPRETATION OF RESULTS

At intervals during the incubation period and at its conclusion, examine the media for macroscopic evidence of microbial growth. If the material being tested renders the medium turbid so that the presence or absence of microbial growth cannot be readily determined by visual examination, 14 days after the beginning of incubation transfer portions (each not less than 1 mL) of the medium to fresh vessels of the same medium, and then incubate the original and transfer vessels for not less than 4 days.

If no evidence of microbial growth is found, the product to be examined complies with the test for sterility. If evidence of microbial growth is found, the product to be examined does not comply with the test for sterility, unless it can be clearly demonstrated that the test was invalid for causes unrelated to the product to be examined. The test may be considered invalid only if one or more of the following conditions are fulfilled:

- a. The data of the microbiological monitoring of the sterility testing facility show a fault.
- b. A review of the testing procedure used during the test in question reveals a fault.
- c. Microbial growth is found in the negative controls.
- d. After determination of the identity of the microorganisms isolated from the test, the growth of this species (or these species) may be ascribed unequivocally to faults with respect to the material and or the technique used in conducting the sterility test procedure.

If the test is declared to be invalid, it is repeated with the same number of units as in the original test. If no evidence of microbial growth is found in the repeat test, the product examined complies with the test for sterility. If microbial growth is found in the repeat test, the product examined does not comply with the test for sterility.

#### APPLICATION OF THE TEST TO PARENTERAL PREPARATIONS, OPHTHALMIC, AND OTHER NONINJECTABLE PREPARATIONS REQUIRED TO COMPLY WITH THE TEST FOR STERILITY

When using the technique of membrane filtration, use, whenever possible, the whole contents of the container, but not less than the quantities indicated in [Tables 2](#) and [3](#), diluting where necessary to about 100 mL with a suitable sterile solution, such as ♦Fluid A (see Diluting and Rinsing Fluids for Membrane Filtration).♦

When using the technique of direct inoculation of media, use the quantities shown in [Tables 2](#) and [3](#), unless otherwise justified and authorized. The tests for bacterial and fungal sterility are carried out on the same sample of the product to be examined. When the volume or the quantity in a single container is insufficient to carry out the tests, the contents of two or more containers are used to inoculate the different media.

♦1 In appropriate cases, periodic testing of the different batches prepared from the same lot of dehydrated medium is acceptable.♦

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Radhakrishna S Tirumalai, Ph.D.</a> Senior Scientist 1-301-816-8339	(MSA05) Microbiology and Sterility Assurance

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#### 81 ANTIBIOTICS—MICROBIAL ASSAYS

The activity (potency) of antibiotics may be demonstrated under suitable conditions by their inhibitory effect on microorganisms. A reduction in antimicrobial activity also will reveal subtle changes not demonstrable by chemical methods. Accordingly, microbial or biological assays remain generally the standard for resolving doubt with respect to possible loss of activity. This chapter summarizes these procedures for the antibiotics recognized in this Pharmacopeia for which microbiological assay remains the definitive method.

Two general methods are employed, the cylinder-plate or "plate" assay and the turbidimetric or "tube" assay. The first depends upon diffusion of the antibiotic from a vertical cylinder through a solidified agar layer in a petri dish or plate to an extent such that growth of the added microorganism is prevented entirely in a circular area or "zone" around the cylinder containing a solution of the antibiotic. The turbidimetric method depends upon the inhibition of growth of a microbial culture in a uniform solution of the antibiotic in a fluid medium that is favorable to its rapid growth in the absence of the antibiotic.

#### APPARATUS

All equipment is to be thoroughly cleaned before and after each use. Glassware for holding and transferring test organisms is sterilized by dry heat or by steam.

#### Temperature Control

Thermostatic control is required in several stages of a microbial assay, when culturing a microorganism and preparing its inoculum, and during incubation in plate and tube assays. Maintain the temperature of assay plates at  $\pm 0.5^{\circ}$  of the temperature selected. Closer control of the temperature ( $\pm 0.1^{\circ}$  of the selected temperature) is imperative during incubation in a tube assay, and may be achieved in either circulated air or water, the greater heat capacity of water lending it some advantage over circulating air.

#### Spectrophotometer

Measuring transmittance within a fairly narrow frequency band requires a suitable spectrophotometer in which the wavelength of the light source can be varied or restricted by the use of a 580-nm filter or a 530-nm filter for reading the absorbance in a tube assay. For the latter purpose, the instrument may be arranged to accept the tube in which incubation takes place (see Turbidimetric Assay Receptacles), to accept a modified cell fitted with a drain that facilitates rapid change of content, or preferably, fixed with a flow-through cell for a continuous flow-through analysis; set the instrument at zero absorbance with clear, uninoculated broth prepared as specified for the particular antibiotic, including the same amount of test solution and formaldehyde as found in each sample.

note—Either absorbance or transmittance measurement may be used for preparing inocula.

#### Cylinder-Plate Assay Receptacles

For assay plates, use glass or plastic petri dishes (approximately 20 × 100 mm) having covers of suitable material. For assay cylinders, use stainless steel or porcelain cylinders with the following dimensions, each dimension having a tolerance of  $\pm 0.1$  mm: outside diameter 8 mm; inside diameter 6 mm; and length 10 mm. Carefully clean cylinders to remove all residues. An occasional acid bath, e.g., with about 2 N nitric acid or with chromic acid (see [Cleaning Glass Apparatus](#) [1051](#)) is needed.

#### Turbidimetric Assay Receptacles

For assay tubes, use glass or plastic test tubes, e.g., 16 × 125 mm or 18 × 150 mm that are relatively uniform in length, diameter, and thickness and substantially free from surface blemishes and scratches. Tubes that are to be placed in the spectrophotometer are matched and are without scratches or blemishes. Cleanse thoroughly to remove all antibiotic residues and traces of cleaning solution, and sterilize tubes that have been used previously, before subsequent use.

#### MEDIA AND DILUENTS



## Media

The media required for the preparation of test organism inocula are made from the ingredients listed herein. Minor modifications of the individual ingredients, or reconstituted dehydrated media, may be substituted, provided the resulting media possess equal or better growth-promoting properties and give a similar standard curve response.

Dissolve the ingredients in water to make 1 L, and adjust the solutions with either 1 N sodium hydroxide or 1 N hydrochloric acid as required, so that after steam sterilization the pH is as specified.

## medium 1

Peptone	6.0 g
Pancreatic Digest of Casein	4.0 g
Yeast Extract	3.0 g
Beef Extract	1.5 g
Dextrose	1.0 g
Agar	15.0 g
Water	1000 mL

pH after sterilization:  $6.6 \pm 0.1$ .

## medium 2

Peptone	6.0 g
Yeast Extract	3.0 g
Beef Extract	1.5 g
Agar	15.0 g
Water	1000 mL

pH after sterilization:  $6.6 \pm 0.1$ .

## medium 3

Peptone	5.0 g
Yeast Extract	1.5 g
Beef Extract	1.5 g
Sodium Chloride	3.5 g
Dextrose	1.0 g
Dibasic Potassium Phosphate	3.68 g
Monobasic Potassium Phosphate	1.32 g
Water	1000 mL

pH after sterilization:  $7.0 \pm 0.05$ .

## medium 4

Same as Medium 2, except for the additional ingredient 1.0 g of Dextrose.

## medium 5

Same as Medium 2, except that the final pH after sterilization is  $7.9 \pm 0.1$ .

## medium 8

Same as Medium 2, except that the final pH after sterilization is  $5.9 \pm 0.1$ .

## medium 9

Pancreatic Digest of Casein	17.0 g
Papaic Digest of Soybean	3.0 g
Sodium Chloride	5.0 g
Dibasic Potassium Phosphate	2.5 g
Dextrose	2.5 g
Agar	20.0 g
Water	1000 mL

pH after sterilization:  $7.2 \pm 0.1$ .

## medium 10

Same as Medium 9, except to use 12.0 g of Agar instead of 20.0 g, and to add 10 mL of Polysorbate 80 after boiling the medium to dissolve the agar.

pH after sterilization:  $7.2 \pm 0.1$ .

## medium 11

Same as Medium 1, except that the final pH after sterilization is  $8.3 \pm 0.1$ .

## medium 13

Dextrose	20.0 g
Peptone	10.0 g
Water	1000 mL

pH after sterilization:  $5.6 \pm 0.1$ .

## medium 19

Peptone	9.4 g
Yeast Extract	4.7 g



Beef Extract	2.4 g
Sodium Chloride	10.0 g
Dextrose	10.0 g
Agar	23.5 g
Water	1000 mL

pH after sterilization:  $6.1 \pm 0.1$ .

#### medium 32

Same as Medium 1, except for the additional ingredient 0.3 g of Manganese Sulfate.

#### medium 34

Glycerol	10.0 g
Peptone	10.0 g
Beef Extract	10.0 g
Sodium Chloride	3.0 g
Water	1000 mL

pH after sterilization:  $7.0 \pm 0.1$ .

#### medium 35

Same as Medium 34, except for the additional ingredient 17.0 g of Agar.

#### medium 36

Pancreatic Digest of Casein	15.0 g
Papaic Digest of Soybean	5.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Water	1000 mL

pH after sterilization:  $7.3 \pm 0.1$ .

#### medium 39

Same as [Medium 3](#), except that the final pH after sterilization is  $7.9 \pm 0.1$ .

#### medium 40

Yeast Extract	20.0 g
Polypeptone	5.0 g
Dextrose	10.0 g
Monobasic Potassium Phosphate	2.0 g
Polysorbate 80	0.1 g
Agar	10.0 g
Water	1000 mL

pH after sterilization:  $6.7 \pm 0.2$ .

#### medium 41

Pancreatic Digest of Casein	9.0 g
Dextrose	20.0 g
Yeast Extract	5.0 g
Sodium Citrate	10.0 g
Monobasic Potassium Phosphate	1.0 g
Dibasic Potassium Phosphate	1.0 g
Water	1000 mL

pH after sterilization:  $6.8 \pm 0.1$ .

#### Phosphate Buffers and Other Solutions

Prepare as follows, or by other suitable means, the potassium phosphate buffers required for the antibiotic under assay. The buffers are sterilized after preparation, and the pH specified in each case is the pH after sterilization.

buffer no. 1, 1 percent, pH 6.0— Dissolve 2.0 g of dibasic potassium phosphate and 8.0 g of monobasic potassium phosphate in 1000 mL of water. Adjust with 18 N phosphoric acid or 10 N potassium hydroxide to a pH of  $6.0 \pm 0.05$ .

buffer no. 3, 0.1 m, pH 8.0— Dissolve 16.73 g of dibasic potassium phosphate and 0.523 g of monobasic potassium phosphate in 1000 mL of water. Adjust with 18 N phosphoric acid or 10 N potassium hydroxide to a pH of  $8.0 \pm 0.1$ .

buffer no. 4, 0.1 m, pH 4.5— Dissolve 13.61 g of monobasic potassium phosphate in 1000 mL of water. Adjust with 18 N phosphoric acid or 10 N potassium hydroxide to a pH of  $4.5 \pm 0.05$ .

buffer no. 6, 10 percent, pH 6.0— Dissolve 20.0 g of dibasic potassium phosphate and 80.0 g of monobasic potassium phosphate in 1000 mL of water. Adjust with 18 N phosphoric acid or 10 N potassium hydroxide to a pH of  $6.0 \pm 0.05$ .

buffer no. 10, 0.2 m, pH 10.5— Dissolve 35.0 g of dibasic potassium phosphate in 1000 mL of water, and add 2 mL of 10 N potassium hydroxide. Adjust with 18 N phosphoric acid or 10 N potassium hydroxide to a pH of  $10.5 \pm 0.1$ .

buffer no. 16, 0.1 m, pH 7.0— Dissolve 13.6 g of dibasic potassium phosphate and 4.0 g of monobasic potassium phosphate in 1000 mL of water. Adjust with 18 N phosphoric acid or 10 N potassium hydroxide to a pH of  $7.0 \pm 0.2$ .



other solutions— Use the substances specified under [Reagents, Indicators, and Solutions](#). For water, use Purified Water. For saline, use Sodium Chloride Injection. Dilute formaldehyde is Formaldehyde Solution diluted with water 1:3.

#### UNITS AND REFERENCE STANDARDS

The potency of antibiotics is designated in either "Units" or "µg" of activity. In each case the "Unit" or "µg" of antibiotic activity is established and defined by the designated federal master standard for that antibiotic. The corresponding USP Reference Standard is calibrated in terms of the master standard. USP Reference Standards for antibiotic substances are held and distributed by the U.S. Pharmacopeial Convention, Inc.

The concept of "µg" of activity originated from the situation where the antibiotic preparation selected as the reference standard was thought to consist entirely of a single chemical entity and was therefore assigned a potency of 1000 "µg" per mg. In several such instances, as a result of the development of manufacturing and purification methods for particular antibiotics, preparations became available that contained more than 1000 "µg" of activity per mg. It was then understood that such preparations had an activity equivalent to a given number of "µg" of the original reference standard. In most instances, however, the "µg" of activity is exactly equivalent numerically to the µg (weight) of the pure substance. Complications arise in some situations, e.g., where an antibiotic exists as the free base and in salt form, and the "µg" of activity has been defined in terms of one such form; where the antibiotic substance consists of a number of components having close chemical similarity but differing antibiotic activity; or where the potencies of a family of antibiotics are expressed in terms of a reference standard consisting of a single member which, however, might itself be heterogeneous. In such cases the "µg" of activity defined in terms of a "Master Standard" is tantamount to a "Unit." The "µg" of activity should therefore not be assumed necessarily to correspond to the µg (weight) of the antibiotic substance.

#### PREPARATION OF THE STANDARD

To prepare a stock solution, dissolve a quantity of the USP Reference Standard of a given antibiotic, accurately weighed, or the entire contents of a vial of USP Reference Standard, where appropriate, in the solvent specified in that table, and then dilute to the required concentration as indicated. Store in a refrigerator, and use within the period indicated. On the day of the assay, prepare from the stock solution five or more test dilutions, the successive solutions increasing stepwise in concentration, usually in the ratio of 1:1.25 for a cylinder-plate assay or smaller for a turbidimetric assay. Use the final diluent specified and a sequence such that the middle or median has the concentration designated.

#### PREPARATION OF THE SAMPLE

From the information available for the preparation to be assayed (the "Unknown"), assign to it an assumed potency per unit weight or volume, and on this assumption prepare on the day of the assay a stock solution and test dilution as specified for each antibiotic but with the same final diluent as used for the USP Reference Standard. The assay with five levels of the Standard requires only one level of the Unknown at a concentration assumed equal to the median level of the Standard.

#### ORGANISMS AND INOCULUM

##### Test Organisms

The test organism for each antibiotic is listed in [Table 2](#), together with its identification number in the American Type Culture Collection. The method of assay is given for each in [Table 1](#). Maintain a culture on slants of the medium and under the incubation conditions specified in [Table 3](#), and transfer weekly to fresh slants. For *K. pneumoniae* use a nonencapsulated culture. For *Enterococcus hirae*, stab cultures may be used.

Table 1. Preparation of Stock Solutions and Test Dilutions of Reference Standards

Antibiotic and Type of Assay [Cylinder-plate (CP) or Turbidimetric (T)]	Stock Solution			Test Dilution	
	Initial Solvent (and initial concentration where specified); Further Diluent, if different	Final Stock Concentration per mL	Use Within	Final Diluent	Median Dose (µg of activity or Units per mL)
Amikacin (T)	Water	1 mg	14 days	Water	10 µg
Amphotericin B (CP)	Dimethyl sulfoxide	1 mg	Same day	B. 10	1.0 µg
Bacitracin Zinc (CP)	0.01 N hydrochloric acid	100 U	Same day	B. 1	1.0 U
Bleomycin (CP)	B. 16	2 U	14 days	B. 16	0.04 U
Candidin (T)	Dimethyl sulfoxide	1 mg	Same day	Water	0.06 µg
Capreomycin (T)	Water	1 mg	7 days	Water	100 µg
Carbenicillin (CP)	B. 1	1 mg	14 days	B. 1	20 µg
Cephalothin (CP)	B. 1	1 mg	5 days	B. 1	1.0 µg
Cephapirin (CP)	B. 1	1 mg	3 days	B. 1	1.0 µg
Chloramphenicol (T)	Alcohol (10 mg/mL); [Water]	1 mg	30 days	Water	2.5 µg
Chlortetracycline (T)	0.01 N hydrochloric acid	1 mg	4 days	Water	0.06 µg
Clloxacillin (CP)	B. 1	1 mg	7 days	B. 1	5.0 µg
Colistimethate Sodium (CP)	Water (10 mg/mL); [B. 6]	1 mg	Same day	B. 6	1.0 µg
Colistin (CP)	Water (10 mg/mL); [B. 6]	1 mg	14 days	B. 6	1.0 µg
Cycloserine (T)	Water	1 mg	30 days	Water	50 µg
Demeclocycline (T)	0.1 N hydrochloric acid	1 mg	4 days	Water	0.1 µg
Dihydrostreptomycin (CP)	B. 3	1 mg	30 days	B. 3	1.0 µg
Dihydrostreptomycin (T)	Water	1 mg	30 days	Water	30 µg
Doxycycline (T)	0.1 N hydrochloric acid	1 mg	5 days	Water	0.1 µg
Erythromycin (CP)	Methanol (10 mg/mL); [B. 3]	1 mg	14 days	B. 3	1.0 µg
Gentamicin (CP)	B. 3	1 mg	30 days	B. 3	0.1 µg
Gramicidin (T)	Alcohol 95%	1 mg	30 days	Alcohol 95%	0.04 µg
Kanamycin (T)	Water	1 mg	30 days	Water	10 µg
Methacycline (T)	Water	1 mg	7 days	Water	0.06 µg
Nafcillin (CP)	B. 1	1 mg	2 days	B. 1	2.0 µg
Natamycin (CP)	Dimethyl sulfoxide	1 mg	Same	B. 10	5.00 µg



			day		
Neomycin (CP)	B. 3	1 mg	14 days	B. 3	1.0 µg
Neomycin (T)	B. 3	100 µg	14 days	B. 3	1.0 µg
Netilmicin (CP)	B. 3	1 mg	7 days	B. 3	0.1 µg
Novobiocin (CP)	Alcohol (10 mg/mL); [ B. 3]	1 mg	5 days	B. 6	0.5 µg
Nystatin (CP)	Dimethylformamide	1,000 U	Same day	B. 6	20 U
Oxytetracycline (T)	0.1 N hydrochloric acid	1 mg	4 days	Water	0.24 µg
Paromomycin (CP)	B. 3	1 mg	21 days	B. 3	1.0 µg
Penicillin G (CP)	B. 1	1,000 U	4 days	B. 1	1.0 U
Polymyxin B (CP)	Water; [ B. 6]	10,000 U	14 days	B. 6	10 U
Rolitetracycline (T)	Water	1 mg	1 day	Water	0.24 µg
Sisomicin (CP)	B. 3	1 mg	14 days	B. 3	0.1 µg
Streptomycin (T)	Water	1 mg	30 days	Water	30 µg
Tetracycline (T)	0.1 N hydrochloric acid	1 mg	1 day	Water	0.24 µg
Thiostrepton (T)	Dimethyl sulfoxide	1 U	Same day	Dimethyl sulfoxide	0.80 U
Ticarcillin (CP)	B. 1	1 mg	1 day	B. 1	5.0 µg
Tobramycin (T)	Water	1 mg	14 days	Water	2.5 µg
Troleandomycin (T)	Isopropyl alcohol-water (4:1)	1 mg	Same day	Water	25 µg
Tylosin (T)	Methanol (10 mg/mL); [ B. 16]	1 mg	30 days	B.3: methanol (1:1)	4 µg
Vancomycin (CP)	Water	1 mg	7 days	B. 4	10 µg

notes—"B" denotes "buffer," and the number following refers to the potassium phosphate buffers defined in this chapter.

For amphotericin B, colistimethate sodium, and nystatin, prepare the USP Reference Standard solutions and the sample test solution simultaneously.

For amphotericin B, further dilute the stock solution with dimethyl sulfoxide to give concentrations of 12.8, 16, 20, 25, and 31.2 µg per mL prior to making the test dilutions. The Test Dilution of the sample should contain the same amount of dimethyl sulfoxide as the test dilutions of the USP Reference Standard.

For bacitracin zinc, each of the Standard test dilutions should contain the same amount of hydrochloric acid as the Test Dilution of the sample.

For neomycin turbidimetric assay, dilute the 100-µg-per-mL stock solution quantitatively with Buffer No. 3 to obtain a solution having a concentration equivalent to 25.0 µg of neomycin per mL. To separate 50-mL volumetric flasks add 1.39, 1.67, 2.00, 2.40, and 2.88 mL of this solution, add 5.0 mL of 0.01 N hydrochloric acid to each flask, dilute with Buffer No. 3 to volume, and mix to obtain solutions having concentrations of 0.69, 0.83, 1.0, 1.2, and 1.44 µg of neomycin per mL. Use these solutions to prepare the standard response line.

For nystatin, further dilute the stock solution with dimethylformamide to give concentrations of 256, 320, 400, 500, and 624 Units per mL prior to making the test dilutions. Prepare the standard response line solutions simultaneously with dilutions of the sample to be tested. The Test Dilution of the sample should contain the same amount of dimethylformamide as the test dilutions of the Standard. Use red low-actinic glassware.

For Polymyxin B, prepare the stock solution by adding 2 mL of water for each 5 mg of the weighed USP Reference Standard material.

Table 2. Test Organisms for Antibiotics Assayed by the Procedure Indicated in Table 1

Antibiotic	Test Organism	ATCC <sup>a</sup> Number
Amikacin	Staphylococcus aureus	29737
Amphotericin B	Saccharomyces cerevisiae	9763
Bacitracin	Micrococcus luteus	10240
Bleomycin	Mycobacterium smegmatis	607
Candididin	Saccharomyces cerevisiae	9763
Capreomycin	Klebsiella pneumoniae	10031
Carbenicillin	Pseudomonas aeruginosa	25619
Cephalothin	Staphylococcus aureus	29737
Cephapirin	Staphylococcus aureus	29737
Chloramphenicol	Escherichia coli	10536
Chlortetracycline	Staphylococcus aureus	29737
Cloxacillin	Staphylococcus aureus	29737
Colistimethate Sodium	Bordetella bronchiseptica	4617
Colistin	Bordetella bronchiseptica	4617
Cycloserine	Staphylococcus aureus	29737
Demeclocycline	Staphylococcus aureus	29737
Dihydrostreptomycin (CP)	Bacillus subtilis	6633
Dihydrostreptomycin (T)	Klebsiella pneumoniae	10031
Doxycycline	Staphylococcus aureus	29737
Erythromycin	Micrococcus luteus	9341
Gentamicin	Staphylococcus epidermidis	12228
Gramicidin	Enterococcus hirae	10541
Kanamycin	Staphylococcus aureus	29737
Methacycline	Staphylococcus aureus	29737
Nafcillin	Staphylococcus aureus	29737



Neomycin (CP)	<i>Staphylococcus epidermidis</i>	12228
Neomycin (T)	<i>Klebsiella pneumoniae</i>	10031
Netilmicin	<i>Staphylococcus epidermidis</i>	12228
Novobiocin	<i>Staphylococcus epidermidis</i>	12228
Nystatin	<i>Saccharomyces cerevisiae</i>	2601
Oxytetracycline	<i>Staphylococcus aureus</i>	29737
Paromomycin	<i>Staphylococcus epidermidis</i>	12228
Penicillin G	<i>Staphylococcus aureus</i>	29737
Polymyxin B	<i>Bordetella bronchiseptica</i>	4617
Rolitetracycline	<i>Staphylococcus aureus</i>	29737
Sisomicin	<i>Staphylococcus epidermidis</i>	12228
Spectinomycin	<i>Escherichia coli</i>	10536
Streptomycin (T)	<i>Klebsiella pneumoniae</i>	10031
Tetracycline	<i>Staphylococcus aureus</i>	29737
Thiostrepton (T)	<i>Enterococcus hirae</i>	10541
Tobramycin	<i>Staphylococcus aureus</i>	29737
Troleandomycin	<i>Klebsiella pneumoniae</i>	10031
Tylosin	<i>Staphylococcus aureus</i>	9144
Vancomycin	<i>Bacillus subtilis</i>	6633

\* American Type Culture Collection, 10801 University Boulevard, Manassas VA 20110-2209. (<http://www.atcc.org>).

Table 3. Preparation of Inoculum

Test Organism & (ATCC No.)	Incubation Conditions			Suggested Inoculum Composition		Antibiotics Assayed
	Medium	Temp. (°)	Time	Medium	Amount (mL per 100 mL)	
<i>Bacillus subtilis</i> (6633)	32	32 to 35	5 days	5	As required	Dihydrostreptomycin
				8	As required	Vancomycin
<i>Bordetella bronchiseptica</i> (4617)	1	32 to 35	24 hr.	10	0.1	Colistimethate Sodium, Colistin, Polymyxin B
<i>Escherichia coli</i> (10536)	1	32 to 35	24 hr.	3	0.7	Chloramphenicol
<i>Klebsiella pneumoniae</i> (10031)	1	36 to 37.5	16 to 24 hr.	3	0.05	Capreomycin
				0.1		Streptomycin, Troleandomycin, Dihydrostreptomycin
				39	2	Neomycin
<i>Micrococcus luteus</i> (9341)	1	32 to 35	24 hr.	11	1.5	Erythromycin
<i>Micrococcus luteus</i> (10240)	1	32 to 35	24 hr.	1	0.3	Bacitracin
<i>Mycobacterium smegmatis</i> (607)	36	36 to 37.5	48 hr.	35	1.0	Bleomycin
<i>Pseudomonas aeruginosa</i> (25619)	1	36 to 37.5	24 hr.	10	0.5	Carbenicillin
<i>Saccharomyces cerevisiae</i> (9763)	19	29 to 31	48 hr.	13	0.2	Candidin
				19	1.0	Amphotericin B
<i>Saccharomyces cerevisiae</i> (2601)	19	29 to 31	48 hr.	19	1.0	Nystatin
<i>Staphylococcus aureus</i> (9144)	3	35 to 39	16 to 18 hr.	39	2-3	Tylosin
<i>Staphylococcus aureus</i> (29737)	1	32 to 35	24 hr.	1	0.1	Cephalothin, Cephapirin, Cloxacillin
				1	0.3	Nafcillin
				1	1.0	Penicillin G
				3	0.1	Amikacin, Chlortetracycline, Demeclocycline, Doxycycline, Methacycline, Oxytetracycline, Rolitetracycline, Tetracycline
				3	0.2	Kanamycin
				3	0.4	Cycloserine
				3	0.15	Tobramycin
<i>Staphylococcus epidermidis</i> (12228)	1	32 to 35	24 hr.	11	0.25	Netilmicin
				1	4.0	Novobiocin
				11	0.03	Gentamicin, Sisomicin
				11	0.4	Neomycin
				11	2.0	Paromomycin
<i>Enterococcus hirae</i> (10541)	3	36 to 37.5	16 to 18 hr.	3	1.0	Gramicidin
	40	36 to	18 to 24	41	0.2	Thiostrepton

37.5

hr.

note—For *Pseudomonas aeruginosa* (ATCC 25619) in the assay of Carbenicillin, use 0.5 mL of a 1:25 dilution of the stock suspension per 100 mL of Medium 10.

#### Preparation of Inoculum

Preparatory to an assay, remove the growth from a recently grown slant or culture of the organism, with 3 mL of sterile saline TS and sterile glass beads. Inoculate the surface of 250 mL of the agar medium specified for that organism in [Table 3](#) and contained on the flat side of a Roux bottle except in the case of *Enterococcus hirae* and *Staphylococcus aureus* (ATCC 9144), which are grown in a liquid medium. Spread the suspension evenly over the surface of the agar with the aid of sterile glass beads, and incubate at the temperature shown for approximately the indicated length of time. At the end of this period, prepare the stock suspension by collecting the surface growth in 50 mL of sterile saline TS, except for *Bleomycin* (use 50 mL of [Medium 34](#)).

Determine by trial the quantity of stock suspension to be used as the Inoculum, starting with the volume suggested in [Table 3](#). The trial tests should be incubated for the times indicated in the section Turbidimetric Method for Procedure. Adjust the quantity of Inoculum on a daily basis, if necessary, to obtain the optimum dose-response relationship from the amount of growth of the test organism in the assay tubes and the length of the time of incubation. At the completion of the incubation periods described in the section Turbidimetric Method for Procedure, tubes containing the median dose of the Standard should have absorbances of at least 0.3 absorbance unit, except for *Amikacin*, *Chlortetracycline*, *Gramicidin*, and *Tetracycline* (0.35 absorbance unit), and *Capreomycin*, *Methacycline*, and *Tobramycin* (0.4 absorbance unit).

For the cylinder-plate assay, determine by trial the proportions of stock suspension to be incorporated in the Inoculum, starting with the volumes indicated in [Table 3](#), that result in satisfactory demarcation of the zones of inhibition of about 14 to 16 mm in diameter and giving a reproducible dose relationship. Prepare the inoculum by adding a portion of stock suspension to a sufficient amount of agar medium that has been melted and cooled to 45° to 50°, and swirling to attain a homogeneous suspension.

#### PROCEDURE

##### Assay Designs

Microbial assays gain markedly in precision by the segregation of relatively large sources of potential error and bias through suitable experimental designs. In a cylinder-plate assay, the essential comparisons are restricted to relationships between zone diameter measurements within plates, exclusive of the variation between plates in their preparation and subsequent handling. To conduct a turbidimetric assay so that the differences in observed turbidity will reflect the differences in the antibiotic concentration requires both greater uniformity in the environment created for the tubes through closer thermostatic control of the incubator and the avoidance of systematic bias by use of a random placement of replicate tubes in separate tube racks, each rack containing one complete set of treatments. The essential comparisons are then restricted to relationships between the observed turbidities within racks.

note—For some purposes, the practice is to design the assay so that a set of treatments consists of not fewer than three tubes for each sample and standard concentration, and each set is placed in a single rack.

Within these restrictions, the assay design recommended is a 1-level assay with a standard curve. For this assay with a standard curve, prepare solutions of 5, 6, or more test dilutions, provided they include one corresponding to the reference concentration (S3), of the Standard and a solution of a single median test level of the Unknown as described under Preparation of Standard and Preparation of the Sample. Consider an assay as preliminary if its computed potency with either design is less than 80% or more than 125% of that assumed in preparing the stock solution of the Unknown. In such a case, adjust its assumed potency accordingly and repeat the assay.

Microbial determinations of potency are subject to inter-assay as well as intra-assay variables, so that two or more independent assays are required for a reliable estimate of the potency of a given assay preparation or Unknown. Starting with separately prepared stock solutions and test dilutions of both the Standard and the Unknown, repeat the assay of a given Unknown on a different day. If the estimated potency of the second assay differs significantly, as indicated by the calculated standard error, from that of the first, conduct one or more additional assays. The combined result of a series of smaller, independent assays spread over a number of days is a more reliable estimate of potency than that from a single large assay with the same total number of plates or tubes.

##### Cylinder-Plate Method

To prepare assay plates using Petri dishes, place 21 mL of Medium 2 in each of the required number of plates, and allow it to harden into a smooth base layer of uniform depth, except for *Amphotericin B* and *Nystatin*, where no separate base layer is used. For *Erythromycin*, *Gentamicin*, *Neomycin B*, *Paromomycin*, and *Sisomicin*, use Medium 11. For *Bleomycin*, use 10 mL of Medium 35. For *Dihydrostreptomycin* use Medium 5. For *Vancomycin*, use 10 mL of Medium 8. For *Carbenicillin*, *Colistimethate Sodium*, *Colistin*, and *Polymyxin B*, use [Medium 9](#). For *Netilmicin*, use 20 mL of Medium 11. Add 4 mL of seed layer inoculum (see Preparation of Inoculum and [Table 3](#)), prepared as directed for the given antibiotic, except for *Bleomycin* (use 6 mL), for *Netilmicin* (use 5 mL), and for *Nystatin* and *Amphotericin B* (use 8 mL), tilting the plate back and forth to spread the inoculum evenly over the surface, and allow it to harden. Drop six assay cylinders on the inoculated surface from a height of 12 mm, using a mechanical guide or other device to insure even spacing on a radius of 2.8 cm, and cover the plates to avoid contamination. After filling the six cylinders on each plate with dilutions of antibiotic containing the test levels specified below, incubate the plates at 32° to 35°, or at the temperature specified below for the individual case, for 16 to 18 hours, remove the cylinders, and measure and record the diameter of each zone of growth inhibition to the nearest 0.1 mm. Incubate the plates at 29° to 31° for *Amphotericin B* and *Nystatin*. Incubate at 34° to 36° for *Novobiocin*. Incubate at 36° to 37.5° for *Carbenicillin*, *Colistimethate Sodium*, *Colistin*, *Dihydrostreptomycin*, *Gentamicin*, *Neomycin*, *Netilmicin*, *Paromomycin*, *Polymyxin B*, *Sisomicin*, and *Vancomycin*.

For the 1-level assay with a standard curve, prepare dilutions representing five test levels of the Standard (S1 to S5) and a single test level of the Unknown U3 corresponding to S3 of the standard curve, as defined under Preparation of the Standard and Preparation of the Sample. For deriving the standard curve, fill alternate cylinders on each of three plates with the median test dilution (S3) of the Standard and each of the remaining nine cylinders with one of the other four dilutions of the Standard. Repeat the process for the three dilutions of the Standard. For each Unknown, fill alternate cylinders on each of three plates with the median test dilution of the Standard (S3), and the remaining nine cylinders with the corresponding test dilution (U3) of the Unknown.

##### Turbidimetric Method

On the day of the assay, prepare the necessary doses by dilution of stock solutions of the Standard and of each Unknown as defined under Preparation of the Standard and Preparation of the Sample. Add 1.0 mL of each dose, except for *Gramicidin*, *Thiostrepton*, and *Tylosin* (use 0.10 mL) to each of 3 prepared test tubes, and place the 3 replicate tubes in a position, selected at random, in a test tube rack or other carrier. Include similarly in each rack 1 or 2 control tubes containing 1 mL of the test diluent (see Table 1) but no antibiotic. Upon completion of the rack of test solutions (with *Candididin*, within 30 minutes of the time when water is added to the dimethyl sulfoxide stock solution), add 9.0 mL of inoculum to each tube in the rack in turn, and place the completed rack immediately in an incubator or a water bath maintained at 36° to 37.5°, except for *Candididin* (incubate at 27° to 29°). Incubate the tubes for 4 to 5 hours, except for *Capreomycin*, *Chloramphenicol*, *Cycloserine*, *Dihydrostreptomycin*, *Spectinomycin*, *Streptomycin*, and *Troleandomycin* (incubate for 3 to 4 hours), *Tylosin* (incubate for 3 to 5 hours), and *Candididin* (incubate for 16 to 18 hours). After incubation add 0.5 mL of dilute formaldehyde to each tube, except for *Tylosin* (heat the rack in a water bath at 80° to 90° for 2 to 6 minutes or in a steam bath for 5 to 10 minutes, and bring to room temperature), taking one rack at a time, and read its transmittance or absorbance in a suitable spectrophotometer fitted with a 530-nm or 580-nm filter (see Spectrophotometer under Apparatus).

For the 1-level assay with a standard curve, prepare dilutions representing 5 test levels of the Standard (S1 to S5) and a single test level (U3) of each of up to 20 Unknowns corresponding to S3 of the Standard. Prepare also an extra S3 as a test of growth. Add 1 mL of each test dilution, except for *Gramicidin*, *Thiostrepton*, and *Tylosin* (use 0.10 mL) to 3 tubes and 1 mL of antibiotic-free diluent to 6 tubes as controls. Distribute one complete set, including 2 tubes of controls, to a tube rack, intermingling them at random. Add 9.0 mL of inoculum, except for *Thiostrepton* (use 10.0 mL of inoculum), incubate, add 0.5 mL of dilute formaldehyde, and complete the assay as directed above. Determine the exact duration of incubation by observation of growth in the reference concentration (median dose) of the dilutions of the Standard (S3).

#### CALCULATION

To calculate the potency from the data obtained either by the cylinder-plate or by the turbidimetric method, proceed in each case as directed under Potencies Interpolated from a



standard Curve (see [Design and Analysis of Biological Assays](#) (111)), using a log transformation, straight-line method with a least-squares fitting procedure, and a test for linearity. Where a number of assays of the same material are made with the same standard curve, calculate the coefficient of variation of results of all of the assays of the material. Where more than one assay is made of the same material with different standard curves, average the two or more values of the potency.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Ahalya Wise, M.S.</a> Scientist 1-301-816-8161	(MDANT05) Monograph Development-Antibiotics

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### 85 BACTERIAL ENDOTOXINS TEST

◆ Portions of this general chapter have been harmonized with the corresponding texts of the European Pharmacopoeia and/or the Japanese Pharmacopoeia. Those portions that are not harmonized are marked with symbols (◆◆) to specify this fact.◆

This chapter provides a test to detect or quantify bacterial endotoxins that may be present in or on the sample of the article(s) to which the test is applied. It uses Limulus Amebocyte Lysate (LAL) obtained from the aqueous extracts of circulating amebocytes of horseshoe crab (Limulus polyphemus or Tachypleus tridentatus) which has been prepared and characterized for use as an LAL Reagent.◆◆

There are two types of techniques for this test: the gel-clot techniques, which are based on gel formation, and the photometric techniques. The latter include a turbidimetric method, which is based on the development of turbidity after cleavage of an endogenous substrate, and a chromogenic method, which is based on the development of color after cleavage of a synthetic peptide-chromogen complex. Proceed by any one of these techniques, unless otherwise indicated in the monograph. In case of dispute, the final decision is based on the gel-clot techniques, unless otherwise indicated in the monograph.

In the gel-clot techniques, the reaction endpoint is determined from dilutions of the material under test in direct comparison with parallel dilutions of a reference endotoxin, and quantities of endotoxin are expressed in USP Endotoxin Units (USP-EU). [note—One USP-EU is equal to one IU of endotoxin.]

Because LAL Reagents have been formulated to be used also for turbidimetric or colorimetric tests, such tests may be used to comply with the requirements. These tests require the establishment of a standard regression curve; the endotoxin content of the test material is determined by interpolation from the curve. The procedures include incubation for a preselected time of reacting endotoxin and control solutions with LAL Reagent and reading of the spectrophotometric light absorbance at suitable wavelengths. In the endpoint turbidimetric procedure the reading is made immediately at the end of the incubation period. In the endpoint colorimetric procedure the reaction is arrested at the end of the preselected time by the addition of an enzyme reaction-terminating agent prior to the readings. In the turbidimetric and colorimetric kinetic assays the absorbance is measured throughout the reaction period and rate values are determined from those readings.

#### APPARATUS AND GLASSWARE

Depyrogenate all glassware and other heat-stable materials in a hot-air oven using a validated process.◆◆ Commonly used minimum time and temperature settings are 30 minutes at 250°. If employing plastic apparatus, such as microplates and pipet tips for automatic pipettors, use only that which has been shown to be free of detectable endotoxin and not to interfere with the test. [note—In this chapter, the term “tube” includes any other receptacle such as a micro-titer well.]

#### PREPARATION OF THE STANDARD ENDOTOXIN STOCK SOLUTION AND STANDARD SOLUTIONS

The [USP Endotoxin RS](#) has a defined potency of 10,000 USP Endotoxin Units (EU) per vial. Constitute the entire contents of 1 vial of the RSE with 5 mL of LAL Reagent Water◆◆, mix intermittently for 30 minutes, using a vortex mixer, and use this concentrate for making appropriate serial dilutions. Preserve the concentrate in a refrigerator for making subsequent dilutions for not more than 14 days. Mix vigorously, using a vortex mixer, for not less than 3 minutes before use. Mix each dilution for not less than 30 seconds before proceeding to make the next dilution. Do not store dilutions, because of loss of activity by adsorption, in the absence of supporting data to the contrary.

#### Preparatory Testing

Use an LAL Reagent of confirmed label sensitivity.

The validity of test results for bacterial endotoxins requires an adequate demonstration that specimens of the article or of solutions, washings, or extracts thereof to which the test is to be applied do not of themselves inhibit or enhance the reaction or otherwise interfere with the test. Validation is accomplished by performing the inhibition or enhancement test described under each of the three techniques indicated. Appropriate negative controls are included. Validation must be repeated if the LAL Reagent source or the method of manufacture or formulation of the article is changed.

#### Preparation of Sample Solutions

Prepare sample solutions by dissolving or diluting drugs or extracting medical devices using LAL Reagent Water. Some substances or preparations may be more appropriately dissolved, diluted, or extracted in other aqueous solutions. If necessary, adjust the pH of the solution (or dilution thereof) to be examined so that the pH of the mixture of the LAL Reagent and sample falls within the pH range specified by the LAL Reagent manufacturer. This usually applies to a product with a pH in the range of 6.0 to 8.0. The pH may be adjusted using an acid, base, or suitable buffer as recommended by the LAL Reagent manufacturer. Acids and bases may be prepared from concentrates or solids with LAL Reagent Water in containers free of detectable endotoxin. Buffers must be validated to be free of detectable endotoxin and interfering factors.

#### DETERMINATION OF MAXIMUM VALID DILUTION (MVD)

The Maximum Valid Dilution is the maximum allowable dilution of a specimen at which the endotoxin limit can be determined. It applies to injections or to solutions for parenteral administration in the form constituted or diluted for administration, or, where applicable, to the amount of drug by weight if the volume of the dosage form for administration could be varied. The general equation to determine MVD is:

$$MVD = (\text{Endotoxin limit} \times \text{Concentration of sample solution}) / \lambda$$

where the concentration of sample solution and  $\lambda$  are as defined below. Where the endotoxin limit concentration is specified in the individual monograph in terms of volume (in EU per mL), divide the limit by  $\lambda$ , which is the labeled sensitivity (in EU per mL) of the LAL Reagent, to obtain the MVD factor. Where the endotoxin limit concentration is specified in the individual monograph in terms of weight or Units of active drug (in EU per mg or in EU per Unit), multiply the limit by the concentration (in mg per mL or in Units per mL) of the drug in the solution tested or of the drug constituted according to the label instructions, whichever is applicable, and divide the product of the multiplication by  $\lambda$ , to obtain the MVD factor. The MVD factor so obtained is the limit dilution factor for the preparation for the test to be valid.

#### ESTABLISHMENT OF ENDOTOXIN LIMITS

The endotoxin limit for parenteral drugs, defined on the basis of dose, is equal to  $K/M$ ,◆◆ where  $K$  is the threshold human pyrogenic dose of endotoxin per kg of body weight, and  $M$  is equal to the maximum recommended human dose of product per kg of body weight in a single hour period.



The endotoxin limit for parenteral drugs is specified in individual monographs in units such as EU/mL, EU/mg, or EU/Unit of biological activity.

## GEL-CLOT TECHNIQUES

The gel-clot techniques detect or quantify endotoxins based on clotting of the LAL Reagent in the presence of endotoxin. The concentration of endotoxin required to cause the lysate to clot under standard conditions is the labeled sensitivity of the LAL Reagent. To ensure both the precision and validity of the test, tests for confirming the labeled LAL Reagent sensitivity and for interfering factors are described under Preparatory Testing for the Gel-Clot Techniques.

### Preparatory Testing for the Gel-Clot Techniques

Test for Confirmation of Labeled LAL Reagent Sensitivity— Confirm the labeled sensitivity using at least 1 vial of the LAL Reagent lot. Prepare a series of two-fold dilutions of the [USP Endotoxin RS](#) in LAL Reagent Water to give concentrations of  $2\lambda$ ,  $\lambda$ ,  $0.5\lambda$ , and  $0.25\lambda$ , where  $\lambda$  is as defined above. Perform the test on the four standard concentrations in quadruplicate and include negative controls. The test for confirmation of lysate sensitivity is to be carried out when a new batch of LAL Reagent is used or when there is any change in the experimental conditions that may affect the outcome of the test.

Mix a volume of the LAL Reagent with an equal volume (such as 0.1-mL aliquots) of one of the standard solutions in each test tube. When single test vials or ampuls containing lyophilized LAL Reagent are used, add solutions directly to the vial or ampul. Incubate the reaction mixture for a constant period according to directions of the LAL Reagent manufacturer (usually at  $37 \pm 1^\circ$  for  $60 \pm 2$  minutes), avoiding vibration. To test the integrity of the gel, take each tube in turn directly from the incubator and invert it through about  $180^\circ$  in one smooth motion. If a firm gel has formed that remains in place upon inversion, record the result as positive. A result is negative if an intact gel is not formed. The test is not valid unless the lowest concentration of the standard solutions shows a negative result in all replicate tests.

The endpoint is the last positive test in the series of decreasing concentrations of endotoxin. Calculate the mean value of the logarithms of the endpoint concentration and then the antilogarithm of the mean value using the following equation:

$$\text{Geometric Mean Endpoint Concentration} = \text{antilog}(\Sigma e / f)$$

where  $\Sigma e$  is the sum of the log endpoint concentrations of the dilution series used, and  $f$  is the number of replicate test tubes. The geometric mean endpoint concentration is the measured sensitivity of the LAL Reagent (in EU/mL). If this is not less than  $0.5\lambda$  and not more than  $2\lambda$ , the labeled sensitivity is confirmed and is used in tests performed with this lysate.

Interfering Factors Test for the Gel-Clot Techniques— Prepare solutions A, B, C, and D as shown in [Table 1](#), and perform the inhibition/enhancement test on the sample solutions at a dilution less than the MVD, not containing any detectable endotoxins, following the procedure in the Test for Confirmation of Labeled LAL Reagent Sensitivity above. The geometric mean endpoint concentrations of solutions B and C are determined using the equation in that test.

Table 1. Preparation of Solutions for the Inhibition/Enhancement Test for Gel-Clot Techniques

Solution	Endotoxin Concentration/Solution to which Endotoxin is Added	Diluent	Dilution Factor	Initial Endotoxin Concentration	Number of Replicates
A <sup>a</sup>	none/sample solution	—	—	—	4
B <sup>b</sup>	$2\lambda$ /sample solution	sample solution	1	$2\lambda$	4
			2	$1\lambda$	4
			4	$0.5\lambda$	4
			8	$0.25\lambda$	4
C <sup>c</sup>	$2\lambda$ /water for BET	LAL Reagent Water	1	$2\lambda$	2
			2	$1\lambda$	2
			4	$0.5\lambda$	2
			8	$0.25\lambda$	2
D <sup>d</sup>	none/LAL Reagent Water	—	—	—	2

a Solution A: a sample solution of the preparation under test that is free of detectable endotoxins.

b Solution B: test for interference.

c Solution C: control for labeled LAL Reagent sensitivity.

d Solution D: negative control of LAL Reagent Water.

This test must be repeated when any condition that is likely to influence the test results changes. The test is not valid unless Solutions A and D show no reaction and the result of Solution C confirms the labeled sensitivity.

If the sensitivity of the lysate determined in the presence of the sample solution under test of Solution B is not less than  $0.5\lambda$  and not greater than  $2\lambda$ , the sample solution does not contain factors which interfere under the experimental conditions used. Otherwise, the sample solution to be examined interferes with the test.

If the sample under test does not comply with the test at a dilution less than the MVD, repeat the test using a greater dilution, not exceeding the MVD. The use of a more sensitive lysate permits a greater dilution of the sample to be examined and this may contribute to the elimination of interference.

Interference may be overcome by suitable treatment, such as filtration, neutralization, dialysis, or heating. To establish that the chosen treatment effectively eliminates interference without loss of endotoxins, perform the assay described below using the preparation to be examined to which [USP Endotoxin RS](#) has been added and which has been subjected to the selected treatment.

### Gel-Clot Limit Test

This test is used when a monograph contains a requirement for endotoxin limits.

Procedure— Prepare Solutions A, B, C, and D as shown in [Table 2](#), and perform the test on these solutions following the procedure in the Test for Confirmation of Labeled LAL Reagent Sensitivity under Preparatory Testing for the Gel-Clot Techniques.

Table 2. Preparation of Solutions for the Gel-Clot Limit Test

Solution*	Endotoxin Concentration/Solution to which Endotoxin is Added	Number of Replicates
A	none/diluted sample solution	2
B	$2\lambda$ /diluted sample solution	2
C	$2\lambda$ /LAL Reagent Water	2
D	none/LAL Reagent Water	2

\* Prepare Solution A and positive product control Solution B using a dilution not greater than the MVD and treatments as directed in the Interfering Factors Test for the Gel-Clot Techniques under Preparatory Testing for the Gel-Clot Techniques. Positive control Solutions B and C contain the standard endotoxin preparation at a concentration corresponding to twice the labeled LAL Reagent sensitivity. The negative control Solution D is LAL Reagent Water.

...terpretation— The test is not valid unless both replicates of positive control Solutions B and C are positive and those of negative control Solution D are negative. The preparation under test complies with the test when a negative result is found for both tubes containing Solution A. The preparation under test does not comply with the test when a positive result is found for both tubes containing Solution A.

Repeat the test when a positive result is found for 1 tube containing Solution A and a negative result for the other one. The preparation under test complies with the test when a negative result is found for both tubes containing Solution A in the repeat result. If the test is positive for the preparation under test at a dilution less than the MVD, the test may be repeated at a dilution not greater than the MVD.

#### Gel-Clot Assay

This assay quantifies bacterial endotoxins in sample solutions by titration to an endpoint.

Procedure— Prepare Solutions A, B, C, and D as shown in [Table 3](#), and test these solutions by following the procedure in the Test for Confirmation of Labeled LAL Reagent Sensitivity under Preparatory Testing for the Gel-Clot Techniques.

Table 3. Preparation of Solutions for the Gel-Clot Assay

Solution	Endotoxin Concentration/Solution to which Endotoxin is Added	Diluent	Dilution Factor	Initial Endotoxin Concentration	Number of Replicates
A <sup>a</sup>	none/sample solution	LAL Reagent Water	1	—	2
			2	—	2
			4	—	2
			8	—	2
B <sup>b</sup>	2 $\lambda$ /sample solution	—	1	2 $\lambda$	2
C <sup>c</sup>	2 $\lambda$ /LAL Reagent Water	LAL Reagent Water	1	2 $\lambda$	2
			2	1 $\lambda$	2
			4	0.5 $\lambda$	2
			8	0.25 $\lambda$	2
D <sup>d</sup>	none/LAL Reagent Water	—	—	—	2

a Solution A: a sample solution under test at the dilution, not to exceed the MVD, with which the Interfering Factors Test for the Gel-Clot Techniques was completed. Subsequent dilution of the sample solution must not exceed the MVD. Use LAL Reagent Water to make dilution series of four tubes containing the sample solution under test at concentrations of 1,  $\frac{1}{2}$ ,  $\frac{1}{4}$ , and  $\frac{1}{8}$  relative to the dilution with which the Interfering Factors Test for the Gel-Clot Techniques was completed. Other dilutions may be used as appropriate.

b Solution B: Solution A containing standard endotoxin at a concentration of 2 $\lambda$  (positive product control).

c Solution C: two series of 4 tubes of LAL Reagent Water containing the standard endotoxin at a concentration of 2 $\lambda$ ,  $\lambda$ , 0.5 $\lambda$ , and 0.25 $\lambda$ , respectively.

d Solution D: LAL Reagent Water (negative control).

Calculation and Interpretation— The test is not valid unless the following conditions are met: (1) both replicates of negative control Solution D are negative; (2) both replicates of positive product control Solution B are positive; and (3) the geometric mean endpoint concentration of Solution C is in the range of 0.5 $\lambda$  to 2 $\lambda$ .

To determine the endotoxin concentration of Solution A, calculate the endpoint concentration for each replicate series of dilutions by multiplying each endpoint dilution factor by  $\lambda$ . The endotoxin concentration in the sample is the geometric mean endpoint concentration of the replicates (see the formula given in the Test for Confirmation of Labeled LAL Reagent Sensitivity under Preparatory Testing for the Gel-Clot Techniques). If the test is conducted with a diluted sample solution, calculate the concentration of endotoxin in the original sample solution by multiplying by the dilution factor. If none of the dilutions of the sample solution is positive in a valid assay, report the endotoxin concentration as less than  $\lambda$  (if the diluted sample was tested, less than  $\lambda$  times the lowest dilution factor of the sample.) If all dilutions are positive, the endotoxin concentration is reported as equal to or greater than the greatest dilution factor multiplied by  $\lambda$  (e.g., initial dilution factor times 8 times  $\lambda$  in [Table 3](#)).

The article meets the requirements of the test if the concentration of endotoxin is less than that specified in the individual monograph.

#### PHOTOMETRIC TECHNIQUES

The turbidimetric method measures increases in turbidity. Depending on the test principle used, this technique is classified as either endpoint-turbidimetric or kinetic-turbidimetric. The endpoint-turbidimetric technique is based on the quantitative relationship between the concentration of endotoxins and the turbidity (absorbance or transmission) of the reaction mixture at the end of an incubation period. The kinetic-turbidimetric technique is a method to measure either the onset time needed to reach a predetermined absorbance of the reaction mixture or the rate of turbidity development.

The chromogenic method measures the chromophore released from a suitable chromogenic peptide by the reaction of endotoxins with the LAL Reagent. Depending on the test principle employed, this technique is classified as either endpoint-chromogenic or kinetic-chromogenic. The endpoint-chromogenic technique is based on the quantitative relationship between the concentration of endotoxins and the release of chromophore at the end of an incubation period. The kinetic-chromogenic technique is a method to measure either the onset time needed to reach a predetermined absorbance of the reaction mixture or the rate of color development.

All photometric tests are carried out at the incubation temperature recommended by the LAL Reagent manufacturer, which is usually 37  $\pm$  1°.

#### Preparatory Testing for the Photometric Techniques

To assure the precision or validity of the turbidimetric and chromogenic techniques, preparatory tests are conducted to verify that the criteria for the standard curve are valid and that the sample solution does not inhibit or enhance the reaction. Revalidation for the test method is required when conditions that are likely to influence the test result change.

Verification of Criteria for the Standard Curve— Using the Standard Endotoxin Solution, prepare at least three endotoxin concentrations to generate the standard curve. Perform the test using at least three replicates of each standard endotoxin concentration according to the manufacturer's instructions for the LAL Reagent (with regard to volume ratios, incubation time, temperature, pH, etc.). If the desired range in the kinetic methods is greater than two logs, additional standards should be included to bracket each log increase within the range of the standard curve. The absolute value of the correlation coefficient, |r|, must be greater than or equal to 0.980 for the range of endotoxin concentrations indicated by the manufacturer of the LAL Reagent.

Interfering Factors Test for the Photometric Techniques— Select an endotoxin concentration at or near the middle of the endotoxin standard curve. Prepare Solutions A, B, C, and D as shown in [Table 4](#). Perform the test on Solutions A, B, C, and D at least in duplicate following the instructions for the LAL Reagent used (with regard to volume of sample and LAL Reagent, volume ratio of sample to LAL Reagent, incubation time, etc.).

Table 4. Preparation of Solutions for the Inhibition/Enhancement Test for Photometric Techniques

Solution	Endotoxin Concentration	Solution to which Endotoxin is Added	Number of Replicates
A <sup>a</sup>	none	sample solution	not less than 2
B <sup>b</sup>	middle concentration of the standard curve	sample solution	not less than 2
C <sup>c</sup>	at least 3 concentrations (lowest concentration is designated $\lambda$ )	LAL Reagent Water	each not less than 2
D <sup>d</sup>	none	LAL Reagent Water	not less than 2



Solution A: the sample solution may be diluted not to exceed MVD.

- b Solution B: the preparation under test at the same dilution as Solution A, containing added endotoxin at a concentration equal to or near the middle of the standard curve.
- c Solution C: the standard endotoxin at the concentrations used in the validation of the method described in Verification of Criteria for the Standard Curve under Preparatory Testing for the Photometric Techniques (positive control series).
- d Solution D: LAL Reagent Water (negative control).

Calculate the mean recovery of the added endotoxin by subtracting the mean endotoxin concentration in the solution (if any) from that containing the added endotoxin. In order to be considered free of interfering factors under the conditions of the test, the measured concentration of the endotoxin added to the sample solution must be within 50% to 200% of the known added endotoxin concentration after subtraction of any endotoxin detected in the solution without added endotoxin.

When the endotoxin recovery is out of the specified ranges, the interfering factors must be removed as described in the Interfering Factors Test for the Gel-Clot Techniques under Preparatory Testing for the Gel-Clot Techniques. Repeating the Interfering Factors Test for the Gel-Clot Techniques validates the treatment.

#### Procedure for the Photometric Techniques

Follow the procedure described in the Interfering Factors Test for the Photometric Techniques under Preparatory Testing for the Photometric Techniques.

#### Calculation for the Photometric Techniques

Calculate the endotoxin concentration of each of the replicates of test Solution A using the standard curve generated by positive control series C. The test is not valid unless the following conditions are met: (1) the results of control series C comply with the requirements for validation defined under Verification of Criteria for the Standard Curve under Preparatory Testing for the Photometric Techniques; (2) the endotoxin recovery, calculated from the concentration found in Solution B after subtracting the endotoxin concentration found in Solution A is within 50 to 200%; and (3) the result of negative control series D does not exceed the limit of the blank value required in the description of the LAL Reagent used.

#### Interpretation of Results from the Photometric Techniques

In photometric assays, the preparation under test complies with the test if the mean endotoxin concentration of the replicates of Solution A, after correction for dilution and concentration, is less than the endotoxin limit for the product.

◆1 LAL Reagent reacts with some  $\beta$ -glucans in addition to endotoxins. Some preparations that are treated will not react with  $\beta$ -glucans and must be used for samples that contain glucans.◆

◆2 For a validity test of the procedure for inactivating endotoxins, see Dry-Heat Sterilization under [Sterilization and Sterility Assurance of Compendial Articles](#) (1211). Use an LAL Reagent having a sensitivity of not less than 0.15 Endotoxin Unit per mL.◆

◆3 Sterile Water for Injection or other water that shows no reaction with the specific LAL Reagent with which it is to be used, at the limit of sensitivity of such reagent.◆

◆4 K is 5 USP-EU/kg for any route of administration other than intrathecal (for which K is 0.2 USP-EU/kg body weight). For radiopharmaceutical products not administered intrathecally the endotoxin limit is calculated as 175/V, where V is the maximum recommended dose in mL. For intrathecally administered radiopharmaceuticals, the endotoxin limit is obtained by the formula 14/V. For formulations (usually anticancer products) administered on a per square meter of body surface, the formula is K/M, where K = 5 EU/kg and M is the (maximum dose/m<sup>2</sup>/hour  $\times$  1.80 m<sup>2</sup>)/70 Kg.◆

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Radhakrishna S Tirumalai, Ph.D.</a> Senior Scientist 1-301-816-8339	(MSA05) Microbiology and Sterility Assurance

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#### 87 BIOLOGICAL REACTIVITY TESTS, IN VITRO

The following tests are designed to determine the biological reactivity of mammalian cell cultures following contact with the elastomeric plastics and other polymeric materials with direct or indirect patient contact or of specific extracts prepared from the materials under test. It is essential that the tests be performed on the specified surface area. When the surface area of the specimen cannot be determined, use 0.1 g of elastomer or 0.2 g of plastic or other material for every mL of extraction fluid. Exercise care in the preparation of the materials to prevent contamination with microorganisms and other foreign matter.

Three tests are described (i.e., the Agar Diffusion Test, the Direct Contact Test, and the Elution Test).<sup>1</sup> The decision as to which type of test or the number of tests to be performed to assess the potential biological response of a specific sample or extract depends upon the material, the final product, and its intended use. Other factors that may also affect the suitability of sample for a specific use are the polymeric composition; processing and cleaning procedures; contacting media; inks; adhesives; absorption, adsorption, and permeability of preservatives; and conditions of storage. Evaluation of such factors should be made by appropriate additional specific tests before determining that a product made from a specific material is suitable for its intended use.

[USP Reference Standards](#) (11)—[USP High-Density Polyethylene RS](#). [USP Positive Bioreaction RS](#).

Cell Culture Preparation— Prepare multiple cultures of L-929 (ATCC cell line CCL 1, NCTC clone 929) mammalian fibroblast cells in serum-supplemented minimum essential medium having a seeding density of about 105 cells per mL. Incubate the cultures at 37  $\pm$  1° in a humidified incubator for not less than 24 hours in a 5  $\pm$  1% carbon dioxide atmosphere until a monolayer, with greater than 80% confluence, is obtained. Examine the prepared cultures under a microscope to ensure uniform, near-confluent monolayers. [note—The reproducibility of the In Vitro Biological Reactivity Tests depends upon obtaining uniform cell culture density.]

Extraction Solvents— [Sodium Chloride Injection](#) (see monograph—use Sodium Chloride Injection containing 0.9% of NaCl). Alternatively, serum-free mammalian cell culture media or serum-supplemented mammalian cell culture media may be used. Serum supplementation is used when extraction is done at 37° for 24 hours.

#### Apparatus—

Autoclave— Employ an autoclave capable of maintaining a temperature of 121  $\pm$  2°, equipped with a thermometer, a pressure gauge, a vent cock, a rack adequate to accommodate the test containers above the water level, and a water cooling system that will allow for cooling of the test containers to about 20°, but not below 20°, immediately following the heating cycle.

Oven— Use an oven, preferably a mechanical convection model, that will maintain operating temperatures in the range of 50° to 70° within  $\pm$  2°.

Incubator— Use an incubator capable of maintaining a temperature of 37  $\pm$  1° and a humidified atmosphere of 5  $\pm$  1% carbon dioxide in air.

Extraction Containers— Use only containers, such as ampuls or screw-cap culture test tubes, or their equivalent, of Type I glass. If used, culture test tubes, or their equivalent, are



Closed with a screw cap having a suitable elastomeric liner. The exposed surface of the elastomeric liner is completely protected with an inert solid disk 50 to 75  $\mu\text{m}$  in thickness. . . suitable disk can be fabricated from polytef.

**Preparation of Apparatus**— Cleanse all glassware thoroughly with chromic acid cleansing mixture and, if necessary, with hot nitric acid followed by prolonged rinsing with Sterile Water for Injection. Sterilize and dry by a suitable process containers and devices used for extraction, transfer, or administration of test material. If ethylene oxide is used as the sterilizing agent, allow not less than 48 hours for complete degassing.

#### Procedure—

Preparation of Sample for Extracts— Prepare as directed in the Procedure under [Biological Reactivity Tests, In Vivo](#) (88).

**Preparation of Extracts**— Prepare as directed for Preparation of Extracts in [Biological Reactivity Tests, In Vivo](#) (88) using either Sodium Chloride Injection (0.9% NaCl) or serum-free mammalian cell culture media as Extraction Solvents. [note—If extraction is done at 37° for 24 hours in an incubator, use cell culture media supplemented by serum. The extraction conditions should not in any instance cause physical changes, such as fusion or melting of the material pieces, other than a slight adherence.]

#### Agar Diffusion Test

This test is designed for elastomeric closures in a variety of shapes. The agar layer acts as a cushion to protect the cells from mechanical damage while allowing the diffusion of leachable chemicals from the polymeric specimens. Extracts of materials that are to be tested are applied to a piece of filter paper.

**Sample Preparation**— Use extracts prepared as directed or use portions of the test specimens having flat surfaces not less than 100  $\text{mm}^2$  in surface area.

Positive Control Preparation— Proceed as directed for Sample Preparation.

Negative Control Preparation— Proceed as directed for Sample Preparation.

**Procedure**— Using 7 mL of cell suspension prepared as directed under Cell Culture Preparation, prepare the monolayers in plates having a 60-mm diameter. Following incubation, aspirate the culture medium from the monolayers, and replace it with serum-supplemented culture medium containing not more than 2% of agar. [note—The quality of the agar must be adequate to support cell growth. The agar layer must be thin enough to permit diffusion of leached chemicals.] Place the flat surfaces of Sample Preparation, Negative Control Preparation, and Positive Control Preparation or their extracts in an appropriate extracting medium, in duplicate cultures in contact with the solidified agar surface. Use no more than three specimens per prepared plate. Incubate all cultures for not less than 24 hours at 37  $\pm$  1°, preferably in a humidified incubator containing 5  $\pm$  1% of carbon dioxide. Examine each culture around each Sample, Negative Control, and Positive Control, under a microscope, using a suitable stain, if desired.

**Interpretation of Results**— The biological reactivity (cellular degeneration and malformation) is described and rated on a scale of 0 to 4 (see [Table 1](#)). Measure the responses of the cell cultures to the Sample Preparation, the Negative Control Preparation, and the Positive Control Preparation. The cell culture test system is suitable if the observed responses to the Negative Control Preparation is grade 0 (no reactivity) and to the Positive Control Preparation is at least grade 3 (moderate). The Sample meets the requirements of the test if the response to the Sample Preparation is not greater than grade 2 (mildly reactive). Repeat the procedure if the suitability of the system is not confirmed.

Table 1. Reactivity Grades for Agar Diffusion Test and Direct Contact Test

Grade	Reactivity	Description of Reactivity Zone
0	None	No detectable zone around or under specimen
1	Slight	Some malformed or degenerated cells under specimen
2	Mild	Zone limited to area under specimen
3	Moderate	Zone extends 0.5 to 1.0 cm beyond specimen
4	Severe	Zone extends greater than 1.0 cm beyond specimen

#### Direct Contact Test

This test is designed for materials in a variety of shapes. The procedure allows for simultaneous extraction and testing of leachable chemicals from the specimen with a serum-supplemented medium. The procedure is not appropriate for very low- or high-density materials that could cause mechanical damage to the cells.

**Sample Preparation**— Use portions of the test specimen having flat surfaces not less than 100  $\text{mm}^2$  in surface area.

Positive Control Preparation— Proceed as directed for Sample Preparation.

Negative Control Preparation— Proceed as directed for Sample Preparation.

**Procedure**— Using 2 mL of cell suspension prepared as directed under Cell Culture Preparation, prepare the monolayers in plates having a 35-mm diameter. Following incubation, aspirate the culture medium from the cultures, and replace it with 0.8 mL of fresh culture medium. Place a single Sample Preparation, a Negative Control Preparation, and a Positive Control Preparation in each of duplicate cultures. Incubate all cultures for not less than 24 hours at 37  $\pm$  1° in a humidified incubator containing 5  $\pm$  1% of carbon dioxide. Examine each culture around each Sample, Negative Control, and Positive Control Preparation, either visually or under a microscope, using a suitable stain, if desired.

**Interpretation of Results**— Proceed as directed for Interpretation of Results under Agar Diffusion Test. The Sample meets the requirements of the test if the response to the Sample Preparation is not greater than grade 2 (mildly reactive). Repeat the procedure if the suitability of the system is not confirmed.

#### Elution Test

This test is designed for the evaluation of extracts of polymeric materials. The procedure allows for extraction of the specimens at physiological or nonphysiological temperatures for varying time intervals. It is appropriate for high-density materials and for dose-response evaluations.

**Sample Preparation**— Prepare as directed in Preparation of Extracts, using either Sodium Chloride Injection (0.9% NaCl) or serum-free mammalian cell culture media as Extraction Solvents. If the size of the Sample cannot be readily measured, a mass of not less than 0.1 g of elastomeric material or 0.2 g of plastic or polymeric material per mL of extraction medium may be used. Alternatively, use serum-supplemented mammalian cell culture media as the extracting medium to simulate more closely physiological conditions. Prepare the extracts by heating for 24 hours in an incubator containing 5  $\pm$  1% of carbon dioxide. Maintain the extraction temperature at 37  $\pm$  1°, because higher temperatures may cause denaturation of serum proteins.

Positive Control Preparation— Proceed as directed for Sample Preparation.

Negative Control Preparation— Proceed as directed for Sample Preparation.

**Procedure**— Using 2 mL of cell suspension prepared as directed under Cell Culture Preparation, prepare the monolayers in plates having a 35-mm diameter. Following incubation, aspirate the culture medium from the monolayers, and replace it with extracts of the Sample Preparation, Negative Control Preparation, or Positive Control Preparation. The serum-supplemented and serum-free cell culture media extracts are tested in duplicate without dilution (100%). The Sodium Chloride Injection extract is diluted with serum-supplemented cell culture medium and tested in duplicate at 25% extract concentration. Incubate all cultures for 48 hours at 37  $\pm$  1° in a humidified incubator preferably containing 5  $\pm$  1% of carbon dioxide. Examine each culture at 48 hours, under a microscope, using a suitable stain, if desired.

**Interpretation of Results**— Proceed as directed for Interpretation of Results under Agar Diffusion Test but using [Table 2](#). The Sample meets the requirements of the test if the response to the Sample Preparation is not greater than grade 2 (mildly reactive). Repeat the procedure if the suitability of the system is not confirmed. For dose-response evaluations, repeat the



procedure, using quantitative dilutions of the sample extract.

Table 2. Reactivity Grades for Elution Test

Grade	Reactivity	Conditions of all Cultures
0	None	Discrete intracytoplasmic granules; no cell lysis
1	Slight	Not more than 20% of the cells are round, loosely attached, and without intracytoplasmic granules; occasional lysed cells are present
2	Mild	Not more than 50% of the cells are round and devoid of intracytoplasmic granules; no extensive cell lysis and empty areas between cells
3	Moderate	Not more than 70% of the cell layers contain rounded cells or are lysed
4	Severe	Nearly complete destruction of the cell layers

\* Further details are given in the following publications of the American Society for Testing and Materials, 1916 Race St., Philadelphia, PA 19103: "Standard Test Method for Agar Diffusion Cell Culture Screening for Cytotoxicity," ASTM Designation F 895-84; "Standard Practice for Direct Contact Cell Culture Evaluation of Materials for Medical Devices," ASTM Designation F 813-83.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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Reference Standards	Lili Wang, Technical Services Scientist 1-301-816-8129 <a href="#">RSTech@usp.org</a>	

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### 88 BIOLOGICAL REACTIVITY TESTS, IN VIVO

The following tests are designed to determine the biological response of animals to elastomers, plastics and other polymeric material with direct or indirect patient contact, or by the injection of specific extracts prepared from the material under test. It is essential to make available the specific surface area for extraction. When the surface area of the specimen cannot be determined, use 0.1 g of elastomer or 0.2 g of plastic or other material for every mL of extraction fluid. Also it is essential to exercise care in the preparation of the materials to be injected or instilled to prevent contamination with microorganisms and other foreign matter. Three tests are described. The Systemic Injection Test and the Intracutaneous Test are used for elastomeric materials, especially to elastomeric closures for which the appropriate [Biological Reactivity Tests, In Vitro](#) 87 have indicated significant biological reactivity.

These two tests are used for plastics and other polymers in addition to a third test, the Implantation Test, to test the suitability of these materials intended for use in fabricating containers and accessories thereto, for use in parenteral preparations, and for use in medical devices, implants, and other systems.

These three tests are applied to materials or medical devices, if there is a need for classification of plastics and other polymers based on in vivo biological reactivity testing.

For the purpose of this chapter, these definitions apply: the Sample is the specimen under test or an extract prepared from such a specimen. A Blank consists of the same quantity of the same extracting medium that is used for the extraction of the specimen under test, treated in the same manner as the extracting medium containing the specimen under test. A

Negative Control<sup>a</sup> is a specimen that gives no reaction under the conditions of the test.

Classification of Plastics— Six Plastic Classes are defined (see [Table 1](#)). This classification is based on responses to a series of in vivo tests for which extracts, materials, and routes of administration are specified. These tests are directly related to the intended end-use of the plastic articles. The choice of extractants is representative of the vehicles in preparations with which the plastics are likely to be in contact. The [Table 1](#) classification facilitates communication among suppliers, users, and manufacturers of plastics by summarizing the tests to be performed for containers for injections and medical devices if a need for classification exists.

Table 1. Classification of Plastics

Plastic Classes <sup>a</sup>						Tests To Be Conducted			
I	II	III	IV	V	VI	Test Material	Animal	Dose	Procedure <sup>b</sup>
x	x	x	x	x	x	Extract of Sample in Sodium	Mouse	50 mL/kg	A (iv)
x	x	x	x	x	x	Chloride Injection	Rabbit	0.2 mL/animal at each of 10 sites	B
x	x	x	x	x	x	Extract of Sample in 1 in 20	Mouse	50 mL/kg	A (iv)
x	x	x	x	x	x	Solution of Alcohol in Sodium Chloride Injection	Rabbit	0.2 mL/animal at each of 10 sites	B
x		x	x			Extract of Sample in	Mouse	10 g/kg	A (ip)
		x	x			Polyethylene Glycol 400	Rabbit	0.2 mL/animal at each of 10 sites	B
x	x	x	x			Extract of Sample in	Mouse	50 mL/kg	A (ip)
	x	x	x			Vegetable Oil	Rabbit	0.2 mL/animal at each of 10 sites	B
x		x				Implant strips of Sample	Rabbit	4 strips/animal	C

a Tests required for each class are indicated by "x" in appropriate columns.

b Legend: A (ip)—Systemic Injection Test (intraperitoneal); A (iv)—Systemic Injection Test (intravenous); B—Intracutaneous Test (intracutaneous); C—Implantation Test (intramuscular implantation).

With the exception of the Implantation Test, the procedures are based on the use of extracts that, depending on the heat resistance of the material, are prepared at one of three standard temperatures: 50°, 70°, and 121°. Therefore, the class designation of a plastic must be accompanied by an indication of the temperature of extraction (e.g., IV-121°, which represents a class IV plastic extracted at 121°, or I-50°, which represents a class I plastic extracted at 50°).

Plastics may be classified as USP Plastic Classes I–VI only on the basis of the response criteria prescribed in [Table 1](#).

This classification does not apply to plastics that are intended for use as containers for oral or topical products, or that may be used as an integral part of a drug formulation. [Table 1](#) does not apply to natural elastomers, which are to be tested in Sodium Chloride Injection and vegetable oils only.

The Systemic Injection Test and the Intracutaneous Test are designed to determine the systemic and local, respectively, biological responses of animals to plastics and other polymers by the single-dose injection of specific extracts prepared from a Sample. The Implantation Test is designed to evaluate the reaction of living tissue to the plastic and other polymers by the implantation of the Sample itself into animal tissue. The proper preparation and placement of the specimens under aseptic conditions are important in the conduct of the Implantation Test.

These tests are designed for application to plastics and other polymers in the condition in which they are used. If the material is to be exposed to any cleansing or sterilization process prior to its end-use, then the tests are to be conducted on a Sample prepared from a specimen preconditioned by the same processing.



actors such as material composition, processing and cleaning procedures, contacting media, inks, adhesives, absorption, adsorption and permeability of preservatives, and condition of storage may also affect the suitability of a material for a specific use. Evaluation of such factors should be made by appropriate additional specific tests to determine the suitability of a material for its intended use.

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**Extracting Media—**

sodium chloride injection (see monograph). Use Sodium Chloride Injection containing 0.9% of NaCl.

1 in 20 solution of alcohol in Sodium Chloride Injection.

polyethylene glycol 400 (see monograph).

vegetable oil— Use freshly refined Sesame Oil (see monograph) or Cottonseed Oil (see monograph) or other suitable vegetable oils.

drug product vehicle (where applicable).

water for injection (see monograph).

**note—**The Sesame Oil or Cottonseed Oil or other suitable vegetable oil meets the following additional requirements. Obtain, if possible, freshly refined oil. Use three properly prepared animals and inject the oil intracutaneously in a dose of 0.2 mL into each of 10 sites per animal, and observe the animals at 24, 48, and 72 hours following injection. Rate the observations at each site on the numerical scale indicated in Table 2. For the 3 rabbits (30 injection sites), at any observation time, the average response for erythema is not greater than 0.5 and for edema is not greater than 1.0, and no site shows a tissue reaction larger than 10 mm in overall diameter. The residue of oil at the injection site should not be misinterpreted as edema. Edematous tissue blanches when gentle pressure is applied.

Table 2. Evaluation of Skin Reactions

Erythema and Eschar Formation	Score
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet-redness) to slight eschar formation (injuries in depth)	4
Edema Formation <sup>*</sup>	Score
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond the area of exposure)	4

\* Excludes noninflammatory (mechanical) edema from the blank or extraction fluid.

**Apparatus—** The apparatus for the tests includes the following.

**AUTOCLAVE—** Use an autoclave capable of maintaining a temperature of  $121 \pm 2.0^\circ$ , equipped with a thermometer, a pressure gauge, a vent cock, a rack adequate to accommodate the test containers above the water level, and a water cooling system that will allow for cooling of the test containers to about, but not below,  $20^\circ$  immediately following the heating cycle.

**OVEN—** Use an oven, preferably a forced-circulation model, that will maintain operating temperatures of  $50^\circ$  or  $70^\circ$  within  $\pm 2^\circ$ .

**EXTRACTION CONTAINERS—** Use only containers, such as ampuls or screw-cap culture test tubes, of Type I glass. If used, culture test tubes are closed with screw caps having suitable elastomeric liners. The exposed surface of the elastomeric liner is completely protected with an inert solid disk 0.05 mm to 0.075 mm in thickness. A suitable disk may be fabricated from a polytef resin.

**Preparation of Apparatus—** Cleanse all glassware thoroughly with chromic acid cleansing mixture, or if necessary with hot nitric acid, followed by prolonged rinsing with water. Clean cutting utensils by an appropriate method (e.g., successive cleaning with acetone and methylene chloride) prior to use in subdividing a specimen. Clean all other equipment by thorough scrubbing with a suitable detergent and prolonged rinsing with water.

Render containers and equipment used for extraction, and in transfer and administration of test material, sterile and dry by a suitable process. [note—If ethylene oxide is used as the sterilizing agent, allow adequate time for complete degassing.]

**Procedure—**

**preparation of sample—** Both the Systemic Injection Test and the Intracutaneous Test may be performed using the same extract, if desired, or separate extracts may be made for each test. Select and subdivide into portions a Sample of the size indicated in Table 3. Remove particulate matter, such as lint and free particles, by treating each subdivided Sample or Negative Control as follows: place the Sample into a clean, glass-stoppered, 100-mL graduated cylinder of Type I glass, and add about 70 mL of Water for Injection. Agitate for about 30 seconds, and drain off the water, repeat this step, and dry those pieces prepared for the extraction with Vegetable Oil in an oven at a temperature not exceeding  $50^\circ$ . [note—Do not clean the Sample with a dry or wet cloth or by rinsing or washing with an organic solvent, surfactant, etc.]

Table 3. Surface Area of Specimen To Be Used<sup>1</sup>

Form of Material	Thickness	Amount of Sample for Each 20 mL of Extracting Medium	Subdivided Into
Film or sheet	<0.5 mm	Equivalent of 120 cm <sup>2</sup> total surface area (both sides combined)	Strips of about 5 x 0.3 cm
	0.5 to 1 mm	Equivalent of 60 cm <sup>2</sup> total surface area (both sides combined)	
Tubing	<0.5 mm (wall)	Length (in cm) = 120 cm <sup>2</sup> /(sum of ID and OD circumferences)	Sections of about 5 x 0.3 cm
	0.5 to 1 mm (wall)	Length (in cm) = 60 cm <sup>2</sup> /(sum of ID and OD circumferences)	
Slabs, tubing, and molded items	>1 mm	Equivalent of 60 cm <sup>2</sup> total surface area (all exposed surfaces combined)	Pieces up to about 5 x 0.3 cm
Elastomers	>1 mm	Equivalent of 25 cm <sup>2</sup> total surface area (all exposed surfaces combined)	Do not subdivide <sup>2</sup>

<sup>1</sup> When surface area cannot be determined due to the configuration of the specimen, use 0.1 g of elastomer or 0.2 g of plastic or other polymers for every 1 mL of extracting fluid.

<sup>2</sup> Molded elastomeric closures are tested intact.

**preparation of extracts—** Place a properly prepared Sample to be tested in an extraction container, and add 20 mL of the appropriate extracting medium. Repeat these directions for



each extracting medium required for testing. Also prepare one 20-mL blank of each medium for parallel injections and comparisons. Extract by heating in an autoclave at 121° for 60 minutes, in an oven at 70° for 24 hours, or at 50° for 72 hours. Allow adequate time for the liquid within the container to reach the extraction temperature. [note—The extraction conditions should not in any instance cause physical changes such as fusion or melting of the Sample pieces, which result in a decrease in the available surface area. A slight adherence of the pieces can be tolerated. Always add the cleaned pieces individually to the extracting medium. If culture tubes are used for autoclave extractions with Vegetable Oil, seal screw caps adequately with pressure-sensitive tape.]

Cool to about room temperature but not below 20°, shake vigorously for several minutes, and decant each extract immediately, using aseptic precautions, into a dry, sterile vessel. Store the extracts at a temperature between 20° and 30°, and do not use for tests after 24 hours. Of importance are the contact of the extracting medium with the available surface area of the plastic and the time and temperature during extraction, the proper cooling, agitation, and decanting process, and the aseptic handling and storage of the extracts following extraction.

#### Systemic Injection Test

This test is designed to evaluate systemic responses to the extracts of materials under test following injection into mice.

**Test Animal**— Use healthy, not previously used albino mice weighing between 17 and 23 g. For each test group use only mice of the same source. Allow water and food, commonly used for laboratory animals and of known composition, *ad libitum*.

**Procedure**— [note—Agitate each extract vigorously prior to withdrawal of injection doses to ensure even distribution of the extracted matter. However, visible particulates should not be injected intravenously.] Inject each of the five mice in a test group with the Sample or the Blank as outlined in [Table 4](#), except to dilute each g of the extract of the Sample prepared with Polyethylene Glycol 400, and the corresponding blank, with 4.1 volumes of Sodium Chloride Injection to obtain a solution having a concentration of about 200 mg of polyethylene glycol per mL.

**Table 4. Injection Procedure—Systemic Injection Test**

Extract or Blank	Dose per kg	Route*	Injection Rate, $\mu\text{L}$ per second
Sodium Chloride Injection	50 mL	IV	100
1 in 20 solution of Alcohol in Sodium Chloride Injection	50 mL	IV	100
Polyethylene Glycol 400	10 g	IP	—
Drug product vehicle	50 mL	IV	100
(where applicable)	50 mL	IP	—
Vegetable Oil	50 mL	IP	—

\* IV = intravenous (aqueous sample and blank); IP = intraperitoneal (oleaginous sample and blank).

Observe the animals immediately after injection, again 4 hours after injection, and then at least at 24, 48, and 72 hours. If during the observation period none of the animals treated with the extract of the Sample shows a significantly greater biological reactivity than the animals treated with the Blank, the Sample meets the requirements of this test. If two or more mice die, or if abnormal behavior such as convulsions or prostration occurs in two or more mice, or if a body weight loss greater than 2 g occurs in three or more mice, the Sample does not meet the requirements of the test. If any animals treated with the Sample show only slight signs of biological reactivity, and not more than one animal shows gross symptoms of biological reactivity or dies, repeat the test using groups of 10 mice. On the repeat test, all 10 animals treated with the Sample show no significant biological reactivity above the Blank animals during the observation period.

#### Intracutaneous Test

This test is designed to evaluate local responses to the extracts of materials under test following intracutaneous injection into rabbits.

**Test Animal**— Select healthy, thin-skinned albino rabbits whose fur can be clipped closely and whose skin is free from mechanical irritation or trauma. In handling the animals, avoid touching the injection sites during observation periods, except to discriminate between edema and an oil residue. [note—Rabbits previously used in unrelated tests, such as the [Pyrogen Test](#) (151), and that have received the prescribed rest period, may be used for this test provided that they have clean, unblemished skin.]

**Procedure**— [note—Agitate each extract vigorously prior to withdrawal of injection doses to ensure even distribution of the extracted matter.] On the day of the test, closely clip the fur on the animal's back on both sides of the spinal column over a sufficiently large test area. Avoid mechanical irritation and trauma. Remove loose hair by means of vacuum. If necessary, swab the skin lightly with diluted alcohol, and dry the skin prior to injection. More than one extract from a given material can be used per rabbit, if you have determined that the test results will not be affected. For each Sample use two animals and inject each intracutaneously, using one side of the animal for the Sample and the other side for the Blank, as outlined in [Table 5](#). [note—Dilute each g of the extract of the Sample prepared with Polyethylene Glycol 400, and the corresponding Blank, with 7.4 volumes of Sodium Chloride Injection to obtain a solution having a concentration of about 120 mg of polyethylene glycol per mL.]

**Table 5. Intracutaneous Test**

Extract or Blank	Number of Sites (per animal)	Dose, $\mu\text{L}$ per site
Sample	5	200
Blank	5	200

Examine injection sites for evidence of any tissue reaction such as erythema, edema, and necrosis. Swab the skin lightly, if necessary, with diluted alcohol to facilitate reading of injection sites. Observe all animals at 24, 48, and 72 hours after injection. Rate the observations on a numerical scale for the extract of the Sample and for the Blank, using [Table 2](#). Reclip the fur as necessary during the observation period. The average erythema and edema scores for Sample and Blank sites are determined at every scoring interval (24, 48, and 72 hours) for each rabbit. After the 72 hour scoring, all erythema scores plus edema scores are totalled separately for each Sample and Blank. Divide each of the totals by 12 (2 animals  $\times$  3 scoring periods  $\times$  2 scoring categories) to determine the overall mean score for each Sample versus each corresponding Blank. The requirements of the test are met if the difference between the Sample and the Blank mean score is 1.0 or less. If at any observation period the average reaction to the Sample is questionably greater than the average reaction to the Blank, repeat the test using three additional rabbits. The requirements of the test are met if the difference between the Sample and the Blank mean score is 1.0 or less.

#### Implantation Test

The implantation test is designed for the evaluation of plastic materials and other polymeric materials in direct contact with living tissue. Of importance are the proper preparation of the implant strips and their proper implantation under aseptic conditions. Prepare for implantation 8 strips of the Sample and 4 strips of USP High-Density Polyethylene RS. Each strip should measure not less than 10  $\times$  1 mm. The edges of the strips should be as smooth as possible to avoid additional mechanical trauma upon implantation. Strips of the specified minimum size are implanted by means of a hypodermic needle (15- to 19-gauge) with intravenous point and a sterile trocar. Use either presterilized needles into which the sterile plastic strips are aseptically inserted, or insert each clean strip into a needle, the cannula and hub of which are protected with an appropriate cover, and then subjected to the appropriate sterilization procedure. [note—Allow for proper degassing if agents such as ethylene oxide are used.]

**Test Animal**— Select healthy, adult rabbits weighing not less than 2.5 kg, and whose paravertebral muscles are sufficiently large in size to allow for implantation of the test strips. Do not use any muscular tissue other than the paravertebral site. The animals must be anesthetized with a commonly used anesthetic agent to a degree deep enough to prevent muscular movements, such as twitching.

**Procedure**— Perform the test in a clean area. On the day of the test or up to 20 hours before testing, clip the fur of the animals on both sides of the spinal column. Remove loose hair by means of vacuum. Swab the skin lightly with diluted alcohol and dry the skin prior to injection.



Implant four strips of the Sample into the paravertebral muscle on one side of the spine of each of two rabbits, 2.5 to 5 cm from the midline and parallel to the spinal column, and about 2.5 cm apart from each other. In a similar fashion implant two strips of USP High-Density Polyethylene RS, in the opposite muscle of each animal. Insert a sterile stylet into the needle to hold the implant strip in the tissue while withdrawing the needle. If excessive bleeding is observed after implantation of a strip, place a duplicate strip at another site.

Keep the animals for a period of not less than 120 hours, and sacrifice them at the end of the observation period by administering an overdose of an anesthetic agent or other suitable agents. Allow sufficient time to elapse for the tissue to be cut without bleeding. Examine macroscopically the area of the tissue surrounding the center portion of each implant strip. Use a magnifying lens and auxiliary light source. Observe the Sample and Control implant sites for hemorrhage, necrosis, discolorations, and infections, and record the observations.

Measure encapsulation, if present, by recording the width of the capsule (from the periphery of the space occupied by the implant Control or Sample to the periphery of the capsule) rounded to the nearest 0.1 mm. Score encapsulation according to [Table 6](#).

Table 6. Evaluation of Encapsulation in the Implantation Test

Capsule Width	Score
None	0
up to 0.5 mm	1
0.6–1.0 mm	2
1.1–2.0 mm	3
Greater than 2.0 mm	4

Calculate the differences between average scores for the Sample and Control sites. The requirements of the test are met if the difference does not exceed 1.0, or if the difference between the Sample and Control mean scores for more than one of the four implant sites does not exceed 1 for any implanted animal.

#### SAFETY TESTS—BIOLOGICALS

The safety test set forth here is intended to detect in an article any unexpected, unacceptable biological reactivity. This *in vivo* test is provided for the safety assessment of biologics (see [Biologics](#) 1041) and biotechnology-derived products.

##### Safety Test

Select five healthy mice not previously used for testing, weighing between 17 and 23 g, unless otherwise directed in the individual monograph or elsewhere in this chapter, and maintained on an adequate balanced diet. Prepare a test solution as directed in the individual monograph. Unless otherwise directed in the individual monograph or elsewhere in this chapter, inject intravenously a dose of 0.5 mL of the test solution into each of the mice, using a 26-gauge needle of suitable length, or of the length specified below as applicable. Observe the animals over the 48 hours following the injection. If, at the end of 48 hours, all of the animals survive and not more than one of the animals shows outward symptoms of a reaction not normally expected of the level of toxicity related to the article, the requirements of this test are met. If one or more animals die or if more than one of the animals shows signs of abnormal or untoward toxicity of the article under test, repeat the test using at least another 10 mice similar to those used in the initial test, but weighing  $20 \pm 1$  g. In either case, if all of the animals survive for 48 hours and show no symptoms of a reaction indicative of an abnormal or undue level of toxicity of the article, the requirements of the test are met.

For biologics, perform the test according to the procedures prescribed in the Federal Regulations (see [Biologics](#) 1041), Section 610.11, using not less than two mice similar to those described above but weighing less than 22 g and not less than two healthy guinea pigs weighing less than 400 g. Unless otherwise directed in the individual monograph, for a liquid product or a freeze-dried product that has been constituted as directed in the labeling, inject a volume of 0.5 mL intraperitoneally into each mouse, and inject a volume of 5.0 mL intraperitoneally into each guinea pig. For freeze-dried products for which the volume of constitution is not indicated in the label, or for nonliquid products other than freeze-dried products, perform the test using the route of administration, test dose, and diluent approved by the Center for Biologics Evaluation and Research (FDA), on the basis of substantial evidence demonstrating that the test variation will assure sensitivity equal to or greater than that of the test described above. Observe the animals for a minimum observation period of 7 days. If all of the animals survive the test period, do not exhibit any response that is not specific for or expected from the product and that may indicate a difference in such product quality, and weigh no less at the end of the test period than at the time of injection, the requirements of the test are met. If the article fails to meet the requirements, the test may be repeated as in the initial test, in the one or both species in which the requirements were not met. If the animals fulfill the criteria specified for the initial test, the article meets the requirements of the test. If the article fails to meet the requirements after the first repeat test, and not less than 50% of the total number of animals of the species in which the requirements of the test were not met in the combined initial and first retests have survived, a second retest may be performed. Use twice the number of animals of the relevant species used in the initial test. If the animals fulfill the criteria specified for the initial test, the requirements of the test are met.

\* USP High-Density Polyethylene RS.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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#### 91 CALCIUM PANTOTHENATE ASSAY

USP Reference Standards 11—[USP Calcium Pantothenate RS](#)

Standard Stock Solution of Calcium Pantothenate— Dissolve 50 mg of [USP Calcium Pantothenate RS](#), previously dried and stored in the dark over phosphorus pentoxide and accurately weighed while protected from absorption of moisture during the weighing, in about 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), then dilute with water to volume. Each mL represents 50 µg of [USP Calcium Pantothenate RS](#). Store under toluene in a refrigerator.

Standard Preparation— On the day of the assay, dilute a measured volume of Standard Stock Solution of Calcium Pantothenate with sufficient water so that it contains, in each mL, between 0.01 µg and 0.04 µg of calcium pantothenate, the exact concentration being such that the responses obtained as directed for Procedure, 2.0 and 4.0 mL of the Standard Preparation being used, are within the linear portion of the log-concentration response curve.

Assay Preparation— Proceed as directed in the individual monograph for preparing a solution expected to contain approximately the equivalent of the calcium pantothenate concentration in the Standard Preparation.



Basal Medium Stock Solution—	
Acid-hydrolyzed Casein Solution	25 mL
Cystine-Tryptophane Solution	25 mL
Polysorbate 80 Solution	0.25 mL
Dextrose, Anhydrous	10 g
Sodium Acetate, Anhydrous	5 g
Adenine-Guanine-Uracil Solution	5 mL
Riboflavin-Thiamine Hydrochloride-Biotin Solution	5 mL
Para-aminobenzoic Acid-Niacin-Pyridoxine Hydrochloride Solution	5 mL
Salt Solution A	5 mL
Salt Solution B	5 mL

Dissolve the anhydrous dextrose and sodium acetate in the solutions previously mixed, and adjust with 1 N sodium hydroxide to a pH of 6.8. Finally, dilute with water to 250 mL, and mix.

Acid-Hydrolyzed Casein Solution— Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8 to 12 hours. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of  $3.5 \pm 0.1$ , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 hour, and filter. Repeat the treatment with activated charcoal. Store under toluene in a refrigerator at a temperature not below  $10^\circ$ . Filter the solution if a precipitate forms during storage.

Cystine-Tryptophane Solution— Suspend 4.0 g of L-cystine and 1.0 g of L-tryptophane (or 2.0 g of D,L-tryptophane) in 700 to 800 mL of water, heat to  $70^\circ$  to  $80^\circ$ , and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a refrigerator at a temperature not below  $10^\circ$ .

Adenine-Guanine-Uracil Solution— Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid, cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 Solution— Dissolve 25 g of polysorbate 80 in alcohol to make 250 mL.

Riboflavin-Thiamine Hydrochloride-Biotin Solution— Prepare a solution containing, in each mL, 20  $\mu$ g of riboflavin, 10  $\mu$ g of thiamine hydrochloride, and 0.04  $\mu$ g of biotin, by dissolving riboflavin, thiamine hydrochloride, and biotin in 0.02 N acetic acid. Store, protected from light, under toluene in a refrigerator.

Para-aminobenzoic Acid-Niacin-Pyridoxine Hydrochloride Solution— Prepare a solution in neutral 25 percent alcohol to contain 10  $\mu$ g of para-aminobenzoic acid, 50  $\mu$ g of niacin, and 40  $\mu$ g of pyridoxine hydrochloride in each mL. Store in a refrigerator.

Salt Solution A— Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid, and store under toluene.

Salt Solution B— Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and store under toluene.

Stock Culture of Lactobacillus plantarum— Dissolve 2.0 g of water-soluble yeast extract in 100 mL of water, add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture, with stirring, on a steam bath, until the agar dissolves. Add approximately 10-mL portions of the hot solution to test tubes, suitably close or cover the tubes, sterilize at  $121^\circ$ , and allow the tubes to cool in an upright position. Prepare stab cultures in 3 or more of the tubes, using a pure culture of Lactobacillus plantarum, incubating for 16 to 24 hours at any selected temperature between  $30^\circ$  and  $37^\circ$  but held constant to within  $\pm 0.5^\circ$ , and finally store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for inoculum if the culture is more than 1 week old.

Culture Medium— To each of a series of test tubes containing 5.0 mL of Basal Medium Stock Solution add 5.0 mL of water containing 0.2  $\mu$ g of calcium pantothenate. Plug the tubes with cotton, sterilize in an autoclave at  $121^\circ$ , and cool.

Inoculum— Make a transfer of cells from the stock culture of Lactobacillus plantarum to a sterile tube containing 10 mL of culture medium. Incubate this culture for 16 to 24 hours at any selected temperature between  $30^\circ$  and  $37^\circ$  but held constant to within  $\pm 0.5^\circ$ . The cell suspension so obtained is the inoculum.

Procedure— To similar test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL, respectively, of the Standard Preparation. To each tube and to 4 similar tubes containing no Standard Preparation add 5.0 mL of Basal Medium Stock Solution and sufficient water to make 10 mL.

To similar test tubes add, in duplicate, volumes of the Assay Preparation corresponding to 3 or more of the levels listed above for the Standard Preparation, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the Basal Medium Stock Solution and sufficient water to make 10 mL. Place one complete set of Standard and Assay tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series suitably to prevent contamination, and heat in an autoclave at  $121^\circ$  for 5 minutes. Cool, add 1 drop of inoculum to each tube, except 2 of the 4 tubes containing no Standard Preparation (to serve as the uninoculated blanks), and mix. Incubate the tubes at a temperature between  $30^\circ$  and  $37^\circ$ , held constant to within  $\pm 0.5^\circ$  until, following 16 to 24 hours of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of standard during a 2-hour period.

Determine the transmittance of the tubes in the following manner: Mix the contents of each tube, and transfer to an optical container if necessary. Place the container in a spectrophotometer that has been set at a specific wavelength between 540 nm and 660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 seconds or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation— Prepare a standard concentration-response curve as follows. For each level of the standard, calculate the response from the sum of the duplicate values of the



transmittance as the difference,  $y = 2.00 - \Sigma$  (of transmittance). Plot this response on the ordinate of cross-section paper against the logarithm of the mL of Standard Preparation per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response,  $y$ , adding together the two transmittances for each level of the Assay Preparation. Read from the standard curve the logarithm of the volume of the Standard Preparation corresponding to each of those values of  $y$  that fall within the range of the lowest and highest points plotted for the standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the Assay Preparation to obtain the difference,  $x$ , for each dosage level. Average the values of  $x$  for each of three or more dosage levels to obtain  $x = M'$ , the log-relative potency of the Assay Preparation. Determine the quantity, in mg, of [USP Calcium Pantothenate RS](#) corresponding to the calcium pantothenate in the portion of material taken for assay as antilog:

$$M = \text{antilog}(M' + \log R)$$

in which  $R$  is the number of mg of calcium pantothenate that was assumed to be present in each mg (or capsule or tablet) of the material taken for assay.

Replication— Repeat the entire determination at least once, using separately prepared Assay Preparations. If the difference between the two log-potencies  $M$  is not greater than 0.08, their mean,  $M$ , is the assayed log-potency of the test material (see [The Confidence Interval and Limits of Potency](#) 111). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of  $M$  that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

\* American Type Culture Collection No. 8014 is suitable. This strain formerly was known as *Lactobacillus arabinosus* 17-5.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Curtis Phinney</a>  1-301-816-8540	(DSN05) Dietary Supplements - Non-Botanicals
Reference Standards	Lili Wang, Technical Services Scientist 1-301-816-8129 <a href="mailto:RSTech@usp.org">RSTech@usp.org</a>	

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### 111 DESIGN AND ANALYSIS OF BIOLOGICAL ASSAYS

#### General

The potency of several Pharmacopeial drugs must be determined by bioassay. A controlling factor in assay design and analysis is the variability of the biological test system, which may vary in its mean response from one laboratory to another, and from time to time in the same laboratory. To control this type of variation, the response to a Pharmacopeial drug is compared with a USP Reference Standard or other suitable standard. For convenience, each such preparation will be called the "Standard" and each preparation under assay, or Sample, the "Unknown," and these will be designated respectively by the symbols S and U. (The Sample is sometimes referred to as the "test preparation.")

After elimination of extraneous variables from the comparison of the Standard and the Unknown, an error variance is computed from the remaining variation, which, while uncontrolled, can nevertheless be measured. The error variance is required in calculating the confidence interval of the assayed potency. The confidence interval, known also as the fiducial interval, is so computed that its upper and lower limits are expected to enclose the true potency of the Unknown in 19 out of 20 assays. Many assay procedures fix the acceptable width of the confidence interval, and two or more independent assays may be needed to meet the specified limit. The confidence limits of the individual component assays usually overlap.

The aim of this chapter is to present a concise account of biometrical procedures for the USP bioassays. Its various sections are interrelated. Although the procedures are planned primarily for the assay of a single Unknown, equations for the joint assay of several Unknowns are given in context throughout the chapter and are summarized in the last section. Proof that an assayed potency meets its required confidence limits may be based also upon other recognized biometric methods that have a precision equivalent to that of the methods outlined herein.

A glossary of the terms used in the equations is provided at the end of this chapter.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Larry N. Callahan, Ph.D.</a> Senior Scientist 1-301-816-8385	(STAT05) Statistics

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#### Steps Preceding the Calculation of Potency

Designs for Minimizing the Error Variance— Variation in response is reduced as much as is practicable by the limitations imposed on body weight, age, previous handling, environment, and similar factors. In a number of assays, the test animals or their equivalent are then assigned at random but in equal numbers to the different doses of the Standard and Unknown. This implies an objective random process, such as throwing dice, shuffling cards, or using a table of random numbers. Assigning the same number of individuals to each treatment simplifies the subsequent calculations materially, and usually leads to the shortest confidence interval for a given number of observations.

In some assays, the potential responses can be assembled into homogeneous sets in advance of treatment. The differences between sets are later segregated, so that they do not affect adversely either the computed potency or its confidence interval. One unit within each set, picked at random, receives each treatment. Examples of randomized sets are the cleared areas on a single plate in the plate assay of an antibiotic, and four successive paired readings in the same rat in the Vasopressin Injection assay. Sets of two occur where each test animal is used twice, as in the assays of Tubocurarine Chloride Injection and Insulin Injection. In these cases, neither the average differences between individuals nor the order of treatment can bias the potency or precision. In the microbial assays for vitamin B12 activity and for calcium pantothenate, replicate tubes are assigned to two or more separate, complete sets, preferably with the tubes arranged at random within each set. This restricts the variation due to position or order within a set to the differences within each complete replicate.

Rejection of Outlying or Aberrant Observations— A response that is questionable because of failure to comply with the procedure during the course of an assay is rejected. Other aberrant values may be discovered only after the responses have been tabulated, but can then be traced to assay irregularities, which justify their omission. The arbitrary rejection or retention of an apparently aberrant response can be a serious source of bias. In general, the rejection of observations solely on the basis of their relative magnitudes is a procedure to be used sparingly. When this is unavoidable, each suspected aberrant response or outlier may be tested against one of two criteria:

1. The first criterion is based upon the variation within a single group of supposedly equivalent responses. On the average, it will reject a valid observation once in 25 or once in 50 trials, provided that relatively few, if any, responses within the group are identical. Beginning with the supposedly erratic value or outlier, designate the responses in order of magnitude from  $y_1$  to  $y_N$ , where  $N$  is the number of observations in the group. Compute the relative gap  $G_1 = (y_2 - y_1)/(y_N - y_1)$  when  $N = 3$  to 7,  $G_2 = (y_3 - y_1)/(y_N - y_1)$  when  $N = 8$  to



13, or  $G_3 = (y_3 - y_1)/(y_N - y_1)$  when  $N = 14$  to 24. If  $G_1$ ,  $G_2$ , or  $G_3$  exceeds the critical value in [Table 1](#) for the observed  $N$ , there is a statistical basis for omitting the outlier.

Table 1

Test for outliers. In samples from a normal population, gaps equal to or larger than the following values of  $G_1$ ,  $G_2$ , and  $G_3$  occur with a probability  $P = 0.02$  where outliers can occur only at one end, or with  $P = 0.04$  where they may occur at either end.

N	3	4	5	6	7				
$G_1$	.976	.846	.729	.644	.586				
N	8	9	10	11	12	13			
$G_2$	.780	.725	.678	.638	.605	.578			
N	14	15	16	17	18	19	20	21	22
$G_3$	.602	.579	.559	.542	.527	.514	.502	.491	.481
									.472
									.464

This criterion is applicable also in a microbial assay where each treatment is represented by a transmittance in each of two separate complete sets. Subtract each transmittance in the first set from its paired value in the second set, and record each difference with its sign, either plus or minus. Beginning with the most divergent difference, designate the  $N$  differences in order of magnitude from  $y_1$  to  $y_N$  and compute the relative gap  $G_1$ ,  $G_2$ , or  $G_3$ . If this exceeds its critical value in [Table 1](#), one of the two transmittances giving the aberrant difference is suspect and may be identified on inspection or by comparison with its expectation (see next column). Repeat the process with the remaining differences if an outlier is suspected in a second pair.

2. The second criterion compares the ranges from a series of  $k = 2$  or more groups. Different groups may receive different treatments, but all  $f$  responses within each group represent the same treatment. Compute the range from each group by subtracting the smallest response from the largest within each of the  $k$  groups. Divide the largest of the  $k$  ranges by the sum of all the ranges in the series. Refer this ratio  $R^*$  to [Table 2](#). If  $k$  is not larger than 10, use the tabular values in the upper part of [Table 2](#); if  $k$  is larger than 10, multiply  $R^*$  by  $(k + 2)$  and interpolate, if necessary, between the tabular values in the lower part of [Table 2](#). If  $R^*$  exceeds the tabular or interpolated value, the group with the largest range is suspect and inspection of its components will usually identify the observation, which is then assumed to be aberrant or an outlier. The process may be repeated with the remaining ranges if an outlier is suspected in a second group.

Table 2

Test for groups containing outliers. Compute the range from the  $f$  observations in each of  $k$  groups, where all groups in the series are equal in size. The observed ratio  $R^*$  of the largest range to the sum of the  $k$  ranges will equal or exceed the following critical values at a probability of  $P = 0.05$ .

No. of	Critical $R^*$ for Ranges Each from $f$ Observations								
	Ranges $k$	2	3	4	5	6	7	8	9
2	0.962	0.862	0.803	0.764	0.736	0.717	0.702	0.691	0.682
3	.813	.667	.601	.563	.539	.521	.507	.498	.489
4	.681	.538	.479	.446	.425	.410	.398	.389	.382
5	.581	.451	.398	.369	.351	.338	.328	.320	.314
6	0.508	0.389	0.342	0.316	0.300	0.288	0.280	0.273	0.267
7	.451	.342	.300	.278	.263	.253	.245	.239	.234
8	.407	.305	.267	.248	.234	.225	.218	.213	.208
9	.369	.276	.241	.224	.211	.203	.197	.192	.188
10	.339	.253	.220	.204	.193	.185	.179	.174	.172
No. of	Critical $(k + 2)R^*$ for Ranges Each from $f$ Observations								
Ranges $k$	2	3	4	5	6	7	8	9	10
10	4.06	3.04	2.65	2.44	2.30	2.21	2.14	2.09	2.05
12	4.06	3.03	2.63	2.42	2.29	2.20	2.13	2.07	2.04
15	4.06	3.02	2.62	2.41	2.28	2.18	2.12	2.06	2.02
20	4.13	3.03	2.62	2.41	2.28	2.18	2.11	2.05	2.01
50	4.26	3.11	2.67	2.44	2.29	2.19	2.11	2.06	2.01

Replacement of Missing Values— As directed in the monographs and in this section, the calculation of potency and its confidence interval from the total response for each dose of each preparation requires the same number of observations in each total. When observations are lost or additional responses have been obtained with the Standard, the balance may be restored by one of the following procedures, so that the usual equations apply.

1. Reduce the number of observations in the larger groups until the number of responses is the same for each treatment. If animals have been assigned at random to each treatment group, either omit one or more responses, selected at random, from each larger group, or subtract the mean of each larger group from its initial total as often as may be necessary. The latter technique is preferred when extra animals have been assigned deliberately to the Standard. When the assay consists of randomized sets, retain only the complete sets.
2. Alternatively, an occasional smaller group may be brought up to size when the number of missing responses is not more than one in any one treatment or 10% in the entire assay. Estimate a replacement for each missing value by either method a or method b. One degree of freedom ( $n$ ) is lost from the error variance  $s^2$  for each replacement by either method, except in a microbial assay where each response is based on the sum of two or more transmittances and only one transmittance is replaced.

(a) If animals have been assigned to treatments at random, add the mean of the remaining responses in the incomplete group to their total. In a microbial assay, when one of two transmittances is missing for a given treatment, add the mean difference between sets, computed from all complete pairs, to the remaining transmittance to obtain the replacement.

(b) If the assay consists of randomized sets, replace the missing value by

$$y' = \frac{fT'_r + kT'_t - T'}{(f-1)(k-1)}, \quad (1)$$

where  $f$  is the number of sets,  $k$  is the number of treatments or doses, and  $T'_r$ ,  $T'_t$ , and  $T'$  are the incomplete totals for the randomized set, treatment, and assay from which an observation is missing.

If the assay consists of  $n'$  Latin squares with  $k$  rows in common, replace a missing value by

$$y' = \frac{k(n'T'_c + T'_r + T'_t) - 2T'}{(k-1)(n'k-2)} \quad (1a)$$



where  $n'$  is the number of Latin squares with  $k$  rows in common,  $K$  is the number of treatments or doses, and  $T_c$ ,  $Tr$ ,  $Tt$ , and  $T'$  are respectively the incomplete totals for the column row, treatment, and assay from which an observation is missing.

If more than one value is missing, substitute the treatment mean temporarily in all but one of the empty places, and compute  $y'$  for the other by Equation 1. Replace each of the initial substitutions in turn by Equation 1, and repeat the process in successive approximations until a stable  $y'$  is obtained for each missing observation.

#### Calculation of Potency from a Single Assay

Directions for calculating potency from the data of a single assay are given in the individual monographs. In those assays that specify graphical interpolation from dosage-response curves but that meet the conditions for assay validity set forth herein, potency may be computed alternatively by the appropriate method in this section.

Planning the assay involves assigning to the Unknown an assumed potency, to permit administering it in dosages equivalent to those of the Standard. The closer the agreement between this original assumption and the result of the assay, the more precise is the calculated potency. The ratio of a given dose of the Standard, in  $\mu\text{g}$  or in USP Units, to the corresponding dose of the Unknown, measured as specified in the monograph, is designated uniformly by  $R$ . The log-relative potency in quantities assumed initially to equal those of the Standard is designated as  $M'$ .

Ideally,  $M'$  should not differ significantly from zero. The log-potency is

$$M = M' + \log R \quad (2)$$

or

$$\text{Potency} = P_* = \text{antilog } M = (\text{antilog } M')R$$

Assay from Direct Determinations of the Threshold Dose—Tubocurarine Chloride Injection and Metocurarine Iodide are assayed from the threshold dose that just produces a characteristic biological response. The ratio of the mean threshold dose for the Standard to that for the Unknown gives the potency directly. The threshold dose is determined twice in each animal, once with the Standard and once with the Unknown. Each dose is converted to its logarithm, the difference ( $x$ ) between the two log-doses is determined for each animal, and potency is calculated from the average of these differences.

In the [Bacterial Endotoxins Test](#) (85), the geometric mean dilution endpoint for the Unknown corresponding to the geometric mean dilution endpoint for the Standard (multiplied by a dilution factor, where applicable) gives the concentration of endotoxin in the test material.

In these assays, the confidence interval depends upon the variability in the threshold dose.

Indirect Assays from the Relationship between the Log-Dose and the Response—Generally, the threshold dose cannot be measured directly; therefore, potency is determined indirectly by comparing the responses following known doses of the Standard with the responses following one or more similar doses of the Unknown. Within a restricted dosage range, a suitable measure of the response usually can be plotted as a straight line against the log-dose, a condition that simplifies the calculation of potency and its confidence interval. Both the slope and position of the log-dose response relationship are determined in each assay by the use of two or more levels of the Standard, or, preferably, of both the Standard and the Unknown.

In the assay of Heparin Sodium, the interval between the dose at which clotting occurs and that which produces no clotting is so small that the dosage-response curve is not determined explicitly. Moving averages are used instead to interpolate the log-dose corresponding to 50% clotting for both the Standard and the Unknown, leading to the log-potency (see Calculation under Heparin Sodium). The precision of the potency is estimated from the agreement between independent assays of the same Unknown.

For a drug that is assayed biologically, the response should plot as a straight line against the log-dose over an adequate range of doses. Where a preliminary test is required or the assay depends upon interpolation from a multi-dose Standard curve, plot on coordinate paper the mean response of the Standard at each dosage level on the ordinate against the log-dose  $x$  on the abscissa. If the trend is basically linear over the required dosage range, the initial response unit may be used directly as  $y$ ; if, instead, the trend is clearly curvilinear, a suitable transformation of each initial reading may bring linearity.

One possible transformation is to logarithms; another, in microbial tube assays, where  $y = (100 - \% \text{ transmittance})$  does not plot linearly against the log-dose  $x$ , is to probits. In this case, if absorbance cannot be read directly, the percent transmittance for each tube or test solution is first converted to absorbance,  $A = 2 - \log(\% \text{ transmittance})$ . Each absorbance value, in turn, is converted to % reduction in bacterial growth as

$$\% \text{ reduction} = 100(\bar{A}_c - A)/\bar{A}_c$$

where  $A_c$  is the mean density for the control tubes (without antibiotic or with excess of vitamin) in the same set or tube rack. Percent reduction is then transformed to a probit (see [Table 3](#)) to obtain a new  $y$  for all later calculation. The probit transformation offers the advantage of extending the working range of linearity even where a portion of the dosage-response relationship is nonlinear in the original units of percent transmittance, provided that the incubation period does not extend beyond the logarithmic phase of growth of the control tubes.

Table 3

Probits (normal deviates + 5) corresponding to percentages in the margins.

	0	1	2	3	4	5	6	7	8	9
0	—	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

The LD<sub>50</sub> in the Safety test for [Iron Dextran Injection](#) is calculated with log-doses and probits. The four doses of the Injection, in mg of iron per kg of body weight, are transformed to  $x_1 = 2.574$ ,  $x_2 = 2.699$ ,  $x_3 = 2.875$ , and  $x_4 = 3.000$ . The probits corresponding to the number of deaths observed in each group of 10 mice are designated  $y_1$ ,  $y_2$ ,  $y_3$ , and  $y_4$ , respectively, and are given in [Table 3](#) for mortalities from 10 to 90 percent. For observed deaths of 0 and 10 adjacent to doses giving an intermediate mortality, use the approximate probits 3.02 and 6.98, respectively; omit the end value (at  $x_1$  or  $x_4$ ) if not adjacent to an intermediate mortality. Since the information in a probit varies with its expectation, assign each probit an approximate relative weight  $w$  for computing the LD<sub>50</sub> of the Injection, as shown in the accompanying table.



No. of Deaths	0 or 10	1 or 9	2 or 8	3 or 7	4 to 6
Weight, w	0.3	0.7	1.0	1.2	1.3

Calculate the weighted means

$$\bar{x} = \Sigma(wx)/\Sigma w$$

and (2a)

$$\bar{y} = \Sigma(wy)/\Sigma w$$

from the sum of the weights,  $\Sigma w$ , of the four (or three) acceptable responses and the corresponding weighted sums of the log-doses,  $\Sigma(wx)$ , and of the probits,  $\Sigma(wy)$ . From the sums of the weighted products,  $\Sigma(wxy)$ , and of the weighted squares,  $\Sigma(wx^2)$ , compute the slope  $b$  of the log-dose-probit line as

$$b = \frac{\Sigma(wxy) - \bar{x}\Sigma(wy)}{\Sigma(wx^2) - \bar{x}\Sigma(wx)} \quad (2b)$$

The LD<sub>50</sub> for this safety test, in mg of iron per kg of body weight, is calculated as

$$LD_{50} = \text{antilog}[\bar{x} + (5 - \bar{y})/b] \quad (2c)$$

In quantal assays not included in this Pharmacopeia, such as the mouse assay for insulin, the calculation with probits involves other adjustments that are omitted here.

When the mean response  $y_t$  for each dose of Standard plots linearly against the log-dose, and the  $k$  doses are spaced at equal intervals on the logarithmic scale, the predicted responses ( $Y_L$  and  $Y_H$ ) at the extreme ends of the line of best fit can be computed directly with the coefficients  $x^*$  in [Table 4](#), which correspond to the  $k$  successive log-doses, as

$$Y_L = \Sigma(x_*\bar{y}_i)/\text{divisor}$$

and (3)

$$Y_H = \Sigma(x_*\bar{y}_i)/\text{divisor}$$

where  $\Sigma$  stands uniformly for "the sum of" the values that follow it. When  $Y_L$  and  $Y_H$  are plotted against the low and high log-doses,  $X_L$  and  $X_H$ , respectively, they may be connected by a straight line with the slope

$$b = (Y_H - Y_L)/(X_H - X_L) \quad (4)$$

At any selected log-dose  $x$  of Standard, the predicted response is

$$Y = \bar{y} + b(x - \bar{x}) \quad (5)$$

where  $x = \Sigma x/k$ , and  $y = (Y_L + Y_H)/2$ , or, for predictions within a set,  $y$  is the mean response for the Standard within the set.

Table 4

Coefficients  $x^*$  for computing the responses  $Y_L$  and  $Y_H$  predicted by least squares at the lowest and highest of  $k$  log-doses when these are spaced at equal intervals.

No. of Doses	Predicted	Coefficient $x^*$ for Mean Response $y_t$ at Log-Dose						Divisor
		1	2	3	4	5	6	
3	$Y_L$	5	2	-1				6
	$Y_H$	-1	2	5				
4	$Y_L$	7	4	1	-2			10
	$Y_H$	-2	1	4	7			
5	$Y_L$	3	2	1	0	-1		5
	$Y_H$	-1	0	1	2	3		
6	$Y_L$	11	8	5	2	-1	-4	21
	$Y_H$	-4	-1	2	5	8	11	

When the log-dose response relationship is linear, but the  $k$  doses (expressed in mL) are spaced substantially in an arithmetic sequence as in [Table 5](#) (which refers to the microbial assays set forth under [Antibiotics—Microbial Assays](#) [81](#)), the slope  $b$  of the straight line of best fit may be computed with the terms in [Table 5](#) and the mean response at each dose  $y_t$ , or  $T_t = f(y_t)$  where the number of  $y_t$ 's is constant at each dose, as

$$b = \Sigma(x_1\bar{y}_i)/e_b'i = \Sigma(x_1T_i)/fe_b'i \quad (6)$$

The coefficients  $x_1$  are convenient multiples of the differences  $(x - \bar{x})$  about the mean log-dose  $x$ , and  $e_b'i$  is the corresponding multiple of  $\Sigma(x - \bar{x})^2$ . The predicted response  $Y$  at a given log-dose  $x$  may be computed by substitution of the assay slope  $b$  in Equation 5 and of the mean  $y$  either of all the responses on the Standard in the entire assay or of those for each set separately.

Table 5

Coefficients  $x_1$  for computing the slope  $b$  of a log-dose response curve when the doses are spaced on an arithmetic scale as shown.

No. of Doses	1	1.5	2	3	4	5	Divisor $e_b'i$	Mean Log-Dose $x$
4	—	-29	-12	12	29	—	14.4663	0.38908
5	-34	—	-9	5	15	23	24.7827	0.41584
5	—	-20	-11	2	11	18	13.3249	0.45105
6	-15	-8	-3	4	9	13	14.1017	0.37588

potencies interpolated from a standard curve— Where the log-dose response curve of the Standard in a given assay is curvilinear and is fitted graphically to the plotted points, the amount of Standard that would be expected to produce each observed response  $y$  of an Unknown is estimated by interpolation from the curve and then adjusted for the known concentration of its test solution.

When the response to the Standard can be plotted linearly against the log-dose, it is fitted numerically by a straight line, as described in the preceding section. For assays in randomized sets, a standard curve is computed with  $b$  for the assay and  $y$  for each set and the response  $y_U$  in each tube of a given Unknown in that set is converted to an estimated log-relative potency,

$$X = (y_U - Y_S)/b \quad (7)$$

where  $Y_S$  is the response predicted by the standard curve at the assumed log-dose  $x$  of the Unknown. The average of the separate estimates from each of  $f$  sets,  $M' = \Sigma X/f$ , is the assayed log-relative potency of the Unknown.

Factorial Assays from the Response to Each Treatment— When some function of the response can be plotted linearly against the log-dose, the assayed potency is computed from the total response for each treatment, and its precision is measured in terms of confidence intervals. This requires that (1) in suitable units the response ( $y$ ) depends linearly upon the log-dose within the dosage range of the assay, and (2) the number ( $f$ ) of responses be the same at each dosage level of both Standard and Unknown. The  $y$ 's are totaled at each dosage level of each preparation. In different combinations, these totals,  $T_t$ , lead directly to the log-relative potency and to tests of assay validity. The factorial coefficients in [Tables 6, 7, and 8](#) determine how they are combined. In a given row, each  $T_t$  is multiplied by the corresponding coefficient and the products summed to obtain  $T_i$ . The  $T_i$ 's in the successive rows carry the same meaning in all assays.

Table 6

Factorial coefficients  $x_1$  for analyzing a balanced bioassay, in which successive log-doses of Standard ( $S_i$ ) and of Unknown ( $U_i$ ) are spaced equally, each with the same number ( $f$ ) of responses totaling  $T_t$ .

Design	Row	Factorial Coefficients $x_1$ for Each Dose								$ei$	$T_i$
		$S_1$	$S_2$	$S_3$	$S_4$	$U_1$	$U_2$	$U_3$	$U_4$		
2,2	a	-1	-1			1	1			4	Ta
	b	-1	1			-1	1			4	Tb
	ab	1	-1			-1	1			4	Tab
3,3	a	-1	-1	-1		1	1	1		6	Ta
	b	-1	0	1		-1	0	1		4	Tb
	ab	1	0	-1		-1	0	1		4	Tab
	q	1	-2	1		1	-2	1		12	Tq
	aq	-1	2	-1		1	-2	1		12	Taq
4,4	a	-1	-1	-1	-1	1	1	1	1	8	Ta
	b	-3	-1	1	3	-3	-1	1	3	40	Tb
	ab	3	1	-1	-3	-3	-1	1	3	40	Tab
	q	1	-1	-1	1	1	-1	-1	1	8	Tq
	aq	-1	1	1	-1	1	-1	-1	1	8	Taq
Value of Constant for Design											
For Computing		Equation No.		Constant		2,2		3,3		4,4	
$M'$		8, 10		c		1		4/3		5	
$L$		26, 29		$c'$		1		8/3		5	

Ta in the first row measures the difference in the average response to the Standard and to the Unknown. Tb in the second row leads directly to the combined slope of the dosage-response curves for both Standard and Unknown. The third to the fifth rows (ab, q, and aq) provide tests for the validity of an assay, as described in a later section. From the totals Ta and Tb, compute the log-relative potency of the Unknown, before adjustment for its assumed potency, as

$$M' = ciT_a/T_b \quad (8)$$

where  $i$  is the interval in logarithms between successive log-doses of both the Standard and the Unknown, and the constant  $c$  is given separately at the bottom of each table. Each  $M'$  is corrected to its log-potency  $M$  by Equation 2.

When doses are spaced unequally on a log scale, as in [Table 8](#), use instead the constant  $c_i$  at the bottom of the table.

Table 7

Factorial coefficients  $x_1$  for analyzing a partially balanced assay, in which successive log-doses of Standard ( $S_i$ ) and of Unknown ( $U_i$ ) are spaced equally, each with the same number ( $f$ ) of responses totaling  $T_t$ . If the number of successive doses of the Unknown exceeds by one the number on the Standard, interchange  $S_i$  and  $U_i$  in the heading and reverse all signs in rows a, ab, and aq.

Design	Row	Factorial Coefficients $x_1$ for Each Dose								$ei$	$T_i$
		$S_1$	$S_2$	$S_3$	$S_4$	$U_1$	$U_2$	$U_3$	$U_4$		
2,1	a	-1	-1			2				6	Ta
	b	-1	1			0				2	Tb
3,2	a	-2	-2	-2		3	3			30	Ta
	b	-2	0	2		-1	1			10	Tb
	ab	1	0	-1		-2	2			10	Tab
	q	1	-2	1		0	0			6	Tq
	aq	-3	-3	-3	-3	4	4	4	4	84	Taq
4,3	a	-3	-3	-3	-3	4	4	4	4	28	Tb
	b	-3	-1	1	3	-2	0	2		70	Tab
	ab	3	1	-1	-3	-5	0	5		60	Tq
	q	3	-3	-3	3	2	-4	2		10	Taq
	aq	-1	1	1	-1	1	-2	1			
Value of Constant for Design											
For Computing		Equation No.		Constant	2,1		3,2		4,3		
$M'$		8, 10		c	1/2		5/6		7/6		
$L$		26, 29		$c'$	3/4		25/12		49/12		

Table 8



Factorial coefficients  $x_1$  for analyzing assays with a 3- or 4-dose sequence of 1.5, 2.0, 3.0, and 4.0, each dose having the same number ( $f$ ) of responses.

Design	Row	Dose of Standard				Dose of Unknown				ei	Ti
		1.5	2.0	3.0	4.0	1.5	2.0	3.0	4.0		
4,4	a	-1	-1	-1	-1	1	1	1	1	8	Ta
	b	-29	-12	12	29	-29	-12	12	29	3940	Tb
	ab	29	12	-12	-29	-29	-12	12	29	3940	Tab
	q	1	-1	-1	1	1	-1	-1	1	8	Tq
	aq	-1	1	1	-1	1	-1	-1	1	8	Taq
3,3	a	-1	-1	-1		1	1	1		6	Ta
	b	-25	-3	28		-25	-3	28		2836	Tb
	ab	25	3	-28		-25	-3	28		2836	Tab
	q	31	-53	22		31	-53	22		8508	Tq
	aq	-31	53	-22		31	-53	22		8508	Taq
3,3	a		-1	-1	-1		1	1	1	6	Ta
	b		-28	3	25		-28	3	25	2836	Tb
	ab		28	-3	-25		-28	3	25	2836	Tab
	q		22	-53	31		22	-53	31	8508	Tq
	aq		-22	53	-31		22	-53	31	8508	Taq
						Value of Constant for Design					
For Computing		Equation No.		Constant		4,4		3,3			
M'		8, 10		ci		7.2332		5.3695			
L		26, 29		c' i2		0.10623		0.06100			

In a fully balanced assay, such as the assay for corticotropin, compute  $M'$  with the coefficients in [Table 6](#). If one preparation has one less dose than the other but the successive log-doses of both Standard and Unknown differ by a constant interval  $i$ , use the factorial coefficients in [Table 7](#), correcting for the actual difference between the observed mean log-doses,  $x_S$  and  $x_U$ , by computing

$$M' = \bar{x}_S - \bar{x}_U + M' \quad (9)$$

In assays where the successive doses are not spaced at equal log-intervals, the log-relative potency of a single Unknown may be computed by Equation 8 with the factorial coefficients and  $ci$  in [Table 8](#).

In an assay of two or more Unknowns against a common Standard, all with dosage-response lines that are parallel within the experimental error, each log-relative potency may be computed with the same assay slope as follows. For each preparation, determine the slope factor  $Tb' = \Sigma(x_1 Tt) or \Sigma(x_1 y)$ , where the values of  $x_1$  are the factorial coefficients for the Standard in the appropriate row b of [Table 6](#) or [8](#). The log-relative potency of each Unknown is

$$M' = ci h' T_a / 2 \Sigma T_b' \quad (10)$$

where  $h'$  is the number of values of  $Tb'$  summed in the denominator.

Assays from Differences in Response— When doses of the Standard and Unknown are paired and the difference in response is computed for each pair, these differences are not affected by variations in the average sensitivity of the paired readings. The paired 2-dose insulin assay corresponds to the first design in [Table 6](#), and requires four equal groups of rabbits each injected twice (see [Insulin Assays](#) [121](#)). The difference ( $y$ ) in the blood sugar response of each rabbit to the two treatments leads to the log-relative potency  $M'$  (see the first two paragraphs of the section, Calculation of Potency from a Single Assay). The Vasopressin Injection assay follows a similar design, substituting two or more randomized sets of four successive pairs of injections into rats for the four treatment groups of rabbits in the insulin assay.

Oxytocin Injection is assayed from blood pressure changes in a single test animal following alternating injections of a single dose of Standard and of one of two doses of the Unknown. The calculation of potency from the differences in the response of the Unknown and to the average of the two adjacent responses to the Standard is equivalent to the first design in [Table 7](#) with S and U reversed, where  $i$  is the log-interval between the two dosage levels of the Unknown.

#### Experimental Error and Tests of Assay Validity

As the term is used here, "experimental error" refers to the residual variation in the response of biological indicators, not to a mistake in procedure or to an outlier that needs replacement. It is measured in terms of the error variance of a single response or other unit, which is designated uniformly as  $s^2$ , despite differences in the definition of the unit. It is required in tests of assay validity and in computing the confidence interval.

Error Variance of a Threshold Dose— The individual threshold dose is measured directly in some assays. In a Digitalis assay, designate each individual threshold dose by the symbol  $z$ , the number or frequency of  $z$ 's by  $f$ , and the total of the  $z$ 's for each preparation by  $T$ , with subscripts S and U for Standard and Unknown, respectively. Compute the error variance of  $z$  as

$$s^2 = [\Sigma z^2 - T_S^2/f_S - T_U^2/f_U]/n \quad (11)$$

with  $n = f_S + f_U - 2$  degrees of freedom. In the assay of Tubocurarine Chloride Injection, each log-threshold dose of the Unknown is subtracted from the corresponding log-dose of the Standard in the same rabbit to obtain an individual difference  $x$ . Since each  $x$  may be either positive or negative (+ or -), it is essential to carry the correct sign in all sums. Designate the total of the  $x$ 's for the animals injected with the Standard on the first day as  $T_1$ , and for those injected with the Standard on the second day as  $T_2$ . Compute the error variance of  $x$

with  $n = N - 2$  degrees of freedom as

$$s^2 = \{\Sigma x^2 - (T_1^2 + T_2^2)/f\}/n \quad (12)$$

where  $N$  is the total number of rabbits that complete the assay, excluding any replacement for a missing value to equalize the size of the two groups.

Error Variance of an Individual Response— In the Pharmacopeial assays, differences in dose that modify the mean response are assumed not to affect the variability in the response. The calculation of the error variance depends upon the design of the assay and the form of the adjustment for any missing values. Each response is first converted to the unit  $y$  used in computing the potency. Determine a single error variance from the combined deviations of the  $y$ 's around their respective means for each dosage level, summed over all levels. Doubtful values of  $y$  may be tested as described under Rejection of Outlying or Aberrant Observations, and proved outliers may be replaced as missing values (see Replacement of Missing Values).

In the simplest design, the units of response are assigned at random to each dosage level, as in the assay for corticotropin. If a missing value is replaced by adding the mean of the



Chaining y's at any given dosage level to their total, the degrees of freedom (n) in the error variance are reduced by one for each replacement but no other change is needed in the calculation. Assuming that f is then the same for all doses or groups, compute the error variance from the variation within doses of all the y's as

$$s^2 = \{\sum y^2 - \sum T_i^2/f\}/n \quad (13)$$

where  $T_i$  is the total at each dose of the f values of y, there are k totals  $T_i$  and the degrees of freedom  $n = \sum f - k$ , with  $\sum f$  diminished by 1 for each replacement.

If variations in f are adjusted by subtracting a group mean from its group total, compute the error variance from the observed y's and the unadjusted totals  $T_i$  as

$$s^2 = \{\sum y^2 - \sum (T_i^2/f)\}/n \quad (14)$$

where  $n = \sum f - k$ .

In the calculation of the result of an assay using the coefficients of [Table 6](#) or [8](#),  $s^2$  may be computed from the response y for each of the h' preparations, including the h Unknowns and the corresponding dosage levels of the Standard. For each preparation, compute  $T' = \sum y$  and the slope factor  $Tb' = \sum (x_1 y)$  where the values of  $x_1$  are the factorial coefficients for the Standard in the appropriate row b of [Table 6](#) or [8](#). The error variance for the assay is

$$s^2 = \{\sum y^2 - \sum T'^2/k - 2(\sum T_b')^2/h'e_b f\}/n \quad (15)$$

where the degrees of freedom  $n = h' (k - 1) - 1$ , and  $e_b$  is the ei from the same table and row as the coefficients  $x_1$ .

The Error Variance in Restricted Designs— In some assays, the individual responses occur in randomized sets of three or more. Examples of sets are litter mates in the assay of vitamin D, the cleared areas within each plate in an antibiotic assay, and the responses following four successive pairs of injections in the vasopressin assay. Arrange the individual y's from these assays in a 2-way table, in which each column represents a different treatment or dose and each row a randomized set. Losses may be replaced as described under Replacement of Missing Values. The k column totals are the  $T_i$ 's required for the analysis of balanced designs. The f row totals ( $T_r$ ) represent a source of variation that does not affect the estimated potency and hence is excluded from the assay error. Compute the approximate error variance from the squares of the individual y's and of the marginal totals as

$$s^2 = \{\sum y^2 - \sum T_r^2/k - \sum T_i^2/f + T^2/N\}/n \quad (16)$$

where  $T = \sum T_r = \sum T_i$ , and the  $n = (k - 1)(f - 1)$  degrees of freedom must be diminished by one for any gap in the original table that has been filled by computation.

When the order of treatment is an additional potential source of variation, its effect can be corrected by the dose regimen for a series of n' Latin squares with k rows in common, such as that for the two Latin squares in the dose regimens 1 to 4 and 5 to 8 in the assay of Glucagon for Injection. List the observed responses y of each test animal in a separate column in the order of dosing. The responses to each of the k doses then occur equally often in each of the k rows and of the n' k columns, where n' is the number of Latin squares. Total the responses y in each row ( $T_r$ ) in each column ( $T_c$ ), and, in a separate listing, for each dose or treatment ( $T_i$ ). An occasional lost reading may be replaced by Equation 1a as described under Replacement of Missing Values. Compute the error variance from the squares of the individual y's and of the marginal and treatment totals as

$$s^2 = \{\sum y^2 - \sum T_r^2/n'k - \sum T_c^2/k - \sum T_i^2/n'k + 2T^2/N\}/n \quad (16a)$$

where  $T = \sum y = \sum T_r = \sum T_c = \sum T_i$ ,  $N = n' k^2$ , and the  $n = (k - 1)(n' k - 2)$  degrees of freedom must be diminished by one for any gap in the original table that has been filled by computation.

In assays where the reactions occur in pairs, the differences between test animals or paired reactions are segregated automatically by calculating the assay with the difference within pair as the response. With insulin, the response is the difference y in the blood sugar of a single rabbit following two injections (see [Insulin Assay](#) (121)). After adjustment for rabbits lost during the assay, compute the error variance of y from the responses in all four groups and from the group totals  $T_i = T_1$  to  $T_4$  as

$$s^2 = \{\sum y^2 - \sum T_i^2/f\}/n \quad (17)$$

where the number of rabbits f is the same in each group and the degrees of freedom,  $n = 4(f - 1)$ , are reduced by one for each replacement of a rabbit lost during the assay. In the Oxytocin Injection assay, each y represents the difference between the blood pressure response to a dose of the Unknown and the average for the two adjacent doses of Standard.

Compute the error variance of y as

$$s^2 = \{\sum y^2 - (T_1^2 + T_2^2)/f\}/n \quad (18)$$

with  $n = 2(f - 1)$  degrees of freedom, where  $T_1$  is the total of the y's for the low dose of the Unknown and  $T_2$  for the high dose.

In a microbial assay calculated by interpolation from a standard curve, convert each difference between two paired responses to units of log-dose, X, by the use of Equation 7. With each difference X as the unit, a composite  $s^2$  is computed from the variation in the f values of X for each Unknown, totaled over the h Unknowns in the assay, as

$$s^2 = \{\sum X^2 - \sum (T_x^2/f)\}/n \quad (19)$$

where  $T_x = \sum X$  for a single Unknown and the degrees of freedom  $n = \sum f - h$ .

Tests of Assay Validity— In addition to the specific requirements in each monograph and a combined log-dose response curve with a significant slope (see the statistic C in the next section), two conditions determine the validity of an individual factorial assay: (1) the log-dose response curve for the Unknown must parallel that for the Standard within the experimental error, and (2) neither curve may depart significantly from a straight line. When the assay has been completely randomized or consists of randomized sets, the necessary tests are computed with the factorial coefficients for ab, q, and aq from [Tables 6](#) to [8](#) and the treatment totals  $T_i$ . Sum the products of the coefficients in each row by the corresponding  $T_i$ 's to obtain the product total  $T_i$ , where the subscript i stands in turn for ab, q, and aq, respectively. Each of the three ratios,  $T_i^2/e_i f$ , is computed with the corresponding value of  $e_i$  from the table and with f equal to the number of y's in each  $T_i$ . That in row ab tests whether the dosage-response lines are parallel, and is the only test available in a 2-dose assay. With three or more doses of both preparations, that in row q is a test of combined curvature in the same direction, and in row aq of separate curvatures in opposite directions. If any ratio in a 3- or 4-dose assay exceeds  $s^2$  as much as three-fold, compute

$$F_3 = \sum (T_i^2/e_i f)/3s^2 \quad (20)$$

For a 2-dose assay, compute instead

$$F_1 = T_{ab}^2/e_{ab} fs^2 \quad (21)$$

and for a 3,2 assay ([Table 7](#)) determine



$$F_2 = \Sigma(T_i^2/e_i f)/2s^2 \quad (22)$$

For a valid assay,  $F_1$ ,  $F_2$ , or  $F_3$  does not exceed the value given in [Table 9](#) (at odds of 1 in 20) for the degrees of freedom  $n$  in  $s^2$ .

Table 9

Values of $t$ , $t_2$ , $F_1$ and $\chi_2$ for different degrees of freedom $n$ that will be exceeded with a probability $P = 0.05$ (or 0.95 for confidence intervals). <sup>†</sup>											
$n$	$t$	$t_2 = F_1$	$F_2$	$F_3$	$\chi_2$	$n$	$t$	$t_2 = F_1$	$F_2$	$F_3$	$\chi_2$
1	12.706	161.45	—	—	3.84	19	2.093	4.381	3.52	3.13	30.1
2	4.303	18.51	19.00	19.16	5.99	20	2.086	4.351	3.49	3.10	31.4
3	3.182	10.128	9.55	9.28	7.82	21	2.080	4.325	3.47	3.07	32.7
4	2.776	7.709	6.94	6.59	9.49	22	2.074	4.301	3.44	3.05	33.9
5	2.571	6.608	5.79	5.41	11.07	23	2.069	4.279	3.42	3.03	35.2
6	2.447	5.987	5.14	4.76	12.59	24	2.064	4.260	3.40	3.01	36.4
7	2.365	5.591	4.74	4.35	14.07	25	2.060	4.242	3.38	2.99	37.7
8	2.306	5.318	4.46	4.07	15.51	26	2.056	4.225	3.37	2.98	38.9
9	2.262	5.117	4.26	3.86	16.92	27	2.052	4.210	3.35	2.96	40.1
10	2.228	4.965	4.10	3.71	18.31	28	2.048	4.196	3.34	2.95	41.3
11	2.201	4.844	3.98	3.59	19.68	29	2.045	4.183	3.33	2.93	42.6
12	2.179	4.747	3.89	3.49	21.03	30	2.042	4.171	3.32	2.92	43.8
13	2.160	4.667	3.81	3.41	22.36	40	2.021	4.085	3.23	2.84	55.8
14	2.145	4.600	3.74	3.34	23.68	60	2.000	4.001	3.15	2.76	79.1
15	2.131	4.543	3.68	3.29	25.00	120	1.980	3.920	3.07	2.68	146.6
16	2.120	4.494	3.63	3.24	26.30		1.960	3.841	3.00	2.60	
17	2.110	4.451	3.59	3.20	27.59						
18	2.101	4.414	3.55	3.16	28.87						

<sup>†</sup> Adapted from portions of Tables III to V of "Statistical Tables for Biological, Agricultural and Medical Research," by R. A. Fisher and F. Yates, published by Oliver and Boyd, Ltd., Edinburgh.

An assay may fail the test for validity and still provide a contributory estimate of potency that can be combined profitably with the result of a second assay of the same Unknown, as described in a later section. An end dosage level for either the Standard or the Unknown, or both, may fall outside the linear zone. With three or more dosage levels and relatively large values of  $T_a$ ,  $T_{ab}$ , and  $T_{aq}$ , the total response  $T_t$  at an end dose of one preparation may approach an upper or lower limit and be responsible for the large values of  $T_{ab}$  and  $T_{aq}$ . This  $T_t$  may be omitted and the assay recomputed with the appropriate design in [Table 7](#). If the assay then meets the test in Equation 20, or 22, the resulting potency,  $M$ , may be combined with that of a second assay in computing the log-potency of the Unknown (see under Combination of Independent Assays). If  $T_a$  is not significant but  $T_q$  shows significant combined curvature, the largest (or smallest) dose of both preparations may be too large (or too small). Their omission may lead to a valid assay with the factorial coefficients for the next smaller design in [Table 6](#) or [8](#). A statistically significant  $T_q$  or  $\Sigma T_q'$  may be neglected and all dosage levels retained without biasing the computed log-potency  $M'$  and its confidence interval by more than 5% when the following inequality is true:

$$\begin{aligned} T_b^2/e_b &> 100T_q^2/e_q \\ \text{or} \\ (\Sigma T_b')^2/e_b &> 100(\Sigma T_q')^2/e_q \end{aligned} \quad (23)$$

where each  $T_b'$  and  $T_q'$  is computed with the  $T_t$ 's (or  $y$ 's) for a single preparation multiplied by the coefficients for the Standard in rows  $b$  and  $q$ , respectively. If both  $T_a$  and  $T_{ab}$  are significant in a 2-dose assay, one  $T_t$  may be outside the linear zone. Sometimes a preliminary or contributory estimate of potency can be computed from the remaining three values of  $T_t$  and the first design in [Table 7](#). In assays of insulin and of other drugs in which the responses are paired, the test for parallelism is so insensitive that it is omitted. If the tubes in each set are arranged systematically instead of at random in a microbial assay, the tests for validity may be subject to bias from positional effects.

#### The Confidence Interval and Limits of Potency

A bioassay provides an estimate of the true potency of an Unknown. This estimate falls within a confidence interval, which is computed so that the odds are not more than 1 in 20 ( $P = 0.05$ ) that the true potency either exceeds the upper limit of the confidence interval or is less than its lower limit. Since this interval is determined by a number of factors that may influence the estimate of potency, the required precision for most bioassays is given in the monograph in terms of the confidence interval, related either to the potency directly or to its logarithm.

General Calculation— Despite their many forms, bioassays fall into two general categories: (1) those where the log-potency is computed directly from a mean or a mean difference, and (2) those where it is computed from the ratio of two statistics.

(1) When the log-potency of an assay is computed as the mean of several estimated log-potencies that are approximately equal in precision, the log-confidence interval is

$$L = 2st/\sqrt{k} \quad (24)$$

where  $s$  is the standard deviation of a single estimated log-potency,  $t$  is read from [Table 9](#) with the  $n$  degrees of freedom in  $s$ , and  $k$  is the number of estimates that have been averaged. The same equation holds where the log-potency is computed as the mean  $x$  of  $k$  differences  $x$ , with  $s$  the standard deviation of a single  $x$ . In either case, the estimated log-potency  $M$  is in the center of its confidence interval, so that its confidence limits are

$$X_M = M + \frac{1}{2}L \text{ and } M - \frac{1}{2}L, \text{ or } X_M = M \pm \frac{1}{2}L \quad (25)$$

The upper and lower limits are converted to their antilogarithms to obtain the limits as explicit potencies.

(2) More often, the log-potency or potency is computed from a ratio, and in these cases the length of the confidence interval is typified by the log-interval in the equation

$$L = 2\sqrt{(C-1)(CM'^2 + c'i^2)} \quad (26)$$

where  $M'$  is the log-relative potency as defined (see Calculation of Potency from a Single Assay),  $i$  is the log-interval between successive doses, and  $c'$  is a constant characteristic of



... assay procedure. The remaining term C depends upon the precision with which the slope of the dosage-response curve has been determined. (This is sometimes expressed ... terms of  $g = (C - 1)/C$ .) In factorial assays, it is computed as

$$C = T_b^2 / (T_b^2 - e_b f s^2 t^2) \quad (27)$$

where  $s^2$  is the error variance of a single observation,  $t^2$  is read from [Table 9](#) with the degrees of freedom in  $s^2$ ,  $f$  is the number of responses in each  $T_t$  used in calculating  $T_b$ , and  $T_b$  and  $e_b$  are computed with the factorial coefficients for row b in [Tables 6 to 8](#). The  $s^2$  in Equation 26 depends upon the design of the assay, as indicated for each drug in the next section. In a valid assay, C is a positive number.

In an assay of two or more Unknowns against a common Standard, all with dosage-response curves that are parallel within the experimental error, C may be computed with the error variance  $s^2$  for the assay and with the assay slope as

$$C = (\Sigma T_b')^2 / \{(\Sigma T_b')^2 - e_b f h' s^2 t^2 / 2\} \quad (28)$$

The slope factor  $T_b' = \Sigma(x_1 T_t)$  or  $\Sigma(x_1 y)$  for each of the  $h'$  preparations, including the Standard, is computed with the factorial coefficients  $x_1$  for the Standard in the appropriate row b of [Table 6](#) or [8](#). If a treatment total  $T_t$  includes one or more replacements for a missing response, replace  $e_b f$  in Equation 27, or  $e_b f h'/2$  in Equation 28, by  $f^2 \Sigma(x_1 t^2)$ , where each  $x_1$  is a factorial coefficient in row b of [Tables 6 to 8](#), in this chapter, and  $f'$  is the number of responses in the corresponding  $T_t$  before adding the replacement. With this C, compute the confidence interval as

$$L = 2\sqrt{(C - 1)(CM^2 + c' t^2 h' / 2)} \quad (29)$$

In assays computed from a ratio, the most probable log-potency M is not in the exact center of the confidence interval. The upper and lower confidence limits in logarithms are

$$X_M = \log R + CM' + \frac{1}{2}L \text{ and } \log R + CM' - \frac{1}{2}L \quad (30)$$

C is often very little larger than unity, and the more precise the assay, the more nearly C approaches 1 exactly.  $R = z_S / z_U$  is the ratio of corresponding doses of the Standard and of the Unknown or the assumed potency of the Unknown. The upper and lower confidence limits in log-potencies are converted separately to their antilogarithms to obtain the corresponding potencies.

Confidence Intervals for Individual Assays— Since the confidence interval may vary in detail from the above general patterns, compute it for each assay by the special directions given under the name of the substance in the paragraphs following.

Antibiotic Assays— The confidence interval may be computed by Equations 24 and 25.

Calcium Pantothenate— For log-potencies obtained by interpolation from the Standard curve, the confidence interval may be computed with Equations 19 and 24. For log-potencies calculated with Equation 8 or 10,  $s^2$  may be computed with Equation 15, C with Equation 27 or 28, and the confidence interval L with Equation 26 or 29.

Corticotropin Injection— Compute the log confidence interval by Equations 26 and 27, with the coefficients and constants in [Table 6](#) for a 3-dose assay, and  $s^2$  as determined by Equation 13 or 14.

Digitalis— Compute the confidence interval as

$$L = 2\sqrt{(C - 1)\{C(\bar{z}_S/\bar{z}_U)^2 + f_U/f_S\}} \quad (31)$$

where  $f_U$  and  $f_S$  are the number of observations on the Unknown and on the Standard, and

$$C = \bar{z}_U^2 / (\bar{z}_U^2 - s^2 t^2 / f_U) \quad (32)$$

is determined with  $s^2$  from Equation 11. The confidence limits for the potency in USP Units are then

$$X_{P_*} = R\{C(\bar{z}_S/\bar{z}_U) \pm \frac{1}{2}L\} \quad (33)$$

in which R is as defined in the Glossary of Symbols.

Glucagon for Injection— Compute the error variance  $s^2$  by Equation 15a, C by Equation 27 with  $e_b f = 16n'$ , and the log confidence interval L by Equation 26 with  $c' i^2 = 0.09062$ .

Chorionic Gonadotropin— Proceed as directed under Corticotropin Injection.

Heparin Sodium— If two independent determinations of the log-potency M differ by more than 0.05, carry out additional assays and compute the error variance among the N values of M as

$$s^2 = \{\Sigma M^2 - (\Sigma M)^2 / N\} / n \quad (34)$$

with  $n = N - 1$  degrees of freedom. Given this value, determine the confidence interval in logarithms (L) by Equation 24.

Insulin Injection— Compute the error variance ( $s^2$ ) of y by Equation 16 and C as

$$C = T_b^2 / (T_b^2 - s^2 t^2 N) \quad (35)$$

where  $t^2$  from [Table 9](#) depends upon  $n = 4(f - 1)$  degrees of freedom in  $s^2$  and  $N = 4f$  is the total number of differences in the four groups. By Equation 26, compute the confidence interval L in logarithms, where  $c' i^2 = 0.09062$ . The upper and lower confidence limits in USP Units of insulin are given by the antilogarithms of  $X_M$  from Equation 30.

Oxytocin Injection— Compute the approximate log confidence interval by Equation 26, in which

$$C = (T_2 - T_1)^2 / \{(T_2 - T_1)^2 - 4(f + 1)s^2 t^2 / 3\} \quad (36)$$

where  $s^2$  is defined by Equation 18, and

$$c' = (4f - 1) / 8(f + 1) \quad (37)$$

Tubocurarine Chloride Injection— Compute the error variance by Equation 12, and the confidence interval by Equation 24.

Vasopressin Injection— Compute the error variance  $s^2$  by Equation 16, C by Equation 35, and the log confidence interval by Equation 26, where  $c' = 1$  and  $i$  is the log-interval separating the two dosage levels.

Vitamin B12 Activity— Proceed as directed under Calcium Pantothenate.

#### Combination of Independent Assays

When the method permits, additional animals can be added to an insufficiently precise assay until the combined results reduce the confidence interval within the limits specified in the monograph. Where two or more independent assays are required, each leading to a log-potency M, the M's are combined in determining the weighted mean potency of the Unknown. Except in the Heparin Sodium assay, where the log-potencies are weighted equally, the relative precisions of the two or more independent M's determine the weight assigned to each value in computing their mean and its confidence interval.

Before combining two or more separate estimates of M, test their mutual consistency. If the M's are consistent, their respective confidence intervals will overlap. Where the intervals do not overlap or where the overlap is small, compute an approximate  $\chi^2_M$ . Assign each of the  $h$  individual assays a weight w, defined as

$$w = 4t^2/L^2 \quad (38)$$

where the length of the confidence interval L is computed with the appropriate equation from the preceding section, and  $t^2$  is read from [Table 9](#) for the degrees of freedom n in the error variance of the assay. Sum the individual weights to obtain  $\Sigma w$ . Then an approximate  $\chi^2$  with  $h - 1$  degrees of freedom is determined as

$$\text{Approx. } \chi^2_M = \Sigma(wM^2) - \{\Sigma(wM)\}^2/\Sigma w \quad (39)$$

For two assays with log-potencies M1 and M2 and weights w1 and w2, Equation 35 reduces to

$$\text{Approx. } \chi^2_M = \frac{w_1 w_2 (M_1 - M_2)^2}{w_1 + w_2} \quad (40)$$

with one degree of freedom. If the approximate  $\chi^2_M$  is well under the critical value for  $\chi^2$  in [Table 9](#), use the weights w in computing the mean log-potency M and its confidence interval, L. If  $\chi^2_M$  approaches or exceeds this critical value, use instead the semi-weights w' (Equation 47) when computing M.

Compute the mean log-potency M of two or more mutually consistent assays as

$$\bar{M} = \Sigma(wM)/\Sigma w \quad (41)$$

This is the most probable single value within a combined confidence interval of length Lc, defined as the square root of

$$L_c^2 = \frac{4t_L^2}{\Sigma w} \left\{ 1 + \frac{4}{\Sigma w^2} \sum \frac{w(\Sigma w - w)}{n'} \right\} \quad (42)$$

where each  $n' = n - 4(h - 2)/(h - 1)$  and  $t_L^2$  is interpolated from [Table 9](#) with the degrees of freedom

$$n_L = \Sigma^2 w / \Sigma(w^2/n) \quad (43)$$

For two assays ( $h = 2$ ) with log-potencies M1 and M2 and weights w1 and w2, respectively, the above equation may be rewritten as

$$L_c^2 = \frac{4t_L^2}{\Sigma w} \left\{ 1 + \frac{4w_1 w_2}{\Sigma w^2} \left[ \frac{1}{n_1} + \frac{1}{n_2} \right] \right\} \quad (43)$$

where  $\Sigma w = w_1 + w_2$ . Where Lc, the confidence interval for a combined estimate, does not exceed the requirement in a monograph, upper and lower confidence limits are taken  $\pm L_c$  above and below M, to obtain approximately a 95% confidence interval.

Where the variation in the assayed potency between the  $h$  independent determinations, as tested by  $\chi^2_M$ , approaches or exceeds  $P = 0.05$ , the several estimates are assigned semi-weights w'. From the weight w, compute the variance of each M as

$$V = 1/w = L^2/4t^2 \quad (44)$$

Calculate the variance of the heterogeneity between assays as

$$v = \frac{\Sigma M^2 - (\Sigma M)^2/h}{h - 1} - \frac{\Sigma V}{h} \quad (45)$$

or if  $h = 2$ ,

$$v = \frac{(M_1 - M_2)^2}{2} - \frac{V_1 + V_2}{2} \quad (46)$$

Where V varies so markedly that v calculated as above is a negative number, compute instead an approximate v by omitting the term following the minus sign in Equations 45 and 46. A semi-weight is defined as

$$w' = 1/(V + v) \quad (47)$$

Substitute w' and  $\Sigma w'$  for w and  $\Sigma w$  in Equation 41 to obtain the semi-weighted mean M. This falls near the middle of a confidence interval of approximate length Lc', where

$$L_c'^2 = 4t^2/\Sigma w' \quad (48)$$

and  $t^2$  from [Table 9](#) has  $\Sigma n$  degrees of freedom.

Where  $\chi^2_M$  in Equation 39, from  $h = 4$  or more estimates of M, exceeds the critical level in [Table 9](#) by more than 50%, and the weights w differ by less than 30%, the h estimates of M may be checked for a suspected outlier with [Table 1](#). Where significant, the outlying M may be omitted in computing M with w'.

Where the potency of a drug is determined repeatedly in a given laboratory by the same bioassay method, successive determinations of both the slope b and the error variance  $s^2$  may



Scatter randomly within the sampling error about a common value for each parameter. Plotting estimates from successive assays on a quality control chart for each statistic and computing the midvalue and control limits defining the allowable random variation make it possible to check continuously the consistency of an assay technique. Where estimates of  $b$  and  $s^2$  from a single assay fall within the control limits, they may be replaced by their laboratory means. Reject any assay in which these statistics fall outside the control limits, or accept it only after close scrutiny with respect to its validity.

#### Joint Assay of Several Preparations

Each monograph describes the assay of a single Unknown against the Standard. Although not provided explicitly, several different Unknowns are often included in the same assay and each is compared separately with the same responses to the Standard. This fact may warrant increasing the number of observations with the Standard. Given  $f$  observations at each dosage level of each of  $h$  different Unknowns, the number of observations at each dosage level of the Standard may be increased advantageously, if  $h$  is large, to

$$f\sqrt{h}.$$

This rule can be applied only approximately where litter differences or their equivalent must be segregated, and in any case is merely suggestive.

If all of several assays conducted concurrently meet the requirements for validity, and have linear log-dose response curves with the same slope  $b$  and the same error variance  $s^2$  about these lines, these two statistics may be considered as characteristic of the assay. Combining all of the evidence from the same assay into a single value of the assay slope results in a more stable and reliable estimate of  $b$  than if each Unknown were analyzed independently. The degrees of freedom and reliability of the error variance  $s^2$  can be increased similarly. Confidence intervals computed with these composite values for  $b$  and  $s^2$  are smaller on the average than if based upon only part of the relevant data. For the calculation or application of such assay estimates, see Equations 10, 15, 16, 19, 28, and 29. The potency estimated with a slope computed from a single Unknown and the Standard agrees within a fraction of the confidence interval with that computed from the combined slope for the entire assay. Since it is based upon more evidence, the latter is considered the better estimate.

#### GLOSSARY

##### Glossary of Symbols

A	absorbance for computing % reduction in bacterial growth from turbidimetric readings.
b	slope of the straight line relating response (y) to log-dose (x) [Equations 2b, 4, 5, 6].
c	constant for computing $M'$ with Equations 8 and 10.
$c'$	constant for computing $L$ with Equations 26 and 29.
$c_i$	constant for computing $M'$ when doses are spaced as in <a href="#">Table 8</a> .
$c'_{i2}$	constant for computing $L$ when doses are spaced as in <a href="#">Table 8</a> .
C	term measuring precision of the slope in a confidence interval [Equations 27, 28, 35, 36].
$\chi_2$	statistical constant for testing significance of a discrepancy [ <a href="#">Table 9</a> ].
$\chi_{M2}$	$\chi_2$ testing the disagreement between different estimates of log-potency [Equations 39, 40].
$eb$	$ei$ from row $b$ in <a href="#">Tables 6 to 8</a> .
$eb' i$	multiple of $\sum(x - \bar{x})^2$ [Table 5; Equation 6].
$ei$	sum of squares of the factorial coefficients in each row of <a href="#">Tables 6 to 8</a> .
$eq$	$ei$ from row $q$ in <a href="#">Tables 6 to 8</a> .
f	number of responses at each dosage level of a preparation; number of replicates or sets.
$f_S$	number of observations on the Standard.
$f_U$	number of observations on the Unknown.
$F1$ to $F3$	observed variance ratio with 1 to 3 degrees of freedom in numerator [ <a href="#">Table 9</a> ].
$G1$ , $G2$ , and $G3$	relative gap in test for outlier [ <a href="#">Table 1</a> ].
h	number of Unknowns in a multiple assay.
$h'$	number of preparations in a multiple assay, including the Standard and $h$ Unknowns; i.e., $h' = h + 1$ .
i	interval in logarithms between successive log-doses, the same for both Standard and Unknown.
k	number of estimated log-potencies in an average [Equation 24]; number of treatments or doses [ <a href="#">Table 4</a> ; Equations 1, 13, 15, 16]; number of ranges or groups in a series [ <a href="#">Table 2</a> ]; number of rows, columns, and doses in a single Latin square [Equations 1a, 16a].
L	length of the confidence interval in logarithms [Equations 24, 26, 29, 38], or in terms of a proportion of the relative potency of the dilutions compared [Equations 31, 33].
$L_c$	length of a combined confidence interval [Equations 42, 43].
$L_c'$	length of confidence interval for a semi-weighted mean $M$ [Equation 48].
LD50	lethal dose killing an expected 50% of the animals under test [Equation 2c].
M	log-potency [Equation 2].
$M'$	log-potency of an Unknown, relative to its assumed potency.
M	mean log-potency.
n	degrees of freedom in an estimated variance $s^2$ or in the statistic $t$ or $\chi_2$ .
$n'$	number of Latin squares with rows in common [Equations 1a, 16a].
N	number; e.g., of observations in a gap test [ <a href="#">Table 1</a> ], or of responses $y$ in an assay [Equation 16].
P	probability of observing a given result, or of the tabular value of a statistic, usually $P = 0.05$ or $0.95$ for confidence intervals [ <a href="#">Tables 1, 2, 9</a> ].



	potency, $P^*$ = antilog M or computed directly.
R	ratio of a given dose of the Standard to the corresponding dose of the Unknown, or assumed potency of the Unknown [Equations 2, 30, 33].
$R^*$	ratio of largest of k ranges in a series to their sum [Table 2].
$s = \sqrt{s_2}$	standard deviation of a response unit, also of a single estimated log-potency in a direct assay [Equation 24].
$s_2$	error variance of a response unit.
Si	a log-dose of Standard [Tables 6, 7].
$\Sigma$	"the sum of."
t	Student's t for n degrees of freedom and probability P = 0.05 [Table 9].
T	total of the responses y in an assay [Equation 16].
$T'$	incomplete total for an assay in randomized sets with one missing observation [Equation 1].
T1	$\Sigma(y)$ for the animals injected with the Standard on the first day [Equations 18, 36].
T2	$\Sigma(y)$ for the animals injected with the Standard on the second day [Equations 18, 36].
Ta	Ti for the difference in the responses to the Standard and to the Unknown [Tables 6 to 8].
Tab	Ti for testing the difference in slope between Standard and Unknown [Tables 6 to 8].
Taq	Ti for testing opposed curvature in the curves for Standard and Unknown [Tables 6 to 8].
Tb	Ti for the combined slope of the dosage-response curves for Standard and Unknown [Tables 6 to 8].
Tb'	$\Sigma(x_1 T_t)$ or $\Sigma(x_1 y)$ for computing the slope of the log-dose response curve [Equations 10, 23, 28].
Ti	sum of products of Tt multiplied by the corresponding factorial coefficients in each row of [Tables 6 to 8].
Tq	Ti for testing similar curvature in the curves for Standard and Unknown [Tables 6 to 8].
Tr	row or set total in an assay in randomized sets [Equation 16].
$Tr'$	incomplete total for the randomized set with a missing observation in Equation 1.
Tt	total of f responses y for a given dose of a preparation [Tables 6 to 8]; Equations 6, 13, 14, 16].
$Tt'$	incomplete total for the treatment with a missing observation in Equation 1.
Ui	a log-dose of Unknown [Tables 6 to 8].
v	variance for heterogeneity between assays [Equation 45].
$V = 1/w$	variance of an individual M [Equations 44 to 47].
w	weight assigned to the M for an individual assay [Equation 38], or to a probit for computing an LD50 [Equations 2a, 2b].
$w'$	semi-weight of each M in a series of assays [Equations 47, 48].
x	a log-dose of drug in a bioassay [Equation 5]; also the difference between two log-threshold doses in the same animal [Equation 12].
$x^*$	coefficients for computing the lowest and highest expected responses YL and YH in a log-dose response curve [Table 4; Equation 3].
x1	a factorial coefficient that is a multiple of $(x - x)$ for computing the slope of a straight line [Table 5; Equation 6].
x	mean log-dose [Equation 5].
xS	mean log-dose for Standard [Equation 9].
xU	mean log-dose for Unknown [Equation 9].
X	log-potency from a unit response, as interpolated from a standard curve [Equations 7a, 7b, 19].
XM	confidence limits for an estimated log-potency M [Equations 25, 30].
XP*	confidence limits for a directly estimated potency $P^*$ (see Digitalis assay) [Equation 33].
y	an observed individual response to a dose of drug in the units used in computing potency and the error variance [Equations 13 to 16]; a unit difference between paired responses in 2-dose assays [Equations 17, 18].
$y_1 \dots y_N$	observed responses listed in order of magnitude, for computing G1, G2, or G3 in Table 1.
y'	replacement for a missing value [Equation 1].
y	mean response in a set or assay [Equation 5].
yt	mean response to a given treatment [Equations 3, 6].
Y	a response predicted from a dosage-response relationship, often with qualifying subscripts [Equations 3 to 5].
z	threshold dose determined directly by titration (see Digitalis assay) [Equation 11].
z	mean threshold dose in a set (see Digitalis assay) [Equations 31, 32, 33].

### 115 DEXPANTHENOL ASSAY

The following procedure is provided for the determination of dexamphenol as an ingredient of multiple-vitamin preparations. It is applicable also to the determination of the dextrorotatory component of racemic panthenol and of other mixtures containing dextrorotatory panthenol.

Media may be prepared as described hereinafter, or dehydrated mixtures yielding similar formulations may be used provided that, when reconstituted as directed by the manufacturer or distributor, they have growth-promoting properties equal to or superior to those obtained from the formulas given herein.

USP Reference Standards (11) — [USP Dexamphenol RS](#).

Standard Stock Solution of Dexamphenol— Dissolve an accurately weighed quantity of [USP Dexamphenol RS](#) in water, dilute with water to obtain a solution having a known concentration of about 800  $\mu$ g per mL, and mix. Store in a refrigerator, protected from light, and use within 30 days.

Standard Preparation— On the day of the assay, prepare a water dilution of the Standard Stock Solution of Dexamphenol to contain 1.2  $\mu$ g of dexamphenol per mL.

Assay Preparation— Proceed as directed in the individual monograph for preparing a solution expected to contain approximately the equivalent of the dexamphenol concentration in the Standard Preparation.

Modified Pantothenate Medium—



Acid-Hydrolyzed Casein Solution	25 mL
Cystine-Tryptophane Solution	25 mL
Polysorbate 80 Solution	0.25 mL
Dextrose, Anhydrous	10 g
Sodium Acetate, Anhydrous	5 g
Adenine-Guanine-Uracil Solution	5 mL
Riboflavin-Thiamine Hydrochloride-Biotin Solution	5 mL
Para-aminobenzoic Acid-Niacin-Pyridoxine Hydrochloride Solution	5 mL
Salt Solution A	5 mL
Salt Solution B	5 mL
Pyridoxal-Calcium Pantothenate Solution	5 mL
Polysorbate 40-Oleic Acid Solution	5 mL

Dissolve the anhydrous dextrose and sodium acetate in the solutions previously mixed, and adjust with 1 N sodium hydroxide to a pH of 6.8. Finally, dilute with water to 250 mL, and mix.

Double-Strength Modified Pantothenate Medium— Prepare as directed under Modified Pantothenate Medium, but make the final dilution to 125 mL instead of 250 mL. Prepare fresh.

Acid-Hydrolyzed Casein Solution— Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8 to 12 hours. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in about 500 mL of water, adjust the solution with 1 N sodium hydroxide to a pH of  $3.5 \pm 0.1$ , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 hour, and filter. Repeat the treatment with activated charcoal. Store under toluene in a refrigerator at a temperature not below  $10^{\circ}$ . Filter the solution if a precipitate forms during storage.

Cystine-Tryptophane Solution— Suspend 4.0 g of L-cystine and 1.0 g of L-tryptophane (or 2.0 g of D,L-tryptophane) in 700 mL to 800 mL of water, heat to  $75 \pm 5^{\circ}$ , and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, add water to make 1000 mL, and mix. Store under toluene in a refrigerator at a temperature not below  $10^{\circ}$ .

Adenine-Guanine-Uracil Solution— Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid, cool, add water to make 200 mL, and mix. Store under toluene in a refrigerator.

Polysorbate 80 Solution— Dissolve 25 g of polysorbate 80 in alcohol to make 250 mL, and mix.

Riboflavin-Thiamine Hydrochloride-Biotin Solution— Prepare a solution containing, in each mL, 20  $\mu$ g of riboflavin, 10  $\mu$ g of thiamine hydrochloride, and 0.04  $\mu$ g of biotin, by dissolving riboflavin, thiamine hydrochloride, and biotin in 0.02 N acetic acid. Store, protected from light, under toluene in a refrigerator.

Para-aminobenzoic Acid-Niacin-Pyridoxine Hydrochloride Solution— Prepare a solution in neutral 25 percent alcohol to contain 10  $\mu$ g of para-aminobenzoic acid, 50  $\mu$ g of niacin, and 40  $\mu$ g of pyridoxine hydrochloride in each mL. Store in a refrigerator.

Salt Solution A— Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid, mix, and store under toluene.

Salt Solution B— Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, mix, and store under toluene.

Pyridoxal-Calcium Pantothenate Solution— Dissolve 40 mg of pyridoxal hydrochloride and 375  $\mu$ g of calcium pantothenate in 10 percent alcohol to make 2000 mL, and mix. Store in a refrigerator, and use within 30 days.

Polysorbate 40-Oleic Acid Solution— Dissolve 25 g of polysorbate 40 and 0.25 g of oleic acid in 20 percent alcohol to make 500 mL, and mix. Store in a refrigerator, and use within 30 days.

Stock Culture of *Pediococcus acidilactici*— Dissolve in about 800 mL of water, with the aid of heat, 6.0 g of peptone, 4.0 g of pancreatic digest of casein, 3.0 g of yeast extract, 1.5 g of beef extract, 1.0 g of dextrose, and 15.0 g of agar. Adjust with 0.1 N sodium hydroxide or 0.1 N hydrochloric acid to a pH between 6.5 and 6.6, adjust the volume with water to 1000 mL, and mix. Add approximately 10-mL portions of the solution to culture tubes, place caps on the tubes, and sterilize at  $121^{\circ}$  for 15 minutes. Cool on a slant, and store in a refrigerator. Prepare a stock culture of *Pediococcus acidilactici* on a slant of this medium. Incubate at  $35^{\circ}$  for 20 to 24 hours, and store in a refrigerator. Maintain the stock culture by monthly transfer onto fresh slants.

Inoculum— Inoculate three 250-mL portions of Modified Pantothenate Medium from a stock culture slant, and incubate at  $35^{\circ}$  for 20 to 24 hours. Centrifuge the suspension from the combined portions, and wash the cells with Modified Pantothenate Medium. Resuspend the cells in sufficient Modified Pantothenate Medium so that a 1:50 dilution, when tested in a 13-mm diameter test tube, gives 80% light transmission at 530 nm. Transfer 1.2-mL portions of this stock suspension to glass ampuls, seal, freeze in liquid nitrogen, and store in a freezer. On the day of the assay, allow the ampuls to reach room temperature, mix the contents, and dilute 1 mL of thawed culture with sterile saline TS to 150 mL. [note—This dilution may be altered, when necessary, to obtain the desired test response.]

Procedure— Prepare in triplicate a series of eight culture tubes by adding the following quantities of water to the tubes within a set: 5.0 mL, 4.5 mL, 4.0 mL, 3.5 mL, 3.0 mL, 2.0 mL, 1.0 mL, and 0.0 mL. To these same tubes, and in the same order, add 0.0 mL, 0.5 mL, 1.0 mL, 1.5 mL, 2.0 mL, 3.0 mL, 4.0 mL, and 5.0 mL of the Standard Preparation.

Prepare in duplicate a series of five culture tubes by adding the following quantities of water to the tubes within a set: 4.0 mL, 3.5 mL, 3.0 mL, 2.0 mL, and 1.0 mL. To these same tubes, and in the same order, add 1.0 mL, 1.5 mL, 2.0 mL, 3.0 mL, and 4.0 mL of the Assay Preparation.

Add 5.0 mL of Double-Strength Modified Pantothenate Medium to each tube, and mix. Cover the tubes with metal caps, and sterilize in an autoclave at  $121^{\circ}$  for 5 minutes. Cool to room temperature in a chilled water bath, and inoculate each tube with 0.5 mL of the Inoculum. Allow to incubate at  $37^{\circ}$  for 16 hours. Terminate growth by heating to a temperature not



below 80°, such as by steaming at atmospheric pressure in a suitable sterilizer, for 5 to 10 minutes. Cool, and concomitantly determine the percentage transmittance of the suspensions, in cells of equal pathlength, on a suitable spectrophotometer, at 530 nm.

**Calculation**— Draw a dose-response curve on arithmetic graph paper by plotting the average response, in percent transmittance, for each set of tubes of the standard curve against the standard level concentrations. The curve is drawn by connecting each adjacent pair of points with a straight line. From this standard curve, determine by interpolation the potency, in terms of dexpanthenol, of each tube containing portions of the Assay Preparation. Divide the potency of each tube by the amount of Assay Preparation added to it, to obtain the individual responses. Calculate the mean response by averaging the individual responses that vary from their mean by not more than 15%, using not less than half the total number of tubes. Calculate the potency of the portion of the material taken for assay, in terms of dexpanthenol, by multiplying the mean response by the appropriate dilution factor.

\* American Type Culture Collection No. 8042 is suitable.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Curtis Phinney</a>  1-301-816-8540	(DSN05) Dietary Supplements - Non-Botanicals
Reference Standards	Lili Wang, Technical Services Scientist 1-301-816-8129 <a href="mailto:RSTech@usp.org">RSTech@usp.org</a>	

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## 121 INSULIN ASSAYS

The most prominent manifestation of insulin activity, an abrupt decrease in blood glucose, was the basis for biologic assay from the time of its first clinical use. The procedure, although relatively cumbersome, has the great merit of accurately reflecting the effect on the diabetic patient. The advent of practical yet sophisticated physicochemical methods (e.g., liquid chromatography) to measure insulin potency quantitatively has resulted in a more accurate and precise compendial test for insulin and insulin products. However, the bioidentity of insulin and insulin products cannot be assessed by these methods. Thus, a qualitative test in rabbits is included in this chapter, and its use is called for in the appropriate monographs.

**The Rabbit Blood Sugar Method**—Quantitative is used to determine the potency of Insulin Reference Standards, for the validation of the stability of new insulin preparations, and to determine the specific activities of insulin analogs.

### RABBIT BLOOD SUGAR METHOD—QUANTITATIVE

USP Reference Standards (11)—[USP Dextrose RS](#), [USP Insulin RS](#), [USP Insulin \(Beef\) RS](#), [USP Insulin Human RS](#), [USP Insulin \(Pork\) RS](#).

**Diluent**— Prepare an aqueous solution containing 0.1% to 0.25% (w/v) of either cresol or phenol, 1.4% to 1.8% (w/v) of glycerin, and sufficient hydrochloric acid to produce a pH between 2.5 and 3.5, unless otherwise directed in the individual monograph.

**Standard Stock Solution**— Dissolve either a suitable quantity of accurately weighed [USP Insulin RS](#) or a vial of lyophilized [USP Insulin RS](#) of the appropriate species in Diluent to make a Standard Stock Solution containing 40 USP Insulin Units per mL and having a pH between 2.5 and 3.5, unless otherwise directed in the individual monograph. Store in a cold place, protected from freezing, and use within 6 months.

**Standard Solutions**— Dilute portions of the Standard Stock Solution with Diluent to make two solutions, one to contain 1.0 USP Insulin Unit per mL (Standard Solution 1), and the other to contain 2.0 USP Insulin Units per mL (Standard Solution 2).

**Assay Stock Solution**— Proceed as directed under Standard Stock Solution, except to use a suitable quantity of the preparation under test in place of [USP Insulin RS](#). The Assay Stock Solution contains about 40 USP Insulin Units per mL.

**Assay Solutions**— Dilute portions of the Assay Stock Solution with Diluent to make two dilutions of the preparation under test, one of which may be expected, on the basis of the assumed potency, to contain 1.0 USP Insulin Unit per mL (Assay Solution 1), and the other to contain 2.0 USP Insulin Units per mL (Assay Solution 2). In the case of neutral insulin injection, adjust to a pH of 2.5 to 3.5 prior to making the dilutions.

**Doses of the Solutions To Be Injected**— Select on the basis of trial or experience the dose of the dilutions to be injected, the volume of which usually will be between 0.30 mL and 0.50 mL. For each animal the volume of the Standard Solution is the same as that of the Assay Solution.

**Preparation of Animal**— Select suitable, healthy rabbits each weighing not less than 1.8 kg. Keep the rabbits in the laboratory for not less than 1 week before use in the assay, maintaining them on an adequate uniform diet, with water available at all times.

**Procedure**— Divide the rabbits into four equal groups of preferably not less than six rabbits each. On the preceding day, approximately 20 hours before the assay, provide each rabbit with an amount of food that will be consumed within 6 hours. Follow the same feeding schedule before each test day. During the assay, withhold all food until after the final blood specimen is taken. Handle the rabbits with care in order to avoid undue excitement, and inject subcutaneously the doses indicated in the following design (see [Table 1](#)), the second injection being made on the day after the first injection, or not more than 1 week later. The time between the first and second injection is the same for all rabbits.

Table 1

Group	First Injection	Second Injection
1	Standard Solution 2	Assay Solution 1
2	Standard Solution 1	Assay Solution 2
3	Assay Solution 2	Standard Solution 1
4	Assay Solution 1	Standard Solution 2

**Blood Samples**— At 1 hour ± 5 minutes and 2½ hours ± 5 minutes after the time of injection, obtain from each rabbit a suitable blood specimen from a marginal ear vein. Blood can also be collected effectively from the central auricular artery.

**Dextrose Determination**— Determine the dextrose content of the blood specimens by a suitable procedure that is adapted to automated analysis. The following procedure may be used.

**Anticoagulant Solution**— Dissolve 1 g of edetate sodium and 200 mg of sodium fluoride in 1 L of water, and mix.

**Dextrose Standard Preparations**— Transfer known concentrations of [USP Dextrose RS](#) to suitable vessels, and dilute quantitatively and stepwise with Anticoagulant Solution (1:9) to obtain a range of Dextrose Standard Preparations containing between 20 and 100 mg per 100 mL, having known concentrations similar to the concentrations in the rabbit blood samples.

**Test Preparations**— Pipet into separate, suitable vessels 0.1 mL of each Blood Sample and 0.9 mL of Anticoagulant Solution.

**Procedure**— Subject the Test Preparations to dialysis across a semipermeable membrane for a sufficient time so that the dextrose passes through the membrane into a saline TS solution containing glucose oxidase, horseradish peroxidase, 3-methyl-2-benzothiazolinone hydrazone hydrochloride TS, and N,N-dimethylaniline. The absorbances of the Test



Preparations are determined at 600 nm in a recording colorimeter. The absorbances of the Dextrose Standard Preparations are similarly determined at the start and the end of each run.

Calculation— Calculate the response of each rabbit to each injection from the sum of the two blood-sugar values, and subtract its response, disregarding the chronological order in which the responses were observed, to obtain the individual differences,  $y$ , as shown in [Table 2](#).

When the data for one or more rabbits are missing in an assay, do not use the confidence interval formulas given here, but seek statistical help. The data can still be analyzed with proper analysis of variance.

When the number of rabbits,  $f$ , carried through the assay is the same in each group, total the  $y$ 's in each group and compute  $T_a = -T_1 + T_2 + T_3 - T_4$  and  $T_b = T_1 + T_2 + T_3 + T_4$ . The logarithm of the relative potency of the test dilutions is  $M' = 0.301T_a / T_b$ . The potency of the injection in USP Units per mg equals the antilog ( $\log R + M'$ ), where  $R = vS / vU$ , in which  $vS$  is the number of USP Units per mL of the Standard solution and  $vU$  is the number of mg of insulin per mL of the corresponding Assay solution.

Determine the 95% confidence interval for the log-relative potency using Fieller's Theorem (see Appendix and [Design and Analysis of Biological Assays](#) (111)). If the confidence interval is more than 0.082, which corresponds at  $P = 0.95$  to confidence limits of about  $\pm 10\%$  of the computed potency, repeat the assay until the combined data of the two or more assays, redetermined as described in Combination of Independent Assays under [Design and Analysis of Biological Assays](#) (111), meet this acceptable limit.

Table 2

Group	Differences	Individual Response ( $y$ )	Total Response ( $T$ )	Standard Deviations of Differences ( $S$ )
1	Standard Solution 2 – Assay Solution 1	$y_1$	$T_1$	$S_1$
2	Assay Solution 2 – Standard Solution 1	$y_2$	$T_2$	$S_2$
3	Assay Solution 2 – Standard Solution 1	$y_3$	$T_3$	$S_3$
4	Standard Solution 2 – Assay Solution 1	$y_4$	$T_4$	$S_4$

## BIOIDENTITY TEST

Proceed as directed for Rabbit Blood Sugar Method—Quantitative with the following modifications:

Procedure— Divide the rabbits into four equal groups of two rabbits each.

Calculation— Proceed as directed for Calculation under Rabbit Blood Sugar Method—Quantitative, but do not determine the confidence interval of the log-relative potency,  $M'$ .

Interpretation— If the potency value obtained is not less than 15 USP Units per mg, the Bioidentity Test requirement is met. If the potency value is less than 15 USP Units per mg, repeat the test using eight more rabbits. If the average potency of the two sets of tests is not less than 15 USP Units per mg, the requirement of the test is met.

## Appendix—Fieller's Theorem for Determining the Confidence Interval for a Ratio

This version of Fieller's Theorem is for the case where the numerator and denominator are uncorrelated. The equation assumes the numerator and denominator are normally distributed and the groups of rabbits are equal-sized.

Then, the 95% confidence interval for the ratio is:

$$(L, U) = \frac{M' \pm \frac{t}{T_b} \sqrt{(1-g)S_N^2 + (M')^2 S_D^2}}{1-g}$$

where  $f$  (degrees of freedom in the standard errors) =  $4(k - 1)$ , where  $k$  is the number of rabbits in a group,  $t$  is the upper 97.5 percentile of the t-distribution with  $f$  degrees of freedom, and

$$g = \frac{t^2 S_D^2}{T_b^2}$$

If  $g \geq 1$ , the denominator is not significantly different from 0 and the formula does not work.

$$S_N = 0.301 \sqrt{k} \sqrt{S_1^2 + S_2^2 + S_3^2 + S_4^2}$$

$$S_D = \sqrt{k} \sqrt{S_1^2 + S_2^2 + S_3^2 + S_4^2}$$

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Larry N. Callahan, Ph.D.</a> Senior Scientist 1-301-816-8385	(BBPP05) Biologics and Biotechnology - Proteins and Polysaccharides

## Introduction

Protein A is coupled to a resin support in order to create protein A affinity chromatography media commonly used in the manufacturing of recombinant therapeutic monoclonal antibodies. Natural protein A is derived from *Staphylococcus aureus* and contains five homologous antibody binding regions and a C-terminal region for cell wall attachment. In addition to naturally derived protein A, recombinant material manufactured in *Escherichia coli*, as well as several engineered versions of the protein, also manufactured recombinantly, have entered the market place. When immobilized on a column, protein A provides a highly efficient and robust purification method for purifying antibodies at various scales. However, protein A ligand from the column can co-elute with the antibody during purification, an effect which is often referred to as protein A leaching. This tendency increases as the chromatography medium ages. Engineered versions of protein A may improve the pH tolerance of the medium, but do not eliminate leaching. It is the current regulatory expectation that leached protein A should be cleared during the purification of antibodies for human use, and manufacturing processes should be validated accordingly. Enzyme-Linked Immunosorbent Assay (ELISA)-based residuals testing is generally employed during process development and validation to assure the efficient removal of residual protein A during process steps following protein A affinity chromatography. In addition, the manufacturer should have a clear understanding and documentation of resin and ligand quality through raw materials qualification and column lifetime studies.

General Chapter 130 describes quality attributes of protein A ligands that are used in chromatography media for the manufacture of therapeutic monoclonal antibodies: Protein A; rProtein A; rProtein A, C-Cys; rProtein A, B4, C-Cys.

## Protein A

C1995H3163N597O697S3

46,760

N-terminal Sequence AQHDEA

C-terminal Sequence IAADNK

Protein A is derived from *Staphylococcus aureus*. The structure is composed of a single polypeptide chain containing four IgG binding domains. With the exception of IgG3, all other human IgGs bind to protein A. Each molecule of Protein A is capable of binding two IgG molecules. It is manufactured as a bulk solution at a concentration of greater than 20 mg protein A per mL with an IgG-binding potency of greater than 95%. Because Protein A is used as an ancillary material in the manufacture of recombinant therapeutic drugs, regulatory requirements differ from those for therapeutic drug products.

Packaging and storage— Store in closed containers at the temperature indicated on the label.

Labeling— Preserve in sealed containers, and store at a temperature of  $-20^{\circ}$  or below.

[USP Reference standards](#) 11— [USP Endotoxin RS](#), [USP Protein A RS](#).

## Identification—

A: SDS-PAGE—It meets the requirements of Identification test A under rProtein A using [USP Protein A RS](#).

B: IgG Binding—It meets the requirements of Identification test B under rProtein A using [USP Protein A RS](#).

[Microbial Enumeration Tests](#) 61 and [Tests for Specified Microorganisms](#) 62— The total aerobic microbial count does not exceed 100 cfu per mL, and the total yeasts and molds count does not exceed 10 cfu per mL.

[Bacterial endotoxins](#) 85— It contains not more than 1 USP Endotoxin Unit per mg of total protein. [note—The Bacterial endotoxins test for Protein A is used to describe the quality of this ancillary material. This test does not define the acceptable level of bacterial endotoxin in the preparation of injectable dosage forms in which Protein A is used.]

Total protein (see [Spectrophotometry and Light-Scattering](#) 851)— Prepare triplicate samples for analysis by diluting Protein A to 3.0 mg per mL in [Water for Injection](#). Measure the absorbance of each sample at 275 nm after correcting for the absorbance using [Water for Injection](#) as the blank. Determine the protein concentration using the equation:

$$\text{Protein concentration (mg per mL)} = (A275 / 0.149)$$

in which A is the absorbance of Protein A at the wavelength of 275 nm and 0.149 is the molar absorptivity. Average the triplicate results, and determine a coefficient of variance (CV):  
the CV is  $\leq 5\%$ .

Limit of common contaminant protein and corresponding assay—

Enterotoxin B— Enterotoxin B is determined using a commercially available microstrip enzyme-immunoassay kit.<sup>1</sup> Wells of the microstrips are coated with sheep antibodies to enterotoxin B. Standard curves are made using the ELISA kit control. The negative controls are wells coated with serum from nonimmunized sheep. The level of enterotoxin is determined from the standard curve. The specification for the enterotoxin B level is  $\leq 1$  ng per mg of total protein.

Chromatographic purity— [note—The size-exclusion chromatographic purity test resolves Protein A from high molecular weight contaminants.]

Mobile phase— Prepare a solution of 50 mM sodium dihydrogen phosphate, pH 6.5 in the following manner. Add  $6.9 \pm 0.1$  g of sodium dihydrogen phosphate into a 1000-mL beaker. Dilute with water to 900 mL, and adjust with 5 M sodium hydroxide to a pH of  $6.50 \pm 0.05$ . Transfer the solution into a 1000-mL volumetric flask, and dilute with water to volume. Pass the solution through a 0.22- $\mu$ m membrane filter.

Column regeneration solution— Prepare a solution of 0.1 M sodium dihydrogen phosphate, pH 3.0 in the following manner. Add  $13.8 \pm 0.1$  g of sodium dihydrogen phosphate into a 1000-mL beaker. Dilute with water to 900 mL, and adjust with hydrochloric acid to a pH of  $3.0 \pm 0.1$ . Transfer the solution into a 1000-mL volumetric flask, and dilute with water to volume. Pass the solution through a 0.22- $\mu$ m membrane filter.

Column storage solution— Mix 100 mL of methanol with 900 mL of water.

Test solution— Dilute Protein A to approximately 1 mg per mL with Mobile phase.

Calibration standards— Using Mobile phase, prepare separate 1 mg per mL solutions of each of the following: thyroglobulin (670 kD), IgG (150 kD), beta lactoglobulin (36 kD), and lysozyme (14 kD).

Standard solution— Prepare a solution containing 1 mg per mL of [USP Protein A RS](#) in Mobile phase.

Chromatographic system (see [Chromatography](#) 621)— The liquid chromatograph is equipped with a 280-nm detector and a 7.8-mm  $\times$  30-cm column that contains packing L33. Equilibrate the column for approximately 30 minutes at 0.5 mL of Mobile phase per minute or until a stable baseline is achieved.

Procedure— Separately inject 100  $\mu$ L of each sample, and run the samples in the following sequence: Calibration standards, thyroglobulin, IgG, beta lactoglobulin, and lysozyme; the Standard solution; and the Test solution. Run the sequence three times isocratically using Mobile phase at 0.5 mL per minute for 30 minutes. Absorbance is detected at 280 nm.



...alyze the 280-nm peak data, and pick the retention time (RT) with the largest peak area. Using the data from the Calibration standards, plot the mean RT versus the log molecular weight to produce the standard curve. The purity should be  $\geq 95\%$  in the main peak. Use the formula from the standard curve to give the log molecular weights of the Test solutions. Convert the log molecular weights of the Test solutions and the Standard solutions to actual molecular weights. The apparent molecular weight of protein A from the Standard solution is between 156 and 205 kDa; and the Protein A from the Test solution is within the same range.

Column cleaning and storage— Rinse the column with 100 mL of Column regeneration solution, and store by flushing with 100 mL of Column storage solution.

rPROTEIN A, C-CYS

C1478H2320N432O503S4

34317.5 Da

N-terminal Sequence AQHDEAQQNA

rProtein A, C-Cys is a recombinant Protein A lacking the C-terminal membrane binding part; instead, a C-terminal cysteine has been introduced for directed immobilization purposes. It has five homologous IgG binding domains identical to the native Protein A and is produced using *Escherichia coli* as the host cell followed by purification with conventional chromatography. rProtein A, C-Cys is manufactured as a bulk solution with an IgG-binding potency of greater than 95%. Because rProtein A, C-Cys is used as an ancillary material in the manufacture of recombinant therapeutic drugs, regulatory requirements differ from those for therapeutic drug products.

Packaging and storage— Store in closed containers at the temperature indicated on the label.

Labeling— Preserve in sealed containers, and store at a temperature of  $-20^{\circ}$  or below.

[USP Reference standards](#) (11) — [USP Endotoxin RS](#). [USP rProtein A, C-Cys RS](#).

Identification—

A: SDS-PAGE—It meets the requirements of Identification test A under rProtein A using [USP rProtein A, C-Cys RS](#).

B: IgG Binding—It meets the requirements of Identification test B under rProtein A using [USP rProtein A, C-Cys RS](#).

[Microbial Enumeration Tests](#) (61) and [Tests for Specified Microorganisms](#) (62)— The total aerobic microbial count does not exceed 100 cfu per mL, and the total yeasts and molds count does not exceed 10 cfu per mL.

[Bacterial endotoxins](#) (85)— It contains not more than 1 USP Endotoxin Unit per mg of total protein. [note—The Bacterial endotoxins test for rProtein A, C-Cys is used to describe the quality of this ancillary material. This test does not define the acceptable level of bacterial endotoxin in the preparation of injectable dosage forms in which rProtein A, C-Cys is used.]

Total protein (see [Spectrophotometry and Light-Scattering](#) (851))— Prepare triplicate samples for analysis by diluting the rProtein A, C-Cys to 3.0 mg per mL in [Water for Injection](#). Measure the absorbance of each sample at 275 nm after correcting for the absorbance using [Water for Injection](#) as the blank. Determine the protein concentration using the equation:

$$\text{Protein concentration (mg per mL)} = (A275 / 0.22)$$

in which A is the absorbance of rProtein A, C-Cys, at the wavelength of 275 nm and 0.22 is the molar absorptivity. Average the triplicate results, and determine a coefficient of variance (CV): the CV is  $\leq 2.5\%$ .

Chromatographic purity— [note—The size-exclusion chromatographic purity test resolves rProtein A, C-Cys from high molecular weight contaminants and low molecular weight contaminants.]

Mobile phase— Prepare a solution of 0.02 M sodium phosphate, pH 7.2 containing 0.15 M sodium chloride in the following manner. Add  $0.96 \pm 0.02$  g of monobasic sodium phosphate hydrate,  $2.32 \pm 0.02$  g of dibasic sodium phosphate dihydrate, and  $8.76 \pm 0.02$  g of sodium chloride into a 1000-mL beaker. Dilute with water to 900 mL, and adjust with 1 M sodium hydroxide to a pH of  $7.2 \pm 0.05$ . Transfer this solution into a 1000-mL volumetric flask, and dilute with water to volume. Pass the solution through a 0.45- $\mu$ m membrane filter.

EDTA solution— Prepare a 20 mM ethylenediaminetetraacetic acid (EDTA) solution by dissolving  $0.74 \pm 0.02$  g of EDTA in 100 mL of Mobile phase.

DTT solution— Prepare a 100 mM dl-dithiothreitol (DTT) solution by dissolving  $1.54 \pm 0.02$  g of DTT in 100 mL of Mobile phase.

Pretreatment solution— Prepare a solution containing a mixture of EDTA solution and DTT solution (1:1, v/v). [note—Prepare fresh just before use.]

Test solution— Dilute rProtein A, C-Cys 1 to 5 in Pretreatment solution, and mix gently. Incubate the sample at  $40^{\circ}$  for 60 minutes.

Chromatographic system (see [Chromatography](#) (621))— The liquid chromatograph is equipped with a 214-nm detector and a 10-mm  $\times$  30-cm column that contains packing L54. Equilibrate the column with at least two column volumes of Mobile phase at a flow rate of 0.4 mL per minute.

Procedure— Inject 100  $\mu$ L of Pretreatment solution, and allow the chromatography to continue for at least two column volumes. Repeat this twice before injecting 100  $\mu$ L of the Test solution. Absorbance is detected at 214 nm. Integrate the main peak from the Test solution run and all other peaks not present in the Pretreatment solution runs. Calculate the percentage of impurities in the portion of the rProtein A, C-Cys taken by the formula:

$$100(r_i / r_s)$$

in which  $r_i$  is the peak response for each impurity; and  $r_s$  is the sum of the responses of all the peaks: the sum of all impurities is not more than 5%; and the Test solution shows a major peak at approximately 35 minutes.

rPROTEIN A

C1917H3039N565O658S3

44.618

N-terminal Sequence FLRPVE

Protein A is a component of the cell wall of *Staphylococcus aureus*. Recombinant Protein A (rProtein A) consists of five homologous immunoglobulin (IgG) binding domains (E, D, A, B, C) followed by a partial X domain sequence. It is expressed in *Escherichia coli* and purified via a column chromatography process. IgG columns are not used in the purification process. It is manufactured as a bulk solution with an IgG-binding potency greater than 95%. Release testing methods and specifications are described below. Because rProtein A is used as an ancillary material in the manufacture of recombinant therapeutic drugs, regulatory requirements differ from those of therapeutic drug products.

Packaging and storage— Store in closed containers at the temperature indicated on the label.

Labeling— The labeling states that the material is of recombinant DNA origin along with the lot number, storage conditions, and the statement "Formulated in [Water for Injection](#)."

[USP Reference standards](#) (11) — [USP Endotoxin RS](#). [USP rProtein A RS](#).

Identification—

A: SDS-PAGE—

Molecular weight marker— Use a suitable molecular weight marker (MWM) containing protein bands between 20 and 200 kDa.

PBS solution— Prepare a solution that contains 8065.0 mg and 200.0 mg of sodium chloride and potassium chloride, respectively, per L of 0.01 M sodium phosphate buffer, pH 7.4.



Sample buffer<sup>2</sup>— Dissolve 0.666 g of tris-hydrochloride, 0.682 g of tris base, 0.800 g of lithium dodecyl sulfate (LDS), 0.006 g of ethylenedinitriol tetraacetic acid (EDTA), and 4 g glycerol in 8 mL of water; add 0.75 mL of 1% Coomassie brilliant blue G-250 (see Coomassie Blue G-250 in Reagents under Reagents, Indicators, and Solutions) solution and 0.25 mL of 1% phenol red solution. Mix well, and adjust the volume with water to 10 mL.

2X Sample buffer— Prepare a mixture of 4X Sample buffer and water (1:1).

1X Sample buffer— Prepare a mixture of 2X Sample buffer and water (1:1).

1 M Dithiothreitol solution— Dissolve 0.154 g of dl-dithiothreitol (DTT) in 1 mL of water.

2X Reducing sample buffer— Mix 180  $\mu$ L of 2X Sample buffer and 20  $\mu$ L of 1 M Dithiothreitol solution.

20X Running buffer<sup>3</sup>— Dissolve 104.6 g of 3-(N-morpholino)propanesulfonic acid (MOPS), 60.6 g of tris base, 10 g of sodium dodecyl sulfate (SDS), and 3.0 g of EDTA in 400 mL of water. Mix well, and adjust with water to 500 mL.

1X Running buffer— Prepare a solution of water and 20X Running buffer (19:1).

Gel staining solution— Prepare a solution of Coomassie brilliant blue R-250 (see Coomassie Brilliant Blue R-250 in Reagents under Reagents, Indicators, and Solutions) having a concentration of 0.5 g per L in a mixture of water, isopropanol, and acetic acid (6.5:2.5:1.0). Filter, and store at room temperature. Silver staining is not recommended.

Destaining solution— Mix 100 mL of acetic acid with 900 mL of water.

Standard preparation— Dilute [USP rProtein A RS](#) to 0.4 mg per mL with PBS solution. Further dilute this solution 1:1 with 2X Reducing sample buffer, and incubate in a closed tube for 5 minutes at 90°. Mix, and quick spin prior to loading.

Test preparation— Dilute rProtein A with PBS solution to 0.4 mg per mL. Proceed as directed under Standard preparation beginning with "Further dilute."

Comix solution— Dilute rProtein A and [USP rProtein A RS](#) with PBS solution to 0.8 mg per mL. This solution contains 0.4 mg per mL of each protein. Proceed as directed under Standard preparation beginning with "Further dilute."

SDS-PAGE gel and apparatus set-up— Assemble gel apparatus following the manufacturer's instructions. Lock the gel tension wedge in place, and fill approximately 200 mL of 1X Running buffer into the inside chamber. If there are no leaks, pour 600 mL of 1X Running buffer into the outer chamber. Gently pull the comb out of the cassette to immerse the wells in 1X Running buffer. Load 10  $\mu$ L of each preparation as directed below under Gel loading onto a 10% Bis-Tris SDS-PAGE gel.<sup>4</sup>

Gel loading— Use the following gel loading scheme when running one Test preparation (see [Table 1](#)). Each Test preparation is run by itself and as part of the Comix solution that contains the rProtein A and [USP rProtein A RS](#).

Table 1

Lane	Sample	Load Volume ( $\mu$ L)	Load Amount ( $\mu$ g)
1	1X Sample buffer	10	N/A
2	MWM	20	N/A
3	Test preparation #1	10	2
4	Comix solution #1	10	4 (total)
5	Test preparation #1	10	2
6	1X Sample buffer	10	N/A
7	Standard preparation	10	2
8	MWM	20	N/A
9	—	—	—
10	—	—	—

Running the gel— Set the voltage to 125 volts, and run at a constant voltage. Run the gels until the bromophenol blue band is approximately 5 mm from the bottom of the gel (approximately 120 to 140 minutes).

Gel staining— Pour approximately 100 mL of Gel staining solution into the staining container. Place the gel into the staining container, and allow the stain to completely cover the gel. Cook the gel and container in a microwave for 30 seconds. Place the staining container on an orbital shaker, and stain the gel for 1 hour with gentle shaking.

Destaining— Drain the Gel staining solution, and add enough Destaining solution to the container to cover the gel. Place the container on an orbital shaker, and shake at low speed. Change the Destaining solution as necessary until a clear background is obtained. After destaining, rinse the gel thoroughly with water, and leave the gel in water for 10 minutes before scanning.

Gel scanning— Apply some water to the glass plate of the scanner, and place the gels on a wetted glass plate. Eliminate any bubbles. Using appropriate settings, scan the gels.

Data analysis— Choose a band between the 20 kD and 30 kD bands of the MWM to calculate the percentage of the retention factor. Draw a line in one lane (lane containing 1X Sample buffer) from the well to the apex (region of greatest intensity) of the chosen band.

The length of this line is denoted as the total distance (DT). For the lanes containing samples draw a line from the well to the apex of each band. For each band the length of this distance is the migration distance (DM) in mm. Record the DT and DM on the report sheet for each peak or band. The total distance should be the same for each lane on a gel. Calculate the percentage of the retention factor (RF) of each major peak or band, and document on the report sheet using the following equation:

$$\%RF = DM / DT \times 100$$

Also for each gel, record the number of bands and approximate molecular weight of each band in each sample.

System suitability— All bands between 20 kD and 70 kD are present. The lane containing 1X Sample buffer does not contain any bands.

Specificity— The rProtein A has one major band and a similar molecular weight that corresponds to those of the [USP rProtein A RS](#). The Comix solution also shows a single major band.

B: IgG Binding—[note—The IgG binding assay is a functional method for determining the percentage of rProtein A capable of binding to immobilized human polyclonal immunoglobulin. Since the percent of functional rProtein A in each lot is not less than 95%, the assay measures unbound protein versus total protein injected. This is done by comparing the absorbance in the flow-through to absorbance from an injection bypassing the column.]

Sample pretreatment (desalting)— In order to remove any buffer components that may contribute to absorbance in the "unbound" IgG column fraction, samples are desalted with Solution A. Desalting may be performed using a suitable desalting column<sup>5</sup> depending on the volumes required.

IgG column— A 1-mL Sepharose column<sup>6</sup> with immobilized human polyclonal IgG (IgG) is required to perform this assay. [note—The IgG column requires washing when it is new, when it has performed several analysis cycles, or after system suitability failure. Column washing procedure is not required for each sample injection.]

Column washing solution A— Prepare a solution of 0.5 M acetic acid, pH 3.4 by adding 28.6 mL of acetic acid into a 1000-mL beaker, diluting to 900 mL with water, and adjusting with ammonium acetate to a pH of 3.4. Transfer the solution into a 1000-mL volumetric flask, and dilute with water to volume. Pass the solution through a 0.45- $\mu$ m membrane filter.

Column washing solution B— Prepare a solution of 50 mM Tris, pH 7.6, 150 mM sodium chloride, and 0.05% Tween 20 by the following procedure. Add 6.06  $\pm$  0.01 g of Tris and 8.77

0.01 g of sodium chloride into a 1000-mL beaker. Dilute with water to 900 mL, and adjust with 0.5 M sodium hydroxide to a pH of  $7.60 \pm 0.05$ . Transfer the solution into a 1000-mL volumetric flask, and dilute with water to volume. Pass the solution through a 0.45- $\mu\text{m}$  membrane filter (buffer solution). Add 0.5 mL of Tween 20 into 1 L of the buffer solution and mix thoroughly.

**Solution A**— Prepare a solution of 20 mM monobasic sodium phosphate and 150 mM sodium chloride, pH 7.6 by the following procedure. Add  $2.76 \pm 0.01$  g monobasic sodium phosphate hydrate and  $8.77 \pm 0.01$  g sodium chloride into a 1000-mL beaker. Dilute with water to 900 mL, and adjust with 5 M sodium hydroxide to a pH of  $7.60 \pm 0.05$ . Transfer the solution into a 1000-mL volumetric flask, and dilute with water to volume. Pass the solution through a 0.45- $\mu\text{m}$  membrane filter.

**Solution B**— Prepare a solution of 100 mM phosphoric acid pH 2.8 by the following procedure. Add 6.8 mL of phosphoric acid into a 1000-mL beaker. Dilute with water to 900 mL, and adjust with 2 M potassium hydroxide to a pH of  $2.80 \pm 0.05$ . Transfer the solution into a 1000-mL volumetric flask, and dilute with water to volume.

**Mobile phase**— Use variable mixtures of Solution A and Solution B as directed for Chromatographic system. Make adjustments if necessary (see System Suitability under [Chromatography \(621\)](#)).

**Standard preparation**— Thaw [USP rProtein A RS](#), and use directly.

**Test preparation**— Prepare a 4.0 to 6.0 mg per mL rProtein A solution in Solution A.

**Chromatographic system**— The liquid chromatograph is equipped with a 280-nm detector and a 1-mL column with immobilized hIgG. The chromatograph is equipped with a bypass valve to allow flow to be diverted from the column. Each analysis consists of a series of two injections, one where the sample is injected onto the column and one where the sample bypasses the column and flows directly into the detector. Perform three replicate analyses. The chromatograph is programmed as follows (see Table 2).

Table 2

Flow Rate (mL per minute)	Time (minutes)	Solution A (%)	Solution B (%)	Valve Position	Elution
1.0	0–6	100	0	column	re-equilibration
1.0	6–12	100→0	0→100	column	re-equilibration
1.0	12–22	100	0	column	equilibration
0.4	22–25	100	0	column	equilibration
0.4 (sample injected)	25–35	100	0	column	isocratic
1.0	35–49	100→0	0→100	column	regeneration
1.0	49–63	0→100	100→0	column	re-equilibration
1	63–65	100	0	bypass	equilibration
0.4	65–68	100	0	bypass	equilibration
0.4 (sample injected)	68–75	100	0	bypass	isocratic

Chromatograph the Standard preparation, record the peak responses, and calculate the percentage of hIgG binding as directed for Procedure: the percentage of hIgG binding  $\geq 95\%$  and the relative standard deviation for replicate analysis is not more than 1%.

**Procedure**— Inject a volume (about 100  $\mu\text{L}$ ) of the Test preparation. Record the chromatogram, and measure the peak responses. Calculate the percentage of hIgG binding activity by the following formula:

$$100 - 100(rC / rB)$$

in which rC is the unbound material peak response from the column injection and rB is the bypass peak response from the bypass injection. Each replicate analysis of the Test preparation is not less than 95% of hIgG binding. Report the average value from three replicate analyses.

[Microbial Enumeration Tests \(61\)](#) and [Tests for Specified Microorganisms \(62\)](#)— The total aerobic microbial count does not exceed 100 cfu per mL, and the total yeasts and molds count does not exceed 10 cfu per mL.

[Bacterial endotoxins \(85\)](#)— It contains not more than 0.5 USP Endotoxin Unit per mg of total protein. [note—The Bacterial endotoxins test for rProtein A is used to describe the quality of this ancillary material. This test does not define the acceptable level of bacterial endotoxin in the preparation of injectable dosage forms in which rProtein A is used.]

Total protein (see [Spectrophotometry and Light-Scattering \(851\)](#))— Prepare triplicate samples for analysis by diluting the rProtein A to 3.0 mg per mL in [Water for Injection](#). Measure the absorbance of each sample at 275 nm after correcting for the absorbance using [Water for Injection](#) as the blank. Determine the protein concentration using the equation:

$$\text{Protein concentration (mg per mL)} = (A275 / 0.165)$$

in which A is the absorbance of rProtein A at the wavelength of 275 nm and 0.165 is the molar absorptivity. Average the triplicate results, and determine a coefficient of variance (CV): the CV is  $\leq 5\%$ .

UV spectral analysis— Dilute rProtein A to 1 mg per mL in [Water for Injection](#). Using a scanning UV spectrophotometer and [Water for Injection](#) as the blank, obtain spectral scans over the range of 240 to 360 nm. From the resulting data, calculate the absorbance value at 270 nm and the ratio of absorbance at 270 to 250 nm (i.e., E270/E250): the absorbance at 270 nm of a 1 mg per mL solution of rProtein A in [Water for Injection](#) is within the range 0.14–0.20.

Chromatographic purity— [note—The size-exclusion chromatographic purity test resolves rProtein A from high molecular weight contaminants.]

Mobile phase— A solution of 0.3 M sodium phosphate, pH 7 is prepared by mixing monobasic and dibasic phosphate solutions in the following manner. Weigh  $21.3 \pm 0.1$  g of dibasic anhydrous sodium phosphate, and dissolve in 500 mL of water to obtain a 0.3 M dibasic sodium phosphate solution (Solution 1). Into a separate container, weigh  $18.0 \pm 0.1$  g monobasic anhydrous sodium phosphate, and dissolve in 500 mL of water to obtain a 0.3 M monobasic sodium phosphate solution (Solution 2). Calibrate a pH meter using pH calibrators at a pH of 7 and 10. Add 400 mL of Solution 1 to a 1-L beaker. Transfer the pH probe to the beaker. Slowly add Solution 2 to the solution until the pH is  $7.0 \pm 0.1$ . Pass the solution through a 0.45- $\mu\text{m}$  membrane filter.

Standard solution— Dilute [USP rProtein A RS](#) to 1 mg per mL in Mobile phase.

Test solution— Dilute rProtein A to 1 mg per mL in Mobile phase.

Chromatographic system (see [Chromatography \(621\)](#))— The liquid chromatograph is equipped with a 214-nm and 280-nm detector and a 9.4-mm  $\times$  25-cm column that contains packing L35. The flow rate is 1 mL per minute. Chromatograph the Standard solution as directed for Procedure: rProtein A shows a single major peak at approximately 9 minutes and the area percentage is  $\geq 98\%$  at 214 nm and  $\geq 95\%$  at 280 nm.

**Procedure**— Inject 100  $\mu\text{L}$  of the Test solution into the chromatograph, run isocratically for 15 minutes, and record the chromatogram. The values for the rProtein A from the Test solution correspond to the specifications of the [USP rProtein A RS](#) from the Standard solution.

Isoforms—

Standard solution— Thaw [USP rProtein A RS](#), and use directly.

Test solution— Dilute rProtein A to 4 mg per mL in [Water for Injection](#).

pl Markers— Use a suitable marker set containing markers between 3 and 10.<sup>7</sup>



IEF gel— Use a suitable gel with the range of between 3 and 10 and a size of  $100 \times 125 \text{ mm}$ .<sup>8</sup>

Procedure— Apply 5- $\mu\text{L}$  aliquots of the *pl* Markers, Test solution, and the Standard solution to the IEF gel, and run under 1W of power for approximately 10 minutes. Remove the sample mask, and apply power with concurrent cooling between  $5^{\circ}$  to  $10^{\circ}$  of the focusing chamber for 40 minutes at a setting of 1000V, 20 mA, 25W. Fix the IEF gel for 1 hour in 20% trichloroacetic acid, then stain using a suitable stain for IEF gels.<sup>9</sup> Finally, wash and dry the gel: the correlation coefficient of the best fit line for the *pl* Markers versus their migration in cm is  $\geq 0.990$ , and the rProtein A from the Standard solution shows a single major band within the *pl* range of 4.6 to 5.2. A single band is seen in the Test solution that corresponds to the *pl* range of the Standard solution.

#### Limit of Triton X-100—

Mobile phase— Prepare a filtered and degassed mixture of water and acetonitrile (60:40).

Test solution— Dilute rProtein A to 5 mg per mL in [Water for Injection](#).

Triton X-100 spike solution— Combine 5 mg per mL of [USP rProtein A RS](#) and 0.15% Triton X-100 (9:1) to obtain a solution having known concentrations of rProtein A and 0.015% Triton X-100.

Chromatographic system (see [Chromatography](#))— The liquid chromatograph is equipped with a 214-nm and a 280-nm detector and a 4.6-mm  $\times$  25-cm column that contains 5- $\mu\text{m}$  packing L11. The flow rate is 1 mL per minute. Chromatograph the Triton X-100 spike solution as directed for Procedure: Triton X-100 has a single major peak at approximately 9 minutes, and the rProtein A shows a smaller or undetectable peak at the same retention time.

Procedure— Inject about 100  $\mu\text{L}$  of the Test solution into the chromatograph, run isocratically for 35 minutes, and record the chromatogram. The absorbance is detected at 223 nm: the Triton X-100 peak is not more than 0.015% (equivalent to the Triton X-100 spike solution).

rPROTEIN A, B<sub>4</sub>, C-CYS

C1177H1854N326O384S1

26747.6 Da

N-terminal Sequence AQGTVDAKFD

rProtein A, B<sub>4</sub>, C-Cys is a recombinant protein derived from the B-domain of Protein A. The Protein A domain has been alkali-stabilized by site-specific mutagenesis and multimerized to a tetramer with a C-terminal cysteine for directed immobilization purposes. rProtein A, B<sub>4</sub>, C-Cys is produced using *Escherichia coli* as the host cell followed by purification with conventional chromatography. rProtein A, B<sub>4</sub>, C-Cys is manufactured as a bulk solution with an IgG-binding potency of greater than 95%. Because rProtein A, B<sub>4</sub>, C-Cys is used as an ancillary material in the manufacture of recombinant therapeutic drugs, regulatory requirements differ from those for therapeutic drug products.

Packaging and storage— Store in closed containers at the temperature indicated on the label.

Labeling— Preserve in sealed containers, and store at a temperature of  $-20^{\circ}$  or below.

[USP Reference standards](#)— [USP Endotoxin RS](#). USP rProtein A, B<sub>4</sub>, C-Cys RS.

#### Identification—

A: SDS-PAGE—It meets the requirements of Identification test A under rProtein A using USP rProtein A, B<sub>4</sub>, C-Cys RS.

B: IgG Binding—It meets the requirements of Identification test B under rProtein A using USP rProtein A, B<sub>4</sub>, C-Cys RS.

[Microbial Enumeration Tests](#) and [Tests for Specified Microorganisms](#)— The total aerobic microbial count does not exceed 100 cfu per mL, and the total yeasts and molds count does not exceed 10 cfu per mL.

[Bacterial endotoxins](#)— It contains not more than 1 USP Endotoxin Unit per mg of total protein. [note—The Bacterial endotoxins test for rProtein A, B<sub>4</sub>, C-Cys is used to describe the quality of this ancillary material. This test does not define the acceptable level of bacterial endotoxin in the preparation of injectable dosage forms in which rProtein A, B<sub>4</sub>, C-Cys is used.]

#### Total protein (see [Spectrophotometry and Light-Scattering](#))—

Formulation buffer solution— Prepare a solution of 0.02 M potassium phosphate, pH 7.0, containing 0.15 M potassium chloride and 2 mM of ethylenediaminetetraacetic acid (EDTA) in the following manner. Add  $2.72 \pm 0.01$  g of monobasic potassium phosphate anhydrous,  $11.18 \text{ g} \pm 0.22$  g of potassium chloride, and  $0.744 \pm 0.02$  g of EDTA into a 1000-mL beaker. Dilute with water to 900 mL, and adjust with 1 M sodium hydroxide to a pH of  $7.00 \pm 0.05$ . Transfer the solution into a 1000-mL volumetric flask, and dilute with water to volume. Pass the solution through a 0.45- $\mu\text{m}$  membrane filter.

Test preparation— Dilute the rProtein A, B<sub>4</sub>, C-Cys to 3.0 mg per mL with Formulation buffer solution.

Procedure— Prepare triplicate samples for analysis. Measure the absorbance of each Test preparation at 275 nm after correcting for the absorbance using the Formulation buffer solution as the blank. Determine the protein concentration using the equation:

$$\text{Protein concentration (mg per mL)} = (A275 / 0.22)$$

in which A is the absorbance of rProtein A, B<sub>4</sub>, C-Cys, at the wavelength of 275 nm and 0.22 is the molar absorptivity. Average the triplicate results, and determine a coefficient of variance (CV): the CV  $\leq 2.5\%$ .

Chromatographic purity— [note—The size-exclusion chromatographic purity test resolves rProtein A, B<sub>4</sub>, C-Cys from high molecular weight contaminants and low molecular weight contaminants.]

Mobile phase— Prepare a solution of 0.02 M sodium phosphate, pH 7.2 containing 0.15 M sodium chloride in the following manner. Add  $0.96 \pm 0.02$  g of monobasic sodium phosphate hydrate,  $2.32 \pm 0.02$  g of dibasic sodium phosphate dihydrate, and  $8.76 \text{ g} \pm 0.02$  g of sodium chloride into a 1000-mL beaker. Dilute with water to 900 mL, and adjust with 1 M sodium hydroxide to a pH of  $7.2 \pm 0.05$ . Transfer this solution into a 1000-mL volumetric flask, and dilute with water to volume. Pass the solution through a 0.45- $\mu\text{m}$  membrane filter.

EDTA solution— Prepare a 20 mM EDTA solution by dissolving  $0.74 \pm 0.02$  g of EDTA in 100 mL of Mobile phase.

DTT solution— Prepare a 100 mM di-dithiothreitol (DTT) solution by dissolving  $1.54 \pm 0.02$  g of DTT in 100 mL of Mobile phase.

Pretreatment solution— Prepare a solution containing a mixture of EDTA solution and DTT solution (1:1, v/v). [note—Prepare fresh just before use.]

Test solution— Dilute rProtein A, B<sub>4</sub>, C-Cys 1 to 5 in Pretreatment solution, and mix gently. Incubate the sample at  $40^{\circ}$  for 60 minutes.

Chromatographic system (see [Chromatography](#))— The liquid chromatograph is equipped with a 214-nm detector and a 10-mm  $\times$  30-cm column that contains packing L54. Equilibrate the column with at least two column volumes of Mobile phase at a flow rate of 0.4 mL per minute.

Procedure— Inject 100  $\mu\text{L}$  of Pretreatment solution, and allow the chromatography to continue for at least two column volumes. Repeat this twice before injecting 100  $\mu\text{L}$  of the Test solution. Absorbance is detected at 214 nm. Integrate the main peak from the Test solution run and all other peaks not present in the Pretreatment solution runs. Calculate the percentage of impurities in the portion of rProtein A, B<sub>4</sub>, C-Cys taken by the formula:

$$100(r_i / r_s)$$

in which  $r_i$  is the peak response for each impurity; and  $r_s$  is the sum of all the responses of all the peaks: the sum of all impurities is not more than 5%; and the Test solution shows a major peak at approximately 37 minutes.



1 A suitable enzyme immunoassay kit is available from TECRA International Pty Ltd., Australia (No. SETVIA96).

2 4X NuPAGE LDS sample buffer is available from Invitrogen (No. NP0007).

3 20X NuPAGE MOPS SDS Running Buffer is available from Invitrogen (No. NP0001).

4 10% Bis-Tris SDS-PAGE gel is available from Invitrogen (No. NP0301).

5 Zeba columns are available from Pierce; Nap-10 columns are available from GE Healthcare.

6 HiTrap IgG Sepharose 6 FF column is available from GE Healthcare (No. 90-1003-97).

7 pI markers in the 3–10 range are available from BioRad (No. 161-0310).

8 IEF gels in the 3–10 range are available from Cambrex (No. 56015).

9 ISS Pro-Blue is available from Integrated Separation Systems.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Anita Y. Sajek, Ph.D.</a> Senior Scientist 1-301-816-8325	(BBPP05) Biologics and Biotechnology - Proteins and Polysaccharides
Reference Standards	Lili Wang, Technical Services Scientist 1-301-816-8129 <a href="mailto:RSTech@usp.org">RSTech@usp.org</a>	
61	<a href="#">Radhakrishna S Tirumalai, Ph.D.</a> Senior Scientist 1-301-816-8339	(MSA05) Microbiology and Sterility Assurance
62	<a href="#">Radhakrishna S Tirumalai, Ph.D.</a> Senior Scientist 1-301-816-8339	(MSA05) Microbiology and Sterility Assurance
85	<a href="#">Radhakrishna S Tirumalai, Ph.D.</a> Senior Scientist 1-301-816-8339	(MSA05) Microbiology and Sterility Assurance

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#### 141 PROTEIN—BIOLOGICAL ADEQUACY TEST

This test is intended for the evaluation of the biological adequacy, as an index to the completeness of the mixture of amino acids contained, of Protein Hydrolysate Injection.

##### Depletion Diet—

	Parts by Weight
Dextrin	83.9
Corn Oil	9.0
Salt Mixture	4.0
Agar	2.0
Cod Liver Oil	1.0
Choline Chloride	0.15
Inositol	0.10
Calcium Pantothenate	0.002
Niacinamide	0.0015
Riboflavin	0.0003
Pyridoxine	0.00025
Thiamine	0.0002
p-Aminobenzoic Acid	0.0002
Folic Acid	0.0002
Menadione	0.0002
Biotin	0.00002

Salt Mixture— Prepare the salt mixture specified in the Depletion Diet as follows:

Sodium Chloride	139.3 g
Potassium Biphosphate	389.0 g
Magnesium Sulfate, Anhydrous	57.3 g
Calcium Carbonate	381.4 g
Ferrous Sulfate	27.0 g
Manganese Sulfate	4.01 g
Potassium Iodide	0.79 g
Zinc Sulfate	0.548 g
Cupric Sulfate	0.477 g
Cobaltous Chloride	0.023 g

Place a portion of the weighed quantity of sodium chloride in a suitable mortar and add, with grinding, the potassium iodide. Set aside the mixture, and mix in a similar manner all the other salts with the remainder of the sodium chloride, adding finally the previously mixed sodium chloride and potassium iodide. Reduce the entire mixture to a fine powder (see

[Powder Fineness](#) 811).

Control Nitrogen Supplement Mixture— Place 50 g of calcium caseinate and 46 g of anhydrous dextrose in a beaker, add sufficient water to make a paste, and finally add 1000 mL of



water. Heat the solution between 70° and 82° for 5 minutes with stirring, and cool. Determine nitrogen on an aliquot using [Nitrogen Determination—Method I](#) or [Method II](#) (461). Store in a refrigerator. Mix before removing portions for analysis or use.

**Depletion and Control Periods**— Select a group of not less than six male rats 2 to 4 months of age and each weighing between 190 g and 225 g. Place the rats in individual cages with free access to water and the Depletion Diet for 12 days. Weigh the depleted rats, and discard any rat that weighs more than 90% of its starting weight.

For the next 3 days substitute as drinking water the Control Nitrogen Supplement Mixture in a quantity equivalent to 0.12 g of nitrogen per rat per day, diluted with water to 20 mL, and offered at the same time each morning either in a dish suitable for preventing spillage or in a reservoir fitted with a drinking tube. Remove all drinking water from the cages of the depleted rats during each feeding, and return it after the supplement has been consumed or is removed. On the third day, weigh each rat. Discard any rats that have not consumed all of the Control Nitrogen Supplement Mixture.

For the next 3 days, replace the Control Nitrogen Supplement Mixture with water ad libitum, and continue the rats on the Depletion Diet. Weigh the rats, and discard any that have not lost weight since the previous weighing.

**Procedure**— Assemble not less than six rats that have completed the depletion and control periods. For 5 days maintain the assembled rats on the Depletion Diet with a daily supplement of 20 mL, accurately measured, of a solution containing the Protein Hydrolysate Injection in an amount equivalent to 0.12 g of nitrogen offered each morning in the same way as the Control Nitrogen Supplement Mixture was offered previously. Withhold water for at least 2 hours prior to offering the supplement and for 4 hours afterward. Then if the supplement has been consumed, offer water ad libitum.

On the afternoon of the fifth day, weigh each rat, and compare the respective final and starting weights. Not fewer than 80% of the group of rats used gain weight or maintain their weight during the test.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Larry N. Callahan, Ph.D.</a> Senior Scientist 1-301-816-8385	(BBPP05) Biologics and Biotechnology - Proteins and Polysaccharides

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### 151 PYROGEN TEST

The pyrogen test is designed to limit to an acceptable level the risks of febrile reaction in the patient to the administration, by injection, of the product concerned. The test involves measuring the rise in temperature of rabbits following the intravenous injection of a test solution and is designed for products that can be tolerated by the test rabbit in a dose not to exceed 10 mL per kg injected intravenously within a period of not more than 10 minutes. For products that require preliminary preparation or are subject to special conditions of administration, follow the additional directions given in the individual monograph or, in the case of antibiotics or biologics, the additional directions given in the federal regulations (see [Biologics](#) (1041)).

#### APPARATUS AND DILUENTS

Render the syringes, needles, and glassware free from pyrogens by heating at 250° for not less than 30 minutes or by any other suitable method. Treat all diluents and solutions for washing and rinsing of devices or parenteral injection assemblies in a manner that will assure that they are sterile and pyrogen-free. Periodically perform control pyrogen tests on representative portions of the diluents and solutions for washing or rinsing of the apparatus. Where Sodium Chloride Injection is specified as a diluent, use Injection containing 0.9 percent of NaCl.

#### TEMPERATURE RECORDING

Use an accurate temperature-sensing device such as a clinical thermometer, or thermistor probes or similar probes that have been calibrated to assure an accuracy of  $\pm 0.1^{\circ}$  and have been tested to determine that a maximum reading is reached in less than 5 minutes. Insert the temperature-sensing probe into the rectum of the test rabbit to a depth of not less than 7.5 cm, and, after a period of time not less than that previously determined as sufficient, record the rabbit's body temperature.

#### TEST ANIMALS

Use healthy, mature rabbits. House the rabbits individually in an area of uniform temperature between 20° and 23° and free from disturbances likely to excite them. The temperature varies not more than  $\pm 3^{\circ}$  from the selected temperature. Before using a rabbit for the first time in a pyrogen test, condition it not more than seven days before use by a sham test that includes all of the steps as directed for Procedure except injection. Do not use a rabbit for pyrogen testing more frequently than once every 48 hours, nor prior to 2 weeks following a maximum rise of its temperature of 0.6° or more while being subjected to the pyrogen test, or following its having been given a test specimen that was adjudged pyrogenic.

#### PROCEDURE

Perform the test in a separate area designated solely for pyrogen testing and under environmental conditions similar to those under which the animals are housed and free from disturbances likely to excite them. Withhold all food from the rabbits used during the period of the test. Access to water is allowed at all times, but may be restricted during the test. If rectal temperature-measuring probes remain inserted throughout the testing period, restrain the rabbits with light-fitting neck stocks that allow the rabbits to assume a natural resting posture. Not more than 30 minutes prior to the injection of the test dose, determine the "control temperature" of each rabbit: this is the base for the determination of any temperature increase resulting from the injection of a test solution. In any one group of test rabbits, use only those rabbits whose control temperatures do not vary by more than 1° from each other, and do not use any rabbit having a temperature exceeding 39.8°.

Unless otherwise specified in the individual monograph, inject into an ear vein of each of three rabbits 10 mL of the test solution per kg of body weight, completing each injection within 10 minutes after start of administration. The test solution is either the product, constituted if necessary as directed in the labeling, or the material under test treated as directed in the individual monograph and injected in the dose specified therein. For pyrogen testing of devices or injection assemblies, use washings or rinsings of the surfaces that come in contact with the parenterally administered material or with the injection site or internal tissues of the patient. Assure that all test solutions are protected from contamination. Perform the injection after warming the test solution to a temperature of  $37 \pm 2^{\circ}$ . Record the temperature at 30-minute intervals between 1 and 3 hours subsequent to the injection.

#### TEST INTERPRETATION AND CONTINUATION

Consider any temperature decreases as zero rise. If no rabbit shows an individual rise in temperature of 0.5° or more above its respective control temperature, the product meets the requirements for the absence of pyrogens. If any rabbit shows an individual temperature rise of 0.5° or more, continue the test using five other rabbits. If not more than three of the eight rabbits show individual rises in temperature of 0.5° or more and if the sum of the eight individual maximum temperature rises does not exceed 3.3°, the material under examination meets the requirements for the absence of pyrogens.



## RADIOACTIVE PHARMACEUTICALS

Test Dose for Preformulated, Ready-to-Use Products Labeled with Radioactivity  
aggregated albumin and other particle-containing products

For the rabbit pyrogen test, dilute the product with Sodium Chloride Injection to not less than 100  $\mu$ Ci per mL, and inject a dose of 3 mL per kg of body weight into each rabbit.

other products

Where Physical Half-life of Radionuclide Is Greater Than 1 Day— Calculate the maximum volume of the product that might be injected into a human subject. This calculation takes into account the maximum recommended radioactive dose of the product, in  $\mu$ Ci, and the radioactive assay, in  $\mu$ Ci per mL, of the product at its expiration date or time. Using this information, calculate the maximum volume dose per kg to a 70-kg human subject.

For the rabbit pyrogen test, inject a minimum of 10 times this dose per kg of body weight into each rabbit. If necessary, dilute with Sodium Chloride Injection. The total injected volume per rabbit is not less than 1 mL and not more than 10 mL of solution.

Where Physical Half-life of Radionuclide is Less Than 1 Day— For products labeled with radionuclides having a half-life of less than 1 day, the dosage calculations are identical to those described in the first paragraph under Other Products. These products may be released for distribution prior to completion of the rabbit pyrogen test, but such test shall be initiated at not more than 36 hours after release.

## Test Dose for Pharmaceutical Constituents or Reagents to Be Labeled

The following test dose requirements pertain to reagents that are to be labeled or constituted prior to use by the direct addition of radioactive solutions such as Sodium Pertechnetate Tc 99m Injection, i.e., "cold kits".

Assume that the entire contents of the vial of nonradioactive reagent will be injected into a 70-kg human subject, or that 1/70 of the total contents per kg will be injected. If the contents are dry, constitute with a measured volume of Sodium Chloride Injection.

For the rabbit pyrogen test, inject (1/7) of the vial contents per kg of body weight into each rabbit. The maximum dose per rabbit is the entire contents of a single vial. The total injected volume per rabbit is not less than 1 mL and not more than 10 mL of solution.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Radhakrishna S Tirumalai, Ph.D.</a> Senior Scientist 1-301-816-8339	(MSA05) Microbiology and Sterility Assurance

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## 161 TRANSFUSION AND INFUSION ASSEMBLIES AND SIMILAR MEDICAL DEVICES

The requirements apply to sterile and nonpyrogenic assemblies or devices in contact directly or indirectly with the cardiovascular system, the lymphatic system, or cerebrospinal fluid. This includes, but is not limited to, solution administration sets, extension sets, transfer sets, blood administration sets, intravenous catheters, implants extracorporeal oxygenator tubings and accessories, dialysers and dialysis tubing and accessories, heart valves, vascular grafts, intramuscular drug delivery catheters, and transfusion and infusion assemblies.

These requirements do not apply to orthopedic products, latex gloves, or wound dressings.

Sterility— Proceed as directed for Sterilized Devices under [Sterility Tests](#) 71.

Bacterial Endotoxins— Proceed as directed under [Bacterial Endotoxins Test](#) 85.

For medical devices, the endotoxin limit is not more than 20.0 USP Endotoxin Units per device except that for those medical devices in contact with the cerebrospinal fluid the limit is not more than 2.15 USP Endotoxin Units per device.

A device that fails this test can be retested once by another Bacterial Endotoxins test. For devices that cannot be tested by the [Bacterial Endotoxins Test](#) 85 because of nonremovable inhibition or enhancement, the [Pyrogen Test](#) 151 is applied.

Preparation of Devices— Select not less than 3 and not more than 10 devices. Rinse or soak the devices with LAL Reagent Water. The volume of rinsing or extracting solution may be adjusted for the size and configuration of the device.

For devices labeled "nonpyrogenic fluid pathway," flush the fluid pathway with extracting fluid that has been heated to  $37 \pm 1.0^{\circ}$ , keeping the extracting fluid in contact with the relevant pathway for not less than 1 hour at controlled room temperature. Extracts may be combined, where appropriate. The endotoxin limit for the rinsing or extracting solution is calculated by the formula:

$$(K \times N) / (V)$$

where K is equal to the amount of endotoxin allowed per device, N is equal to the number of devices tested, and V is equal to the total volume of the extract or rinse. If the undiluted rinsing or extracting solution is unsuitable for the [Bacterial Endotoxins Test](#) 85, repeat the inhibition or enhancement test after neutralization and removal of the interfering substances or after the solution has been diluted by a factor not exceeding the Maximum Valid Dilution. The Maximum Valid Dilution for devices is calculated by dividing the endotoxin limit by the labeled sensitivity  $\lambda$  of the LAL reagent used.

Pyrogen— For samples that cannot be tested by the Bacterial Endotoxins Test because of nonremovable inhibition or enhancement of the test, the [Pyrogen Test](#) 151 is applied. Select 10 devices, and obtain a pooled effluent, utilizing preparation methods appropriate to the device as directed for Bacterial Endotoxins, but with volumes of rinse or extraction fluid not to exceed 40 mL of sterile saline TS per device. The requirements of the [Pyrogen Test](#) 151 are met.

Other Requirements— The portions of medical devices that are made of plastics or other polymers meet the requirements specified for Biological Tests—Plastics and Other Polymers under [Containers—Plastics](#) 661; those made of elastomers meet the requirements under [Elastomeric Closures for Injections](#) 381. If a class designation for elastomers, plastics, or other polymers is needed, perform the appropriate in vivo tests indicated in the general test chapter [Biological Reactivity Tests, In Vivo](#) 88.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Radhakrishna S Tirumalai, Ph.D.</a> Senior Scientist 1-301-816-8339	(GTMDB05) General Toxicology and Medical Device Biocompatibility

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## 171 VITAMIN B12 ACTIVITY ASSAY

USP Reference Standards 11 — [USP Cyanocobalamin RS](#).

Assay Preparation— Place a suitable quantity of the material to be assayed, previously reduced to a fine powder if necessary and accurately measured or weighed, in an appropriate vessel containing, for each g or mL of material taken, 25 mL of an aqueous extracting solution prepared just prior to use to contain, in each 100 mL, 1.29 g of disodium phosphate, 1.1

g of anhydrous citric acid, and 1.0 g of sodium metabisulfite. Autoclave the mixture at 121° for 10 minutes. Allow any undissolved particles of the extract to settle, and filter or centrifuge, if necessary. Dilute an aliquot of the clear solution with water so that the final test solution contains vitamin B12 activity approximately equivalent to that of the Standard Cyanocobalamin Solution which is added to the assay tubes.

Standard Cyanocobalamin Stock Solution— To a suitable quantity of [USP Cyanocobalamin RS](#), accurately weighed, add sufficient 25 percent alcohol to make a solution having a known concentration of 1.0 µg of cyanocobalamin per mL. Store in a refrigerator.

Standard Cyanocobalamin Solution— Dilute a suitable volume of Standard Cyanocobalamin Stock Solution with water to a measured volume such that after the incubation period as described for Procedure, the difference in transmittance between the inoculated blank and the 5.0-mL level of the Standard Cyanocobalamin Solution is not less than that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 ng and 0.04 ng per mL of Standard Cyanocobalamin Solution. Prepare a fresh standard solution for each assay.

Basal Medium Stock Solution— Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed, carefully dissolving the cystine and tryptophane in the hydrochloric acid before adding the next eight solutions in the resulting solution. Add 100 mL of water, mix, and dissolve the dextrose, sodium acetate, and ascorbic acid. Filter, if necessary, add the polysorbate 80 solution, adjust the solution to a pH between 5.5 and 6.0 with 1 N sodium hydroxide, and add purified water to make 250 mL.

I-Cystine	0.1 g
I-Tryptophane	0.05 g
1 N Hydrochloric Acid	10 mL
Adenine-Guanine-Uracil Solution	5 mL
Xanthine Solution	5 mL
Vitamin Solution I	10 mL
Vitamin Solution II	10 mL
Salt Solution A	5 mL
Salt Solution B	5 mL
Asparagine Solution	5 mL
Acid-hydrolyzed Casein Solution	25 mL
Dextrose, Anhydrous	10 g
Sodium Acetate, Anhydrous	5 g
Ascorbic Acid	1 g
Polysorbate 80 Solution	5 mL

Acid-Hydrolyzed Casein Solution— Prepare as directed under [Calcium Pantothenate Assay](#) 91.

Asparagine Solution— Dissolve 2.0 g of l-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine—Guanine—Uracil Solution— Prepare as directed under [Calcium Pantothenate Assay](#) 91.

Xanthine Solution— Suspend 0.20 g of xanthine in 30 mL to 40 mL of water, heat to about 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Salt Solution A— Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL. Add 2 drops of hydrochloric acid, and store under toluene.

Salt Solution B— Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL. Add 2 drops of hydrochloric acid, and store under toluene.

Polysorbate 80 Solution— Dissolve 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

Vitamin Solution I— Dissolve 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 µg of biotin, and 20 mg of niacin in 0.02 N glacial acetic acid to make 400 mL. Store, protected from light, under toluene in a refrigerator.

Vitamin Solution II— Dissolve 20 mg of para-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in dilute neutralized alcohol (1 in 4) to make 400 mL. Store, protected from light, in a refrigerator.

Tomato Juice Preparation— Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend about 5 g per L of analytical filter-aid in the supernatant, and filter, with the aid of reduced pressure, through a layer of the filter-aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture Medium— [note—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.] Dissolve 0.75 g of water-soluble yeast extract, 0.75 g of dried peptone, 1.0 g of anhydrous dextrose, and 0.20 g of potassium biphosphate in 60 mL to 70 mL of water. Add 10 mL of Tomato Juice Preparation and 1 mL of Polysorbate 80 Solution. Adjust the solution with 1 N sodium hydroxide to a pH of 6.8, and add water to make 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 minutes. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension Medium— Dilute a measured volume of Basal Medium Stock Solution with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed above for the Culture Medium.

Stock Culture of *Lactobacillus leichmannii*— To 100 mL of Culture Medium add 1.0 g to 1.5 g of agar, and heat the mixture, with stirring, on a steam bath, until the agar dissolves. Place approximately 10-mL portions of the hot solution in test tubes, cover the tubes suitably, sterilize at 121° for 15 minutes in an autoclave (exhaust line temperature), and allow the



uses to cool in an upright position. Inoculate three or more of the tubes, by stab transfer of a pure culture of *Lactobacillus leichmannii*.\* (Before first using a fresh culture in this assay, make not fewer than 10 successive transfers of the culture in a 2-week period.) Incubate 16 to 24 hours at any selected temperature between 30° and 40° but held constant to within  $\pm 0.5^{\circ}$ , and finally store in a refrigerator.

Prepare fresh stab cultures at least three times each week, and do not use them for preparing the inoculum if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture, to the point where definite turbidity in the liquid inoculum can be observed 2 to 4 hours after inoculation. A slow-growing culture seldom gives a suitable response curve, and may lead to erratic results.

**Inoculum**— [note—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an inoculum comparable to a fresh culture.] Make a transfer of cells from the Stock Culture of *Lactobacillus leichmannii* to 2 sterile tubes containing 10 mL of the Culture Medium each. Incubate these cultures for 16 to 24 hours at any selected temperature between 30° and 40° but held constant to within  $\pm 0.5^{\circ}$ . Under aseptic conditions, centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of sterile Suspension Medium, and combine. Using sterile Suspension Medium, adjust the volume so that a 1 in 20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1 in 400 dilution of the adjusted suspension using Basal Medium Stock Solution, and use it for the test inoculum. (This dilution may be altered, when necessary, to obtain the desired test response.)

**Calibration of Spectrophotometer**— Check the wavelength of the spectrophotometer periodically, using a standard wavelength cell or other suitable device. Before reading any tests, calibrate the spectrophotometer for 0% and 100% transmittance, using water and with the wavelength set at 530 nm.

**Procedure**— Cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 hours, hard-glass test tubes, about 20 mm  $\times$  150 mm in size, and other necessary glassware because of the high sensitivity of the test organism to minute amounts of vitamin B12 activity and to traces of many cleansing agents.

To test tubes add, in duplicate, 1.0 mL, 1.5 mL, 2.0 mL, 3.0 mL, 4.0 mL, and 5.0 mL, respectively, of the Standard Cyanocobalamin Solution. To each of these tubes and to four similar empty tubes add 5.0 mL of Basal Medium Stock Solution and water to make 10 mL.

To similar test tubes add, in duplicate, respectively, 1.0 mL, 1.5 mL, 2.0 mL, 3.0 mL, and 4.0 mL of the Assay Preparation. To each tube add 5.0 mL of Basal Medium Stock Solution and water to make 10 mL. Place one complete set of standard and assay tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes suitably to prevent bacterial contamination, and sterilize the tubes and contents in an autoclave at 121° for 5 minutes, arranging to reach this temperature in not more than 10 minutes by preheating the autoclave, if necessary. Cool as rapidly as practicable to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, since packing tubes too closely in the autoclave, or overloading it, may cause variation in the heating rate.

Aseptically add 0.5 mL of Inoculum to each tube so prepared, except two of the four containing no Standard Cyanocobalamin Solution (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40° held constant to within  $\pm 0.5^{\circ}$ , for 16 to 24 hours.

Terminate growth by heating to a temperature not lower than 80° for 5 minutes. Cool to room temperature. After agitating its contents, place the container in a spectrophotometer that has been set at a wavelength of 530 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 seconds or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5% or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

**Calculation**— Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the standard, calculate the response from the sum of the duplicate values of the transmittances ( $\Sigma$ ) as the difference,  $y = 2.00 - \Sigma$ . Plot this response on the ordinate of cross-section paper against the logarithm of the mL of Standard Cyanocobalamin Solution per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response,  $y$ , adding together the two transmittances for each level of the Assay Preparation. Read from the standard curve the logarithm of the volume of the Standard Preparation corresponding to each of those values of  $y$  that falls within the range of the lowest and highest points plotted for the standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the Assay Preparation to obtain the difference,  $x$ , for each dosage level. Average the values of  $x$  for each of three or more dosage levels to obtain  $x = M'$ , the log-relative potency of the Assay Preparation. Determine the quantity, in  $\mu\text{g}$ , of [USP Cyanocobalamin RS](#) corresponding to the cyanocobalamin in the portion of material taken for assay by the equation  $\text{antilog } M = \text{antilog } (M' + \log R)$ , in which  $R$  is the number of  $\mu\text{g}$  of cyanocobalamin that was assumed to be present in each mg (or capsule or tablet) of the material taken for assay.

**Replication**— Repeat the entire determination at least once, using separately prepared Assay Preparations. If the difference between the two log potencies  $M$  is not greater than 0.08, their mean,  $M$ , is the assayed log-potency of the test material (see Vitamin B12 Activity Assay under [Design and Analysis of Biological Assays](#) (111)). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of  $M$  that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

\* Pure cultures of *Lactobacillus leichmannii* may be obtained as No. 7830 from the American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Curtis Phinney</a> 1-301-816-8540	(DSN05) Dietary Supplements - Non-Botanicals
Reference Standards	Lili Wang, Technical Services Scientist 1-301-816-8129 <a href="mailto:RSTech@usp.org">RSTech@usp.org</a>	

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### 181 IDENTIFICATION—ORGANIC NITROGENOUS BASES

This test is for the identification of tertiary amine compounds.

Dissolve 50 mg of the substance under test, if in bulk, in 25 mL of 0.01 N hydrochloric acid, or shake a quantity of powdered tablets or the contents of capsules equivalent to 50 mg of the substance with 25 mL of 0.01 N hydrochloric acid for 10 minutes. Transfer the liquid to a separator, if necessary filtering it and washing the filter and the residue with several small



solutions of water. In a second separator dissolve 50 mg of the corresponding USP Reference Standard in 25 mL of 0.01 N hydrochloric acid. Treat each solution as follows. Add 2 mL of 1 N sodium hydroxide and 4 mL of carbon disulfide, and shake for 2 minutes. Centrifuge if necessary to clarify the lower phase, and filter it through a dry filter, collecting the filtrate in a small flask provided with a glass stopper.

Determine the absorption spectra of the filtered solutions of both standard and sample without delay, in 1-mm cells between 7  $\mu$ m and 15  $\mu$ m, with a suitable IR spectrophotometer, using carbon disulfide in a matched cell as the blank. The spectrum of the solution prepared from the sample shows all of the significant absorption bands present in the spectrum of the solution prepared from the Reference Standard.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Antonio Hernandez-Cardoso, B.S.</a> Scientist, Latin American Specialist 1-301-816-8308	(GC05) General Chapters 05

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## 191 IDENTIFICATION TESTS—GENERAL

Under this heading are placed tests that are frequently referred to in the Pharmacopeia for the identification of official articles. [note—The tests are not intended to be applicable to mixtures of substances unless so specified.]

Acetate— When acetic acid or an acetate is warmed with sulfuric acid and alcohol, ethyl acetate, recognizable by its characteristic odor, is evolved. With neutral solutions of acetates, ferric chloride TS produces a deep red color that is destroyed by the addition of mineral acids.

Aluminum— With 6 N ammonium hydroxide, solutions of aluminum salts yield a gelatinous, white precipitate that is insoluble in an excess of 6 N ammonium hydroxide. 1 N sodium hydroxide or sodium sulfide TS produces the same precipitate, which dissolves in an excess of either of these reagents.

Ammonium— Ammonium salts are decomposed by the addition of an excess of 1 N sodium hydroxide, with the evolution of ammonia, recognizable by its odor and by its alkaline effect upon moistened red litmus paper exposed to the vapor. Warming the solution accelerates the decomposition.

Antimony— With hydrogen sulfide, solutions of antimony (III) compounds, strongly acidified with hydrochloric acid, yield an orange precipitate of antimony sulfide that is insoluble in 6 N ammonium hydroxide, but is soluble in [ammonium sulfide TS](#).

Barium— Solutions of barium salts yield a white precipitate with 2 N sulfuric acid. This precipitate is insoluble in hydrochloric acid and in nitric acid. Barium salts impart a yellowish-green color to a nonluminous flame that appears blue when viewed through green glass.

Benzoate— In neutral solutions, benzoates yield a salmon-colored precipitate with [ferric chloride TS](#). In moderately concentrated solutions, benzoates yield a precipitate of benzoic acid upon acidification with 2 N sulfuric acid. This precipitate is readily soluble in ethyl ether.

Bicarbonate— See Carbonate.

Bismuth— When dissolved in a slight excess of nitric acid or hydrochloric acid, bismuth salts yield a white precipitate upon dilution with water. This precipitate is colored brown by hydrogen sulfide, and the resulting compound dissolves in a warm mixture of equal parts of nitric acid and water.

Bisulfite— See Sulfite.

Borate— To 1 mL of a borate solution, acidified with hydrochloric acid to litmus, add 3 or 4 drops of [iodine TS](#) and 3 or 4 drops of polyvinyl alcohol solution (1 in 50); an intense blue color is produced. When a borate is treated with sulfuric acid, methanol is added, and the mixture is ignited, it burns with a green-bordered flame.

Bromide— Solutions of bromides, upon the addition of chlorine TS, dropwise, liberate bromine, which is dissolved by shaking with chloroform, coloring the chloroform red to reddish brown. Silver nitrate TS produces in solutions of bromides a yellowish-white precipitate that is insoluble in nitric acid and is slightly soluble in 6 N ammonium hydroxide.

Calcium— Solutions of calcium salts form insoluble oxalates when treated as follows. To a solution of the calcium salt (1 in 20) add 2 drops of [methyl red TS](#), and neutralize with 6 N ammonium hydroxide. Add 3 N hydrochloric acid, dropwise, until the solution is acid to the indicator. Upon the addition of [ammonium oxalate TS](#), a white precipitate is formed. This precipitate is insoluble in 6 N acetic acid but dissolves in hydrochloric acid. Calcium salts moistened with hydrochloric acid impart a transient yellowish-red color to a nonluminous flame.

Carbonate— Carbonates and bicarbonates effervesce with acids, evolving a colorless gas that, when passed into [calcium hydroxide TS](#), produces a white precipitate immediately. A cold solution (1 in 20) of a soluble carbonate is colored red by phenolphthalein TS, while a similar solution of a bicarbonate remains unchanged or is only slightly colored.

Chlorate— Solutions of chlorates yield no precipitate with [silver nitrate TS](#). The addition of sulfuric acid to this mixture produces a white precipitate that is insoluble in nitric acid, but is soluble in 6 N ammonium hydroxide. Upon ignition, chlorates yield chlorides, recognizable by appropriate tests. When sulfuric acid is added to a dry chlorate, decrepitation occurs, and a greenish yellow-gas is evolved. [Caution—Use only a small amount of chlorate for this test, and exercise extreme caution in performing it.]

Chloride— With [silver nitrate TS](#), solutions of chlorides yield a white, curdy precipitate that is insoluble in nitric acid but is soluble in a slight excess of 6 N ammonium hydroxide. When testing amine (including alkaloidal) hydrochlorides that do not respond to the above test, add one drop of diluted nitric acid and 0.5 mL of [silver nitrate TS](#) to a solution of the substance being examined containing, unless otherwise directed in the monograph, about 2 mg of chloride ion in 2 mL; a white, curdy precipitate is formed. Centrifuge the mixture without delay, and decant the supernatant layer. Wash the precipitate with three 1-mL portions of nitric acid solution (1 in 100), and discard the washings. Add [ammonia TS](#) dropwise to this precipitate. It dissolves readily. When a monograph specifies that an article responds to the test for dry chlorides, mix the solid to be tested with an equal weight of manganese dioxide, moisten with sulfuric acid, and gently heat the mixture; chlorine, which is recognizable by the production of a blue color with moistened starch iodide paper, is evolved.

Citrate— To 15 mL of pyridine add a few mg of a citrate salt, dissolved or suspended in 1 mL of water, and shake. To this mixture add 5 mL of acetic anhydride, and shake: a light red color is produced.

Cobalt— Solutions of cobalt salts (1 in 20) in 3 N hydrochloric acid yield a red precipitate when heated on a steam bath with an equal volume of a hot, freshly prepared solution of 1-nitroso-2-naphthol (1 in 10) in 9 N acetic acid. Solutions of cobalt salts, when saturated with potassium chloride and treated with potassium nitrite and acetic acid, yield a yellow precipitate.

Copper— Solutions of cupric compounds, acidified with hydrochloric acid, deposit a red film of metallic copper upon a bright, untarnished surface of metallic iron. An excess of 6 N ammonium hydroxide, added to a solution of a cupric salt, produces first a bluish precipitate and then a deep blue-colored solution. With [potassium ferrocyanide TS](#), solutions of cupric salts yield a reddish-brown precipitate, insoluble in diluted acids.

Hypophosphite— When strongly heated, hypophosphites evolve spontaneously flammable phosphine. Hypophosphites in solution yield a white precipitate with [mercuric chloride TS](#). This precipitate becomes gray when an excess of hypophosphite is present. Solutions of hypophosphites, acidified with sulfuric acid, and warmed with [cupric sulfate TS](#) yield a red precipitate.

Iodide— Solutions of iodides, upon the addition of chlorine TS, dropwise, liberate iodine, which colors the solution yellow to red. When the solution is shaken with chloroform, the latter is colored violet. The iodine thus liberated gives a blue color with [starch TS](#). Silver nitrate TS produces, in solutions of iodides, a yellow, curdy precipitate that is insoluble in nitric acid and in 6 N ammonium hydroxide.

Iron— Ferrous and ferric compounds in solution yield a black precipitate with [ammonium sulfide TS](#). This precipitate is dissolved by cold 3 N hydrochloric acid with the evolution of hydrogen sulfide.



nitric Salts— Acid solutions of ferric salts yield a dark blue precipitate with [potassium ferrocyanide TS](#). With an excess of 1 N sodium hydroxide, a reddish-brown precipitate is formed. With [ammonium thiocyanate TS](#), solutions of ferric salts produce a deep red color that is not destroyed by dilute mineral acids.

Ferrous Salts— Solutions of ferrous salts yield a dark blue precipitate with [potassium ferricyanide TS](#). This precipitate is insoluble in 3 N hydrochloric acid but is decomposed by 1 N sodium hydroxide. With 1 N sodium hydroxide, solutions of ferrous salts yield a greenish-white precipitate, the color rapidly changing to green and then to brown when shaken.

Lactate— When solutions of lactates are acidified with sulfuric acid, potassium permanganate TS is added, and the mixture is heated, acetaldehyde is evolved. This can be detected by allowing the vapor to come into contact with a filter paper that has been moistened with a freshly prepared mixture of equal volumes of 20% aqueous morpholine and sodium nitroferricyanide TS: a blue color is produced.

Lead— With 2 N sulfuric acid, solutions of lead salts yield a white precipitate that is insoluble in 3 N hydrochloric or 2 N nitric acid, but is soluble in warm 1 N sodium hydroxide and in [ammonium acetate TS](#). With [potassium chromate TS](#), solutions of lead salts, free or nearly free from mineral acids, yield a yellow precipitate that is insoluble in 6 N acetic acid but is soluble in 1 N sodium hydroxide.

Lithium— With [sodium carbonate TS](#), moderately concentrated solutions of lithium salts, made alkaline with sodium hydroxide, yield a white precipitate on boiling. The precipitate is soluble in [ammonium chloride TS](#). Lithium salts moistened with hydrochloric acid impart an intense crimson color to a nonluminous flame. Solutions of lithium salts are not precipitated by 2 N sulfuric acid or soluble sulfates (distinction from strontium).

Magnesium— Solutions of magnesium salts in the presence of ammonium chloride yield no more than a slightly hazy precipitate when neutralized with [ammonium carbonate TS](#), but on the subsequent addition of [dibasic sodium phosphate TS](#), a white, crystalline precipitate, which is insoluble in 6 N ammonium hydroxide, is formed.

Manganese— With [ammonium sulfide TS](#), solutions of manganous salts yield a salmon-colored precipitate that dissolves in acetic acid.

Mercury— When applied to bright copper foil, solutions of mercury salts, free from an excess of nitric acid, yield a deposit that upon rubbing, becomes bright and silvery in appearance. With hydrogen sulfide, solutions of mercury compounds yield a black precipitate that is insoluble in [ammonium sulfide TS](#) and in boiling 2 N nitric acid.

Mercuric Salts— Solutions of mercuric salts yield a yellow precipitate with 1 N sodium hydroxide. They yield also, in neutral solutions with [potassium iodide TS](#), a scarlet precipitate that is very soluble in an excess of the reagent.

Mercurous Salts— Mercurous compounds are decomposed by 1 N sodium hydroxide, producing a black color. With hydrochloric acid, solutions of mercurous salts yield a white precipitate that is blackened by 6 N ammonium hydroxide. With [potassium iodide TS](#), a yellow precipitate, that may become green upon standing, is formed.

Nitrate— When a solution of a nitrate is mixed with an equal volume of sulfuric acid, the mixture is cooled, and a solution of ferrous sulfate is superimposed, a brown color is produced at the junction of the two liquids. When a nitrate is heated with sulfuric acid and metallic copper, brownish-red fumes are evolved. Nitrates do not decolorize acidified potassium permanganate TS (distinction from nitrites).

Nitrite— When treated with dilute mineral acids or with 6 N acetic acid, nitrites evolve brownish-red fumes. The solution colors starch-iodide paper blue.

Oxalate— Neutral and alkaline solutions of oxalates yield a white precipitate with [calcium chloride TS](#). This precipitate is insoluble in 6 N acetic acid but is dissolved by hydrochloric acid. Hot acidified solutions of oxalates decolorize potassium permanganate TS.

Permanganate— Solutions of permanganates acidified with sulfuric acid are decolorized by hydrogen peroxide TS and by sodium bisulfite TS, in the cold, and by oxalic acid TS, in hot solution.

Peroxide— Solutions of peroxides slightly acidified with sulfuric acid yield a deep blue color upon the addition of [potassium dichromate TS](#). On shaking the mixture with an equal volume of ethyl ether and allowing the liquids to separate, the blue color is found in the ethyl ether layer.

Phosphate— [note—Where the monograph specifies the identification test for Phosphate, use the tests for orthophosphates, unless the instructions specify the use of the pyrophosphate tests or indicate that the product is to be ignited before performing the test.] With [silver nitrate TS](#), neutral solutions of orthophosphates yield a yellow precipitate that is soluble in 2 N nitric acid and in 6 N ammonium hydroxide. With [ammonium molybdate TS](#), acidified solutions of orthophosphates yield a yellow precipitate that is soluble in 6 N ammonium hydroxide. This precipitate may be slow to form. With silver nitrate TS, pyrophosphates obtained by ignition yield a white precipitate that is soluble in 2 N nitric acid and in 6 N ammonium hydroxide. With [ammonium molybdate TS](#), a yellow precipitate that is soluble in 6 N ammonium hydroxide is formed.

Potassium— Potassium compounds impart a violet color to a nonluminous flame, but the presence of small quantities of sodium masks the color unless the yellow color produced by sodium is screened out by viewing through a blue filter that blocks emission at 589 nm (sodium) but is transparent to emission at 404 nm (potassium). Traditionally, cobalt glass has been used, but other suitable filters are commercially available. In neutral, concentrated or moderately concentrated solutions of potassium salts (depending upon the solubility and the potassium content), sodium bitartrate TS produces a white crystalline precipitate that is soluble in 6 N ammonium hydroxide and in solutions of alkali hydroxides and carbonates. The formation of the precipitate, which is usually slow, is accelerated by stirring or rubbing the inside of the test tube with a glass rod. The addition of a small amount of glacial acetic acid or alcohol also promotes the precipitation.

Salicylate— In moderately dilute solutions of salicylates, ferric chloride TS produces a violet color. The addition of acids to moderately concentrated solutions of salicylates produces a white, crystalline precipitate of salicylic acid that melts between 158° and 161°.

Silver— With hydrochloric acid, solutions of silver salts yield a white, curdy precipitate that is insoluble in nitric acid, but is readily soluble in 6 N ammonium hydroxide. A solution of a silver salt to which 6 N ammonium hydroxide and a small quantity of [formaldehyde TS](#) are added deposits, upon warming, a mirror of metallic silver upon the sides of the container.

Sodium— Unless otherwise specified in an individual monograph, prepare a solution to contain 0.1 g of the sodium compound in 2 mL of water. Add 2 mL of 15% potassium carbonate, and heat to boiling. No precipitate is formed. Add 4 mL of [potassium pyroantimonate TS](#), and heat to boiling. Allow to cool in ice water and, if necessary, rub the inside of the test tube with a glass rod. A dense precipitate is formed. Sodium compounds impart an intense yellow color to a nonluminous flame.

Sulfate— With [barium chloride TS](#), solutions of sulfates yield a white precipitate that is insoluble in hydrochloric acid and in nitric acid. With [lead acetate TS](#), neutral solutions of sulfates yield a white precipitate that is soluble in [ammonium acetate TS](#). Hydrochloric acid produces no precipitate when added to solutions of sulfates (distinction from thiosulfates).

Sulfite— When treated with 3 N hydrochloric acid, sulfites and bisulfites yield sulfur dioxide, which blackens filter paper moistened with [mercurous nitrate TS](#).

Tartrate— Dissolve a few mg of a tartrate salt in 2 drops of sodium metaperiodate solution (1 in 20). Add a drop of 1 N sulfuric acid, and after 5 minutes add a few drops of sulfuric acid followed by a few drops of fuchsin-sulfurous acid TS: a reddish-pink color is produced within 15 minutes.

Thiocyanate— With [ferric chloride TS](#), solutions of thiocyanates yield a red color that is not destroyed by moderately concentrated mineral acids.

Thiosulfate— With hydrochloric acid, solutions of thiosulfates yield a white precipitate that soon turns yellow, and sulfur dioxide, which blackens filter paper moistened with [mercurous nitrate TS](#). The addition of [ferric chloride TS](#) to solutions of thiosulfates produces a dark violet color that quickly disappears.

Zinc— In the presence of sodium acetate, solutions of zinc salts yield a white precipitate with hydrogen sulfide. This precipitate is insoluble in acetic acid, but is dissolved by 3 N hydrochloric acid. Ammonium sulfide TS produces a similar precipitate in neutral and in alkaline solutions. With [potassium ferrocyanide TS](#), zinc salts in solution yield a white precipitate that is insoluble in 3 N hydrochloric acid.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	Antonio Hernandez-Cardoso, B.S. Scientist, Latin American Specialist 1-301-816-8308	(GC05) General Chapters 05



### 193 IDENTIFICATION— TETRACYCLINES

The following chromatographic procedures are provided to confirm the identity of Pharmacopeial drug substances that are of the tetracycline type, such as doxycycline, oxytetracycline, and tetracycline, and to confirm the identity of such compounds in their respective Pharmacopeial dosage forms. Two procedures are provided, one based on paper chromatography (Method I) and the other on thin-layer chromatography (Method II). Method I is to be used unless otherwise directed in the individual monograph.

Standard Solution— Unless otherwise directed in the individual monograph, dissolve the USP Reference Standard for the drug substance being identified in the same solvent and at the same concentration as for the Test Solution.

Test Solution— Prepare as directed in the individual monograph.

#### METHOD I

pH 3.5 Buffer— Dissolve 13.4 g of anhydrous citric acid and 16.3 g of dibasic sodium phosphate in 1000 mL of water, and mix.

Developing Solvent— On the day of use, mix 10 volumes of chloroform, 20 volumes of nitromethane, and 3 volumes of pyridine.

Mixed Test Solution— Mix equal volumes of the Standard Solution and the Test Solution.

Chromatographic Sheet— Draw a spotting line 2.5 cm from one edge of a 20-cm × 20-cm sheet of filter paper (Whatman No. 1, or equivalent). Impregnate the sheet with pH 3.5 Buffer by passing it through a trough filled with pH 3.5 Buffer, and remove the excess solvent by firmly pressing the sheet between nonfluorescent blotting papers.

Procedure— To a suitable chromatographic chamber, prepared for ascending chromatography (see [Chromatography \(621\)](#)) add Developing Solvent to a depth of 0.6 cm. Apply at 1.5-cm intervals 2  $\mu$ L each of the Standard Solution, the Test Solution, and the Mixed Test Solution to the spotting line of the Chromatographic Sheet. Allow the sheet to dry partially, and while still damp place it in the chromatographic chamber with the bottom edge touching the Developing Solvent. When the solvent front has risen about 10 cm, remove the sheet from the chamber, and expose the sheet to ammonia vapor. Examine the chromatogram under long-wavelength UV light. Record the positions of the major yellow fluorescent spots: the RF value of the principal spot obtained from the Test Solution and from the Mixed Test Solution corresponds to that obtained from the Standard Solution.

#### METHOD II

Resolution Solution— Unless otherwise directed in the individual monograph, prepare a solution in methanol containing 0.5 mg each of USP Chlortetracycline Hydrochloride RS, USP Doxycycline Hydrate RS, USP Oxytetracycline RS, and USP Tetracycline Hydrochloride RS per mL.

Developing Solvent— Prepare a mixture of 0.5 M oxalic acid, previously adjusted with ammonium hydroxide to a pH of 2.0, acetonitrile, and methanol (80:20:20).

Chromatographic Plate— Use a suitable thin-layer chromatographic plate (see [Thin-layer Chromatography under Chromatography \(621\)](#)) coated with a 0.25-mm layer of octylsilanized chromatographic silica gel mixture. Activate the plate by heating it at 130° for 20 minutes, allow to cool, and use while still warm.

Procedure— Separately apply 1  $\mu$ L each of the Standard Solution, the Test Solution, and the Resolution Solution to the Chromatographic Plate. Allow the spots to dry, and develop the chromatogram in the Developing Solvent until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow to air-dry. Expose the plate to ammonia vapors for 5 minutes, and promptly locate the spots on the plate by viewing under long-wavelength UV light: the chromatogram of the Resolution Solution shows clearly separated spots, and the principal spot obtained from the Test Solution corresponds in RF value, intensity, and appearance to that obtained from the Standard Solution.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Ahalya Wise, M.S.</a> Scientist 1-301-816-8161	(MDANT05) Monograph Development-Antibiotics

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### 197 SPECTROPHOTOMETRIC IDENTIFICATION TESTS

Spectrophotometric tests contribute meaningfully toward the identification of many compendial chemical substances. The test procedures that follow are applicable to substances that absorb IR and/or UV radiation (see [Spectrophotometry and Light-Scattering \(851\)](#)).

The IR absorption spectrum of a substance, compared with that obtained concomitantly for the corresponding USP Reference Standard, provides perhaps the most conclusive evidence of the identity of the substance that can be realized from any single test. The UV absorption spectrum, on the other hand, does not exhibit a high degree of specificity. Conformance with both IR absorption and UV absorption test specifications, as called for in a large proportion of compendial monographs, leaves little doubt, if any, regarding the identity of the specimen under examination.

#### INFRARED ABSORPTION

Six methods are indicated for the preparation of previously dried test specimens and Reference Standards for analysis. The reference [\(197K\)](#) in a monograph signifies that the substance under examination is mixed intimately with potassium bromide. The reference [\(197M\)](#) in a monograph signifies that the substance under examination is finely ground and dispersed in mineral oil. The reference [\(197F\)](#) in a monograph signifies that the substance under examination is suspended neat between suitable (for example, sodium chloride or potassium bromide) plates. The reference [\(197S\)](#) signifies that a solution of designated concentration is prepared in the solvent specified in the individual monograph, and the solution is examined in 0.1-mm cells unless a different cell path length is specified in the individual monograph. The reference [\(197A\)](#) signifies that the substance under examination is intimately in contact with an internal reflection element for attenuated total reflectance (ATR) analysis. The reference [\(197E\)](#) signifies that the substance under examination is pressed as a thin sample against a suitable plate for IR microscopic analysis. The ATR [\(197A\)](#) and the [\(197E\)](#) techniques can be used as alternative methods for [\(197K\)](#), [\(197M\)](#), [\(197F\)](#), and [\(197S\)](#) where testing is performed qualitatively and the Reference Standard spectra are similarly obtained.

Record the spectra of the test specimen and the corresponding USP Reference Standard over the range from about 2.6  $\mu$ m to 15  $\mu$ m (3800 cm<sup>-1</sup> to 650 cm<sup>-1</sup>) unless otherwise specified in the individual monograph. The IR absorption spectrum of the preparation of the test specimen, previously dried under conditions specified for the corresponding Reference Standard unless otherwise specified, or unless the Reference Standard is to be used without drying, exhibits maxima only at the same wavelengths as that of a similar preparation of the corresponding USP Reference Standard.

Differences that may be observed in the spectra so obtained sometimes are attributed to the presence of polymorphs, which are not always acceptable (see Procedure under [Spectrophotometry and Light-Scattering \(851\)](#)). Unless otherwise directed in the individual monograph, therefore, continue as follows. If a difference appears in the IR spectra of the analyte and the standard, dissolve equal portions of the test specimen and the Reference Standard in equal volumes of a suitable solvent, evaporate the solution to dryness in similar containers under identical conditions, and repeat the test on the residues.



## ULTRAVIOLET ABSORPTION

The reference **197U** in a monograph signifies that a test solution and a Standard solution are examined spectrophotometrically, in 1-cm cells, over the spectral range from 200 to 400 nm unless otherwise specified in the individual monograph.

Dissolve a portion of the substance under examination in the designated Medium to obtain a test solution having the concentration specified in the monograph for Solution. Similarly prepare a Standard solution containing the corresponding USP Reference Standard.

Record and compare the spectra concomitantly obtained for the test solution and the Standard solution. Calculate absorptivities and/or absorbance ratios where these criteria are included in an individual monograph. Unless otherwise specified, absorbances indicated for these calculations are those measured at the maximum absorbance at about the wavelength specified in the individual monograph. Where the absorbance is to be measured at about the specified wavelength other than that of maximum absorbance, the abbreviations (min) and (sh) are used to indicate a minimum and shoulder, respectively, in an absorption spectrum. The requirements are met if the UV absorption spectra of the test solution and the Standard solution exhibit maxima and minima at the same wavelengths and absorptivities and/or absorbance ratios are within specified limits.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Gary E. Ritchie, M.Sc.</a> Scientific Fellow 1-301-816-8353	(GC05) General Chapters 05

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**201 THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

## GENERAL PROCEDURE

The following procedure is applicable as an aid in verifying the identities of many compendial drug substances as such and in their respective dosage forms.

Prepare a test solution as directed in the individual monograph. On a line parallel to and about 2 cm from the edge of a suitable thin-layer chromatographic plate, coated with a 0.25-mm layer of chromatographic silica gel mixture (see [Chromatography \(621\)](#)) apply 10  $\mu$ L of this solution and 10  $\mu$ L of a Standard solution prepared from the USP Reference Standard for the drug substance being identified, in the same solvent and at the same concentration as the test solution, unless otherwise directed in the individual monograph. Allow the spots to dry, and develop the chromatogram in a solvent system consisting of a mixture of chloroform, methanol, and water (180:15:1), unless otherwise directed in the individual monograph, until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow the solvent to evaporate. Unless otherwise directed in the individual monograph, locate the spots on the plate by examination under short-wavelength UV light. The RF value of the principal spot obtained from the test solution corresponds to that obtained from the Standard solution.

## PROCEDURE FOR BACITRACIN, NEOMYCIN, AND POLYMYXIN B

The following thin-layer chromatographic procedure is applicable as an aid in verifying the identities of bacitracin, neomycin, and polymyxin B active ingredients and in dosage forms when present singly and in two- and three-component mixtures. The reference **201BNP** in a monograph signifies that this procedure is intended.

Prepare a Test Solution as follows, unless otherwise directed in the individual monograph.

## Test Solution—

for drug substances— Dissolve a portion of Bacitracin, Bacitracin Zinc, Neomycin Sulfate, or Polymyxin B Sulfate in 0.1 N hydrochloric acid to obtain a solution containing about 500 USP Bacitracin Units per mL, 3.5 mg of neomycin (base) per mL, or 10,000 USP Polymyxin B Units per mL.

for solutions— Where the Solution contains neomycin and polymyxin B, dilute a portion of it with 0.1 N hydrochloric acid to obtain a solution containing the equivalent of about 3.5 mg of neomycin (base) per mL. Where the Solution contains polymyxin B but not neomycin, dilute a portion of it with 0.1 N hydrochloric acid to obtain a solution containing about 10,000 USP Polymyxin B Units per mL.

for creams, lotions, and ointments— Where the Cream, Lotion, or Ointment contains Bacitracin or Bacitracin Zinc, transfer a portion of it equivalent to about 500 USP Bacitracin Units, to a 15-mL centrifuge tube. Where the Cream, Lotion, or Ointment contains neomycin, but not Bacitracin or Bacitracin Zinc, transfer a portion of it equivalent to about 3.5 mg of neomycin (base) per mL to a 15-mL centrifuge tube. Add 4 mL of chloroform to the centrifuge tube, and shake well to disperse the Cream, Lotion, or Ointment. Add 1 mL of 0.1 N hydrochloric acid, vortex for 4 minutes, centrifuge, and use the clear supernatant.

note—The Modified Test Solution as described below in the Modified Procedure may be used in lieu of the Test Solution.

Standard Bacitracin Solution— Dissolve a portion of [USP Bacitracin Zinc RS](#) in 0.1 N hydrochloric acid to obtain a solution containing 500 USP Bacitracin Units per mL.

Standard Neomycin Solution— Dissolve a portion of [USP Neomycin Sulfate RS](#) in 0.1 N hydrochloric acid to obtain a solution containing the equivalent of 3.5 mg of neomycin (base) per mL.

Standard Polymyxin B Solution— Dissolve a portion of [USP Polymyxin B Sulfate RS](#) in 0.1 N hydrochloric acid to obtain a solution containing 10,000 USP Polymyxin B Units per mL. Where the article under test also contains Bacitracin or Bacitracin Zinc, dissolve a portion of USP Polymyxin B Sulfate RS in 0.1 N hydrochloric acid to obtain a solution containing 500J USP Polymyxin B Units per mL, J being the ratio of the labeled amount of USP Polymyxin B Units to the labeled amount of USP Bacitracin Units in each g of Cream, Lotion, or Ointment.

Developing Solvent Solution— Prepare a mixture of methanol, isopropyl alcohol, methylene chloride, ammonium hydroxide, and water (4:2:2:1.5).

Procedure— Apply 10  $\mu$ L of the Test Solution and each of the relevant Standard Solutions to a suitable thin-layer chromatographic plate (see [Chromatography \(621\)](#)) coated with a 0.25-mm layer of chromatographic silica gel. Place the plate in a presaturated chromatographic chamber, and develop the chromatogram with the Developing Solvent System until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the chamber, and dry at 105° for 10 minutes. Spray the plate with a 0.2% solution of ninhydrin in butyl alcohol, and heat at 105° for 5 minutes. The RF value of each principal spot in the chromatogram of the Test Solution corresponds to that of the principal spot in the chromatogram obtained from each relevant Standard Solution as appropriate for the labeled active ingredient or ingredients specified on the label. If the chromatogram of the Test Solution yields excessive streaking, proceed as directed for Modified Procedure.

Modified Procedure— Transfer the Test Solution to a 15-mL centrifuge tube, add 10 mL of saturated aqueous picric acid solution (1.2%, w/v), vortex for 1 minute, centrifuge for 10 minutes, and discard the supernatant. Wash the residue with 1-mL portions of water until no yellow color is observed in the washing. Discard the washings, and dry the residue under stream of nitrogen at 50°. Dissolve the residue in 1 mL of acetone, add 1 mL of a freshly prepared solution of sulfuric acid in acetone (1 in 100), shake, centrifuge for 5 minutes, and discard the supernatant. Rinse the residue with 1 mL of acetone, centrifuge briefly, and discard the washing. Repeat the washing until no yellow color is observed. Dry the residue under a stream of nitrogen at 50°. Dissolve the residue in 0.5 mL of 0.1 N hydrochloric acid (Modified Test Solution). Repeat the Procedure using this Modified Test Solution instead of the Test Solution. The RF value of each principal spot in the chromatogram of the Modified Test Solution corresponds to that of the principal spot in the chromatogram obtained from each relevant Standard Solution as appropriate for the active ingredient or ingredients specified on the label.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Horacio N. Pappa, Ph.D.</a> Senior Scientist and Latin American Liaison 1-301-816-8319	(GC05) General Chapters 05

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Pharmacopeial Forum: Volume No. 28(4) Page 1225

## 206 ALUMINUM

This procedure is provided to demonstrate that the content of aluminum (Al) does not exceed the limit given in the individual monograph of a substance labeled as intended for use in hemodialysis. [note—The Standard Preparations and the Test Preparation may be modified, if necessary, to obtain solutions of suitable concentrations adaptable to the linear or working range of the instrument.]

Nitric Acid Diluent— Transfer 40 mL of nitric acid to a 1000-mL volumetric flask, and dilute with water to volume.

Standard Preparations— Treat some aluminum wire with 6 N hydrochloric acid at 80° for a few minutes. Dissolve about 100 mg of the treated wire, accurately weighed, in a mixture of 10 mL of hydrochloric acid and 2 mL of nitric acid by heating at about 80° for approximately 30 minutes. Continue heating until the volume is reduced to about 4 mL. Cool to room temperature, and add 4 mL of water. Evaporate to about 2 mL by heating. Cool, and transfer this solution, with the aid of water, to a 100-mL volumetric flask, dilute with water to volume, and mix. Transfer 10.0 mL of this solution to a second 100-mL volumetric flask, dilute with water to volume, and mix. Transfer 1.0 mL of this solution to a third 100-mL volumetric flask, dilute with water to volume, and mix. The concentration of aluminum in this Standard Preparation is about 1.0 µg per mL. If a more diluted Standard Preparation is required, transfer 1.0-, 2.0-, and 4.0-mL portions of this solution to separate 100-mL volumetric flasks, dilute with Nitric Acid Diluent to volume, and mix. These solutions contain 0.01, 0.02, and 0.04 µg of Al per mL, respectively.

Test Preparation— Unless otherwise directed in the monograph, transfer an accurately weighed amount (in g) of the test substance, as specified in the monograph, to a 100-mL plastic volumetric flask, add 50 mL of water, and sonicate for 30 minutes. Add 4 mL of nitric acid, dilute with water to volume, and mix.

Procedure— Determine the absorbances of the Standard Preparations and the Test Preparation at the aluminum emission line at 309.3 nm with a suitable atomic absorption spectrophotometer (see [Spectrophotometry and Light-Scattering \( 851 \)](#)) equipped with an aluminum hollow-cathode lamp and a flameless electrically heated furnace, using the Nitric Acid Diluent as the blank. Plot the absorbances of the Standard Preparations versus the content of Al, in µg per mL, drawing a straight line best fitting the three points. From the graph so obtained, determine the quantity, in µg, of Al in each mL of the Test Preparation. Calculate the amount of Al in the specimen taken, in µg per g, by multiplying this value by 100/W, where W is the weight, in g, of the substance taken to prepare the Test Preparation.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Kahkashan Zaidi, Ph.D.</a> Senior Scientist 1-301-816-8269	(GC05) General Chapters 05

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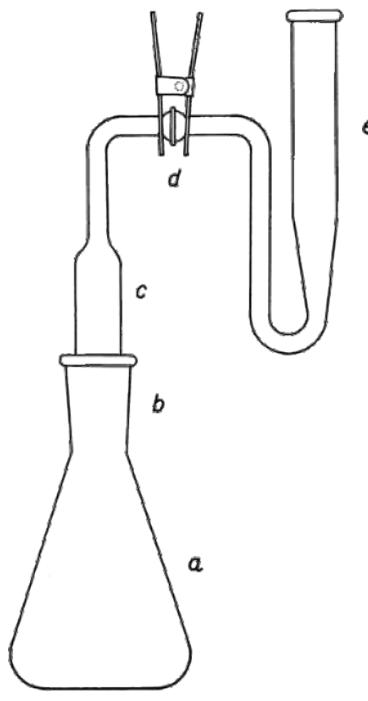
## 211 ARSENIC

This procedure is designed to determine the presence of trace amounts of arsenic (As) by converting the arsenic in a substance under test to arsine, which is then passed through a solution of silver diethyldithiocarbamate to form a red complex. The red color so produced is compared, either visually or spectrophotometrically, to the color produced similarly in a control containing an amount of arsenic equivalent to the limit given in the individual monograph. Limits are stated in terms of arsenic (As). The content of arsenic does not exceed the limit given in the individual monograph.

Two methods are provided, the methods differing only in the preliminary treatment of the test substance and the standard. Generally, Method I is used for inorganic materials, while Method II is used for organic materials.

## Apparatus—

The apparatus (see [illustration](#)) consists of an arsine generator (a) fitted with a scrubber unit (c) and an absorber tube (e) with standard-taper or ground glass ball-and-socket joints (b and d) between the units. However, any other suitable apparatus, embodying the principle of the assembly described and illustrated, may be used.



Arsenic Test Apparatus

**Arsenic Trioxide Stock Solution**— Dissolve 132.0 mg of arsenic trioxide, previously dried at  $105^{\circ}$  for 1 hour and accurately weighed, in 5 mL of sodium hydroxide solution (1 in 5) in a 1000-mL volumetric flask. Neutralize the solution with 2 N sulfuric acid, add 10 mL more of 2 N sulfuric acid, then add recently boiled and cooled water to volume, and mix.

**Standard Arsenic Solution**— Transfer 10.0 mL of Arsenic Trioxide Stock Solution to a 1000-mL volumetric flask, add 10 mL of 2 N sulfuric acid, then add recently boiled and cooled water to volume, and mix. Each mL of Standard Arsenic Solution contains the equivalent of 1  $\mu$ g of arsenic (As). Keep this solution in an all-glass container, and use within 3 days.

#### METHOD I

**Standard Preparation**— Pipet 3.0 mL of Standard Arsenic Solution into a generator flask, and dilute with water to 35 mL.

**Test Preparation**— Unless otherwise directed in the individual monograph, transfer to the generator flask the quantity, in g, of the test substance calculated by the formula:

$$3.0/L$$

in which L is the arsenic limit in ppm, dissolve in water, and dilute with water to 35 mL.

**Procedure**— Treat the Standard Preparation and the Test Preparation similarly as follows. Add 20 mL of 7 N sulfuric acid, 2 mL of [potassium iodide TS](#), 0.5 mL of stronger acid stannous chloride TS, and 1 mL of isopropyl alcohol, and mix. Allow to stand at room temperature for 30 minutes. Pack the scrubber tube (c) with two pledgets of cotton that have been soaked in saturated lead acetate solution, freed from excess solution by expression, and dried in vacuum at room temperature, leaving a 2-mm space between the two pledgets. Lubricate the joints (b and d) with a suitable stopcock grease designed for use with organic solvents, and connect the scrubber unit to the absorber tube (e). Transfer 3.0 mL of [silver diethyldithiocarbamate TS](#) to the absorber tube. Add 3.0 g of granular zinc (No. 20 mesh) to the mixture in the flask, immediately connect the assembled scrubber unit, and allow the evolution of hydrogen and the color development to proceed at room temperature for 45 minutes, swirling the flask gently at 10-minute intervals. Disconnect the absorber tube from the generator and scrubber units, and transfer the absorbing solution to a 1-cm absorption cell. Any red color produced by the Test Preparation does not exceed that produced by the Standard Preparation. If necessary or desirable, determine the absorbance at the wavelength of maximum absorbance between 535 and 540 nm, with a suitable spectrophotometer or colorimeter, using [silver diethyldithiocarbamate TS](#) as the blank.

**Interfering Chemicals**— Metals or salts of metals, such as chromium, cobalt, copper, mercury, molybdenum, nickel, palladium, and silver, may interfere with the evolution of arsine. Antimony, which forms stibine, produces a positive interference in the color development with [silver diethyldithiocarbamate TS](#); when the presence of antimony is suspected, the red colors produced in the two silver diethyldithiocarbamate solutions may be compared at the wavelength of maximum absorbance between 535 and 540 nm, with a suitable colorimeter, since at this wavelength the interference due to stibine is negligible.

#### METHOD II

notes—

(1) Caution—Some substances may react with explosive violence when digested with hydrogen peroxide. Exercise safety precautions at all times.

(2) If halogen-containing compounds are present, use a lower temperature while heating the test specimen with sulfuric acid, avoid boiling the mixture, and add the hydrogen peroxide with caution, before charring begins, to prevent loss of trivalent arsenic.

(3) If the test substance reacts too rapidly and begins charring with 5 mL of sulfuric acid before heating, use instead 10 mL of cooled dilute sulfuric acid (1 in 2), and add a few drops of the hydrogen peroxide before heating.

**Standard Preparation**— Pipet 3.0 mL of Standard Arsenic Solution into a generator flask, add 2 mL of sulfuric acid, mix, and add the total amount of 30 percent hydrogen peroxide used in preparing the Test Preparation. Heat the mixture to strong fuming, cool, add cautiously 10 mL of water, and again heat to strong fumes. Repeat this procedure with another 10 mL of water to remove any traces of hydrogen peroxide. Cool, and dilute with water to 35 mL.

**Test Preparation**— Unless otherwise directed in the individual monograph, transfer to a generator flask the quantity, in g, of the test substance calculated by the formula:

$$3.0 / L$$

in which L is the arsenic limit in ppm. Add 5 mL of sulfuric acid and a few glass beads, and digest in a fume hood, preferably on a hot plate and at a temperature not exceeding  $120^{\circ}$ , until charring begins. (Additional sulfuric acid may be necessary to wet some specimens completely, but the total volume added should not exceed 10 mL.) Cautiously add, dropwise, 30 percent hydrogen peroxide, allowing the reaction to subside and again heating between drops. Add the first few drops very slowly with sufficient mixing, in order to prevent a rapid reaction. Discontinue heating if foaming becomes excessive. When the reaction has abated, heat cautiously, rotating the flask occasionally to prevent the specimen from caking on glass exposed to the heating unit. Maintain oxidizing conditions at all times during the digestion by adding small quantities of the hydrogen peroxide solution whenever the mixture



turns brown or darkens. Continue the digestion until the organic matter is destroyed, gradually raising the temperature of the hot plate until fumes of sulfur trioxide are copiously evolved, and the solution becomes colorless or retains only a light straw color. Cool, add cautiously 10 mL of water, mix, and again evaporate to strong fuming, repeating this procedure to remove any trace of hydrogen peroxide. Cool, add cautiously 10 mL of water, wash the sides of the flask with a few mL of water, and dilute with water to 35 mL.

Procedure— Proceed as directed for Procedure under Method I.

Interfering Chemicals— See Interfering Chemicals under Method I.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Kahkashan Zaidi, Ph.D.</a> Senior Scientist 1-301-816-8269	(GC05) General Chapters 05

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### 221 CHLORIDE AND SULFATE

The following limit tests are provided as general procedures for use where limits for chloride and sulfate are specified in the individual monographs.

Perform the tests and the controls in glass cylinders of the same diameter and matched as closely as practicable in other respects (see Visual Comparison under [Spectrophotometry and Light-Scattering](#) 851). Use the same quantities of the same reagents for both the solution under test and the control solution containing the specified volume of chloride or sulfate. If, after acidification, the solution is not perfectly clear, pass it through a filter paper that gives negative tests for chloride and sulfate. Add the precipitant, silver nitrate TS or barium chloride TS as required, to both the test solution and the control solution in immediate sequence.

Where the individual monograph calls for applying the test to a specific volume of a solution of the substance, and the limit for chloride or sulfate corresponds to 0.20 mL or less of 0.020 N hydrochloric acid or sulfuric acid, respectively, apply the test to the solution without further dilution. In such cases maintain the same volume relationships for the control solution as specified for the solution under test. In applying the test to the salts of heavy metals, which normally show an acid reaction, omit the acidification and do not neutralize the solution. Dissolve bismuth salts in a few mL of water and 2 mL of nitric acid before treating with the precipitant.

Chloride— Dissolve the specified quantity of the substance under test in 30 to 40 mL of water, or, where the substance is already in solution, add water to make a total volume of 30 to 40 mL, and, if necessary, neutralize the solution with nitric acid to litmus. Add 1 mL each of nitric acid and of [silver nitrate TS](#) and sufficient water to make 50 mL. Mix, and allow to stand for 5 minutes protected from direct sunlight. Unless otherwise specified in the monograph, compare the turbidity, if any, with that produced in a solution containing the volume of 0.020 N hydrochloric acid specified in the monograph.

Sulfate— Dissolve the specified quantity of the substance under test in 30 to 40 mL of water, or, where the substance is already in solution, add water to make a total volume of 30 to 40 mL, and, if necessary, neutralize the solution with hydrochloric acid to litmus. Add 1 mL of 3 N hydrochloric acid, 3 mL of [barium chloride TS](#), and sufficient water to make 50 mL. Mix, and allow to stand for 10 minutes. Unless otherwise specified in the monograph, compare the turbidity, if any, with that produced in a solution containing the volume of 0.020 N sulfuric acid specified in the monograph.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Kahkashan Zaidi, Ph.D.</a> Senior Scientist 1-301-816-8269	(GC05) General Chapters 05

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### 223 DIMETHYLANILINE

The following limit test is provided as a general procedure, when specified in the individual monographs for the gas chromatographic determination in compendial articles of traces of dimethylaniline, a hydrochloric acid scavenger that may have been carried over during processing.

Internal Standard Solution— Unless otherwise specified in the individual monograph, prepare a solution of naphthalene in cyclohexane containing about 50 µg per mL.

Standard Preparation— Unless otherwise specified in the individual monograph, transfer 50.0 mg of N,N-dimethylaniline to a 50-mL volumetric flask, add 25 mL of 1 N hydrochloric acid, swirl to dissolve, dilute with water to volume, and mix. Transfer 5.0 mL of the resulting solution to a 250-mL volumetric flask, dilute with water to volume, and mix. To a suitable centrifuge tube add 1.0 mL of this solution, 5.0 mL of 1 N sodium hydroxide, and 1.0 mL of Internal Standard Solution, shake vigorously for 1 minute, and centrifuge. Use the clear supernatant as the Standard Preparation.

Test Preparation— Unless otherwise specified in the individual monograph, transfer 1.0 g of the substance to be tested to a suitable centrifuge tube, add 5 mL of 1 N sodium hydroxide, swirl to dissolve the specimen, add 1.0 mL of Internal Standard Solution, shake vigorously for 1 minute, and centrifuge. Use the clear supernatant as the Test Preparation.

Chromatographic System (see [Chromatography](#) 621)— The gas chromatograph is equipped with a flame-ionization detector and a 2-mm × 2-m column packed with 3% liquid phase G3 on silanized packing S1A and is maintained at 120°. Nitrogen is used as the carrier gas, flowing at the rate of about 30 mL per minute.

Procedure— Inject equal volumes (within the range of 2 µL to 20 µL) of the Standard Preparation and the Test Preparation into the chromatograph, record the chromatograms, and measure the areas for the major peaks. The ratio of the response of any dimethylaniline peak to the response of the naphthalene peak obtained from the Test Preparation is not greater than that obtained from the Standard Preparation (0.002%).

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Ahalya Wise, M.S.</a> Scientist 1-301-816-8161	(MDANT05) Monograph Development-Antibiotics

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### 226 4-EPIANHYDRO-TETRACYCLINE

This chromatographic procedure is provided to demonstrate that the content of 4-epianhydrotetracycline, a degradation product of tetracycline, does not exceed the limit given in the



individual monograph.

EDTA Buffer— Dissolve 37.2 g of edetate disodium in 800 mL of water, adjust with ammonium hydroxide to a pH of 7.8, dilute with water to 1000 mL, and mix.

Support Phase— Add 5 mL of EDTA Buffer to 10 g of acid-washed chromatographic siliceous earth for column chromatography, and mix until the siliceous earth is uniformly moistened.

Test Solution— Prepare as directed in the individual monograph.

Procedure— Prepare a 15-mm  $\times$  170-mm chromatographic tube with a 4-mm  $\times$  50-mm outlet by packing it, in increments, with Support Phase, firmly tamping down each increment, until the tube is filled to a height of about 10 cm. In a beaker, prepare a mixture of 1 g of acid-washed chromatographic siliceous earth for column chromatography and 1 mL of Test Solution. Transfer the mixture to the top of the column. Dry-wash the beaker with Support Phase, and transfer to the column to provide an additional 1-cm layer on top of the mixture containing the Test Solution. Within 30 minutes, pass chloroform through the column, and collect successive fractions of 5.0 mL, 5.0 mL, 10.0 mL, 10.0 mL, and 5.0 mL. Observe the column during elution, and note the appearance of two separate yellow bands. The fraction or fractions containing the first yellow band contain the anhydrotetracyclines. Discard these fractions. The fractions after the first yellow band contain the 4-epianhydrotetracycline. Determine the absorbance of each 4-epianhydrotetracycline fraction at the wavelength of maximum absorbance at about 438 nm, with a suitable spectrophotometer, diluting each fraction, if necessary, with chloroform, and using chloroform as the blank. Calculate the quantity, in mg, of 4-epianhydrotetracycline in each fraction by the formula:

$$AVD/20.08$$

in which A is the absorbance, V is the volume, in mL, of the fraction taken, D is the dilution factor, if the fraction was diluted, and 20.08 is the absorptivity of 4-epianhydrotetracycline at 438 nm. From the sum of the quantities of 4-epianhydrotetracycline found in the fractions, calculate the percentage of 4-epianhydrotetracycline in relation to the tetracycline hydrochloride equivalent contained in the Test Solution.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Ahalya Wise, M.S.</a> Scientist 1-301-816-8161	(MDANT05) Monograph Development-Antibiotics

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## 231 HEAVY METALS

This test is provided to demonstrate that the content of metallic impurities that are colored by sulfide ion, under the specified test conditions, does not exceed the Heavy metals limit specified in the individual monograph in percentage (by weight) of lead in the test substance, as determined by concomitant visual comparison (see Visual Comparison in the section Procedure under [Spectrophotometry and Light-Scattering](#) (851)) with a control prepared from a Standard Lead Solution. [note—Substances that typically will respond to this test are lead, mercury, bismuth, arsenic, antimony, tin, cadmium, silver, copper, and molybdenum.]

Determine the amount of heavy metals by Method I, unless otherwise specified in the individual monograph. Method I is used for substances that yield clear, colorless preparations under the specified test conditions. Method II is used for substances that do not yield clear, colorless preparations under the test conditions specified for Method I, or for substances that, by virtue of their complex nature, interfere with the precipitation of metals by sulfide ion, or for fixed and volatile oils. Method III, a wet-digestion method, is used only in those cases where neither Method I nor Method II can be used.

### Special Reagents

Lead Nitrate Stock Solution— Dissolve 159.8 mg of lead nitrate in 100 mL of water to which has been added 1 mL of nitric acid, then dilute with water to 1000 mL. Prepare and store this solution in glass containers free from soluble lead salts.

Standard Lead Solution— On the day of use, dilute 10.0 mL of Lead Nitrate Stock Solution with water to 100.0 mL. Each mL of Standard Lead Solution contains the equivalent of 10  $\mu$ g of lead. A comparison solution prepared on the basis of 100  $\mu$ L of Standard Lead Solution per g of substance being tested contains the equivalent of 1 part of lead per million parts of substance being tested.

### Method I

pH 3.5 Acetate Buffer— Dissolve 25.0 g of ammonium acetate in 25 mL of water, and add 38.0 mL of 6 N hydrochloric acid. Adjust, if necessary, with 6 N ammonium hydroxide or 6 N hydrochloric acid to a pH of 3.5, dilute with water to 100 mL, and mix.

Standard Preparation— Into a 50-mL color-comparison tube pipet 2 mL of Standard Lead Solution (20  $\mu$ g of Pb), and dilute with water to 25 mL. Using a pH meter or short-range pH indicator paper as external indicator, adjust with 1 N acetic acid or 6 N ammonium hydroxide to a pH between 3.0 and 4.0, dilute with water to 40 mL, and mix.

Test Preparation— Into a 50-mL color-comparison tube place 25 mL of the solution prepared for the test as directed in the individual monograph; or, using the designated volume of acid where specified in the individual monograph, dissolve in and dilute with water to 25 mL the quantity, in g, of the substance to be tested, as calculated by the formula:

$$2.0/(1000L)$$

in which L is the Heavy metals limit, as a percentage. Using a pH meter or short-range pH indicator paper as external indicator, adjust with 1 N acetic acid or 6 N ammonium hydroxide to a pH between 3.0 and 4.0, dilute with water to 40 mL, and mix.

Monitor Preparation— Into a third 50-mL color-comparison tube place 25 mL of a solution prepared as directed for Test Preparation, and add 2.0 mL of Standard Lead Solution. Using a pH meter or short-range pH indicator paper as external indicator, adjust with 1 N acetic acid or 6 N ammonium hydroxide to a pH between 3.0 and 4.0, dilute with water to 40 mL, and mix.

Procedure— To each of the three tubes containing the Standard Preparation, the Test Preparation, and the Monitor Preparation, add 2 mL of pH 3.5 Acetate Buffer, then add 1.2 mL of thioacetamide-glycerin base TS, dilute with water to 50 mL, mix, allow to stand for 2 minutes, and view downward over a white surface: the color of the solution from the Test Preparation is not darker than that of the solution from the Standard Preparation, and the color of the solution from the Monitor Preparation is equal to or darker than that of the solution from the Standard Preparation. [note—if the color of the Monitor Preparation is lighter than that of the Standard Preparation, use Method II instead of Method I for the substance being tested.]

### Method II

note—This method does not recover mercury.

pH 3.5 Acetate Buffer— Prepare as directed under Method I.

Standard Preparation— Prepare as directed under Method I.

Test Preparation— Use a quantity, in g, of the substance to be tested as calculated by the formula:



2.0 / (1000L)

in which L is the Heavy metals limit, in percentage. Transfer the weighed quantity of the substance to a suitable crucible, add sufficient sulfuric acid to wet the substance, and carefully ignite at a low temperature until thoroughly charred. (The crucible may be loosely covered with a suitable lid during the charring.) Add to the carbonized mass 2 mL of nitric acid and 5 drops of sulfuric acid, and heat cautiously until white fumes no longer are evolved. Ignite, preferably in a muffle furnace, at 500° to 600°, until the carbon is completely burned off. Cool, add 4 mL of 6 N hydrochloric acid, cover, digest on a steam bath for 15 minutes, uncover, and slowly evaporate on a steam bath to dryness. Moisten the residue with 1 drop of hydrochloric acid, add 10 mL of hot water, and digest for 2 minutes. Add 6 N ammonium hydroxide dropwise until the solution is just alkaline to litmus paper, dilute with water to 25 mL, and adjust with 1 N acetic acid to a pH between 3.0 and 4.0, using short-range pH indicator paper as an external indicator. Filter if necessary, rinse the crucible and the filter with 10 mL of water, combine the filtrate and rinsing in a 50-mL color-comparison tube, dilute with water to 40 mL, and mix.

Procedure— To each of the tubes containing the Standard Preparation and the Test Preparation, add 2 mL of pH 3.5 Acetate Buffer, then add 1.2 mL of thioacetamide–glycerin base TS, dilute with water to 50 mL, mix, allow to stand for 2 minutes, and view downward over a white surface<sup>\*</sup>: the color of the solution from the Test Preparation is not darker than that of the solution from the Standard Preparation.

### Method III

pH 3.5 Acetate Buffer— Prepare as directed under Method I.

Standard Preparation— Transfer a mixture of 8 mL of sulfuric acid and 10 mL of nitric acid to a clean, dry, 100-mL Kjeldahl flask, and add a further volume of nitric acid equal to the incremental volume of nitric acid added to the Test Preparation. Heat the solution to the production of dense, white fumes; cool; cautiously add 10 mL of water; and, if hydrogen peroxide was used in treating the Test Preparation, add a volume of 30 percent hydrogen peroxide equal to that used for the substance being tested. Boil gently to the production of dense, white fumes. Again cool, cautiously add 5 mL of water, mix, and boil gently to the production of dense, white fumes and to a volume of 2 to 3 mL. Cool, dilute cautiously with a few mL of water, add 2.0 mL of Standard Lead Solution (20 µg of Pb), and mix. Transfer to a 50-mL color-comparison tube, rinse the flask with water, adding the rinsing to the tube until the volume is 25 mL, and mix.

Test Preparation— Unless otherwise indicated in the individual monograph, use a quantity, in g, of the substance to be tested as calculated by the formula:

2.0/(1000L)

in which L is the Heavy metals limit, as a percentage.

If the substance is a solid— Transfer the weighed quantity of the test substance to a clean, dry, 100-mL Kjeldahl flask. [note—A 300-mL flask may be used if the reaction foams excessively.] Clamp the flask at an angle of 45°, and add a sufficient quantity of a mixture of 8 mL of sulfuric acid and 10 mL of nitric acid to moisten the substance thoroughly. Warm gently until the reaction commences, allow the reaction to subside, and add portions of the same acid mixture, heating after each addition, until a total of 18 mL of the acid mixture has been added. Increase the amount of heat, and boil gently until the solution darkens. Cool, add 2 mL of nitric acid, and heat again until the solution darkens. Continue the heating, followed by addition of nitric acid until no further darkening occurs, then heat strongly to the production of dense, white fumes. Cool, cautiously add 5 mL of water, boil gently to the production of dense, white fumes, and continue heating until the volume is reduced to a few mL. Cool, cautiously add 5 mL of water, and examine the color of the solution. If the color is yellow, cautiously add 1 mL of 30 percent hydrogen peroxide, and again evaporate to the production of dense, white fumes and a volume of 2 to 3 mL. If the solution is still yellow, repeat the addition of 5 mL of water and the peroxide treatment. Cool, dilute cautiously with a few mL of water, and rinse into a 50-mL color-comparison tube, taking care that the combined volume does not exceed 25 mL.

If the substance is a liquid— Transfer the weighed quantity of the test substance to a clean, dry, 100-mL Kjeldahl flask. [note—A 300-mL flask may be used if the reaction foams excessively.] Clamp the flask at an angle of 45°, and cautiously add a few mL of a mixture of 8 mL of sulfuric acid and 10 mL of nitric acid. Warm gently until the reaction commences, allow the reaction to subside, and proceed as directed for if the substance is a solid, beginning with “add portions of the same acid mixture.”

Monitor Preparation— Proceed with the digestion, using the same amount of sample and the same procedure as directed in the subsection If the substance is a solid in the section Test Preparation, until the step “Cool, dilute cautiously with a few mL of water.” Add 2.0 mL of Lead Standard Solution (20 µg of lead), and mix. Transfer to a 50-mL color comparison tube, rinse the flask with water, adding the rinsing to the tube until the volume is 25 mL, and mix.

Procedure— Treat the Test Preparation, the Standard Preparation, and the Monitor Preparation as follows. Using a pH meter or short-range pH indicator paper as external indicator, adjust the solution to a pH between 3.0 and 4.0 with ammonium hydroxide (a dilute ammonia solution may be used, if desired, as the specified range is approached), dilute with water to 40 mL, and mix.

To each tube add 2 mL of pH 3.5 Acetate Buffer, then add 1.2 mL of thioacetamide–glycerin base TS, dilute with water to 50 mL, mix, allow to stand for 2 minutes, and view downward over a white surface<sup>\*</sup>: the color of the Test Preparation is not darker than that of the Standard Preparation, and the color of the Monitor Preparation is equal to or darker than that of the Standard Preparation.

\* In those countries or jurisdictions where thioacetamide cannot be used, add 10 mL of freshly prepared [hydrogen sulfide TS](#) to each of the tubes, mix, allow to stand for 5 minutes, and view downward over a white surface.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Kahkashan Zaidi, Ph.D.</a> Senior Scientist 1-301-816-8269	(GC05) General Chapters 05

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241 IRON

This limit test is provided to demonstrate that the content of iron, in either the ferric or the ferrous form, does not exceed the limit for iron specified in the individual monograph. The determination is made by concomitant visual comparison with a control prepared from a standard iron solution.

### Special Reagents—

STANDARD IRON SOLUTION— Dissolve 863.4 mg of ferric ammonium sulfate [FeNH4(SO4)2·12H2O] in water, add 10 mL of 2 N sulfuric acid, and dilute with water to 100.0 mL. Pipet 10 mL of this solution into a 1000-mL volumetric flask, add 10 mL of 2 N sulfuric acid, dilute with water to volume, and mix. This solution contains the equivalent of 0.01 mg (10 µg) of iron per mL.

AMMONIUM THIOCYANATE SOLUTION— Dissolve 30 g of ammonium thiocyanate in water to make 100 mL.

Standard Preparation— Into a 50-mL color-comparison tube pipet 1 mL of Standard Iron Solution (10 µg of Fe), dilute with water to 45 mL, add 2 mL of hydrochloric acid, and mix.

Test Preparation— Into a 50-mL color comparison tube place the solution prepared for the test as directed in the individual monograph and if necessary dilute with water to 45 mL; or, dissolve in water, and dilute with water to 45 mL the quantity, in g, of the substance to be tested, as calculated by the formula:

1.0 / (1000L)



in which L is the Iron limit in percentage. Add 2 mL of hydrochloric acid, and mix.

Procedure— To each of the tubes containing the Standard Preparation and the Test Preparation add 50 mg of ammonium peroxysulfate crystals and 3 mL of Ammonium Thiocyanate Solution, and mix: the color of the solution from the Test Preparation is not darker than that of the solution from the Standard Preparation.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Kahashan Zaidi, Ph.D.</a> Senior Scientist 1-301-816-8269	(GC05) General Chapters 05

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### 251 LEAD

The imposition of stringent limits on the amounts of lead that may be present in pharmaceutical products has resulted in the use of two methods, of which the one set forth following depends upon extraction of lead by solutions of dithizone. For determination of the content of heavy metals generally, expressed as a lead equivalent, see [Heavy Metals](#) 231.

Select all reagents for this test to have as low a content of lead as practicable, and store all reagent solutions in containers of borosilicate glass. Rinse thoroughly all glassware with warm dilute nitric acid (1 in 2), followed by water.

#### Special Reagents—

AMMONIA-CYANIDE SOLUTION— Dissolve 2 g of potassium cyanide in 15 mL of ammonium hydroxide, and dilute with water to 100 mL.

AMMONIUM CITRATE SOLUTION— Dissolve 40 g of citric acid in 90 mL of water. Add 2 or 3 drops of phenol red TS, then cautiously add ammonium hydroxide until the solution acquires a reddish color. Remove any lead that may be present by extracting the solution with 20-mL portions of Dithizone Extraction Solution (see below), until the dithizone solution retains its orange-green color.

DILUTED STANDARD LEAD SOLUTION— Dilute an accurately measured volume of Standard Lead Solution (see [Heavy Metals](#) 231) [containing 10 µg of lead per mL], with 9 volumes of dilute nitric acid (1 in 100) to obtain a solution that contains 1 µg of lead per mL.

DITHIZONE EXTRACTION SOLUTION— Dissolve 30 mg of dithizone in 1000 mL of chloroform, and add 5 mL of alcohol. Store the solution in a refrigerator.

Before use, shake a suitable volume of the dithizone extraction solution with about half its volume of dilute nitric acid (1 in 100), discarding the nitric acid.

HYDROXYLAMINE HYDROCHLORIDE SOLUTION— Dissolve 20 g of hydroxylamine hydrochloride in sufficient water to make approximately 65 mL. Transfer to a separator, add 5 drops of [thymol blue TS](#), then add ammonium hydroxide until the solution assumes a yellow color. Add 10 mL of sodium diethylthiocarbamate solution (1 in 25), mix, and allow to stand for 5 minutes. Extract this solution with successive 10- to 15-mL portions of chloroform until a 5-mL portion of the chloroform extract does not assume a yellow color when shaken with [cupric sulfate TS](#). Add 3 N hydrochloric acid until the solution is pink (if necessary, add 1 or 2 drops more of [thymol blue TS](#)), and then dilute with water to 100 mL.

POTASSIUM CYANIDE SOLUTION— Dissolve 50 g of potassium cyanide in sufficient water to make 100 mL. Remove the lead from this solution by extraction with successive portions of Dithizone Extraction Solution, as described under Ammonium Citrate Solution above, then extract any dithizone remaining in the cyanide solution by shaking with chloroform. Finally dilute the cyanide solution with sufficient water so that each 100 mL contains 10 g of potassium cyanide.

STANDARD DITHIZONE SOLUTION— Dissolve 10 mg of dithizone in 1000 mL of chloroform. Keep the solution in a glass-stoppered, lead-free bottle, suitably wrapped to protect it from light, and store in a refrigerator.

Test Preparation— [note—If, in the following preparation, the substance under test reacts too rapidly and begins charring with 5 mL of sulfuric acid before heating, use instead 10 mL of cooled dilute sulfuric acid (1 in 2), and add a few drops of the hydrogen peroxide before heating.] Where the monograph does not specify preparation of a solution, prepare a Test Preparation as follows. [Caution—Exercise safety precautions in this procedure, as some substances may react with explosive violence when digested with hydrogen peroxide.] Transfer 1.0 g of the substance under test to a suitable flask, add 5 mL of sulfuric acid and a few glass beads, and digest on a hot plate in a hood until charring begins. Other suitable means of heating may be substituted. (Add additional sulfuric acid, if necessary, to wet the substance completely, but do not add more than a total of 10 mL.) Add, dropwise and with caution, 30 percent hydrogen peroxide, allowing the reaction to subside and again heating between drops. Add the first few drops very slowly, mix carefully to prevent a rapid reaction, and discontinue heating if foaming becomes excessive. Swirl the solution in the flask to prevent unreacted substance from caking on the walls of the flask. [note—Add peroxide whenever the mixture turns brown or darkens.] Continue the digestion until the substance is completely destroyed, copious fumes of sulfur trioxide are evolved, and the solution is colorless. Cool, cautiously add 10 mL of water, evaporate until sulfur trioxide again is evolved, and cool. Repeat this procedure with another 10 mL of water to remove any traces of hydrogen peroxide. Cautiously dilute with 10 mL of water, and cool.

Procedure— Transfer the Test Preparation, rinsing with 10 mL of water, or the volume of the prepared sample specified in the monograph to a separator, and, unless otherwise directed in the monograph, add 6 mL of Ammonium Citrate Solution and 2 mL of Hydroxylamine Hydrochloride Solution. (For the determination of lead in iron salts use 10 mL of Ammonium Citrate Solution.) Add 2 drops of phenol red TS, and make the solution just alkaline (red in color) by the addition of ammonium hydroxide. Cool the solution if necessary, and add 2 mL of Potassium Cyanide Solution. Immediately extract the solution with 5-mL portions of Dithizone Extraction Solution, draining off each extract into another separator, until the dithizone solution retains its green color. Shake the combined dithizone solutions for 30 seconds with 20 mL of dilute nitric acid (1 in 100), and discard the chloroform layer. Add to the acid solution 5.0 mL of Standard Dithizone Solution and 4 mL of Ammonia-Cyanide Solution, and shake for 30 seconds: the color of the chloroform layer is of no deeper shade of violet than that of a control made with a volume of Diluted Standard Lead Solution equivalent to the amount of lead permitted in the sample under examination, and the same quantities of the same reagents and in the same manner as in the test with the sample.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Kahashan Zaidi, Ph.D.</a> Senior Scientist 1-301-816-8269	(GC05) General Chapters 05

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### 261 MERCURY

#### Method I

note—Mercuric dithizone is light-sensitive. Perform this test in subdued light.

Reagents—

dithizone stock solution— Dissolve 40 mg of dithizone in 1000 mL of chloroform.

dithizone titrant— Dilute 30.0 mL of Dithizone Stock Solution with chloroform to 100.0 mL. This solution contains approximately 12 mg of dithizone per L.



Mercury stock solution— Transfer 135.4 mg of mercuric chloride to a 100-mL volumetric flask, and dilute with 1 N sulfuric acid to volume. This solution contains the equivalent of 15 mg of Hg in 100 mL.

mercury solution for standardizing dithizone titrant— Transfer 2.0 mL of Mercury Stock Solution to a 100-mL volumetric flask, and dilute with 1 N sulfuric acid to volume. Each mL of this solution contains the equivalent of 20 µg of Hg.

The following solutions are called for in the limit test for mercury that is specified in the monographs on Ferrous Fumarate, Ferrous Sulfate, and Dried Ferrous Sulfate.

hydroxylamine hydrochloride solution— Prepare as directed in the test for [Lead](#) (251).

standard mercury solution— On the day of use, quantitatively dilute 1.0 mL of Mercury Stock Solution with 1 N sulfuric acid to 1000 mL. Each mL of the resulting solution contains the equivalent of 1 µg of mercury.

dithizone extraction solution— Prepare as directed in the test for [Lead](#) (251).

diluted dithizone extraction solution— Just prior to use, dilute 5 mL of Dithizone Extraction Solution with 25 mL of chloroform.

Standardization of Dithizone Titrant— Transfer 1.0 mL of Mercury Solution for Standardizing Dithizone Titrant to a 250-mL separator, and add 100 mL of 1 N sulfuric acid, 90 mL of water, 1 mL of glacial acetic acid, and 10 mL of hydroxylamine hydrochloride solution (1 in 5). Titrate the solution with Dithizone Titrant from a 10-mL microburet, shaking the mixture 20 times after each addition and allowing the chloroform layer to separate, then discarding the chloroform layer. Continue until a final addition of Dithizone Titrant is green in color after shaking. Calculate the quantity, in µg, of Hg equivalent to each mL of Dithizone Titrant by the formula:

$$20 / V$$

in which V is the volume, in mL, of Dithizone Titrant added.

Test Preparation— Transfer about 2 g of the substance under test, accurately weighed, to a glass-stoppered, 250-mL conical flask, add 20 mL of a mixture of equal volumes of nitric acid and sulfuric acid, attach a suitable condenser, reflux the mixture for 1 hour, cool, cautiously dilute with water, and boil until fumes of nitrous acid no longer are noticeable. Cool the solution, cautiously dilute with water, transfer to a 200-mL volumetric flask, dilute with water to volume, mix, and filter.

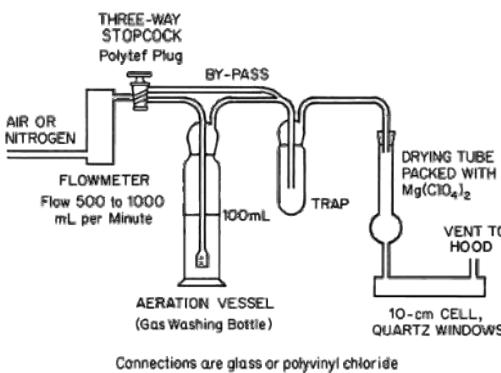
Procedure— Transfer 50.0 mL of Test Preparation to a 250-mL separator, and extract with successive small portions of chloroform until the last chloroform extract remains colorless.

Discard the chloroform extract, and add to the extracted Test Preparation 50 mL of 1 N sulfuric acid, 90 mL of water, 1 mL of glacial acetic acid, and 10 mL of hydroxylamine hydrochloride solution (1 in 5). Proceed as directed under Standardization of Dithizone Titrant, beginning with "Titrate the solution." Calculate the amount of mercury.

#### Method Ia and Method Ib

Mercury Detection Instrument— Use any suitable atomic absorption spectrophotometer equipped with a fast-response recorder and capable of measuring the radiation absorbed by mercury vapors at the mercury resonance line of 253.6 nm. [note—Wash all glassware associated with the test with nitric acid, and rinse thoroughly with water before use.]

Aeration Apparatus— The apparatus (see [accompanying diagram](#)) consists of a flowmeter capable of measuring flow rates from 500 to 1000 mL per minute, connected via a three-way stopcock fitted with a polytef plug to an aeration vessel (250-mL gas washing bottle), followed by a trap, a drying tube packed with magnesium perchlorate, a 10-cm × 25-mm flow-through cell with quartz windows, and terminating with a vent to a fume hood.



#### Mercury Aeration Apparatus

##### Reagents—

Potassium Permanganate Solution— Dissolve 5 g of potassium permanganate in 100 mL of water.

Hydroxylamine Hydrochloride Solution— Dissolve 10 g of hydroxylamine hydrochloride in 100 mL of water.

Stannous Chloride Solution— Dissolve 10 g of SnCl2·2H2O in 20 mL of warm hydrochloric acid, and add 80 mL of water. Prepare fresh each week.

Standard Mercury Solution— Prepare from Mercury Stock Solution as directed under Method I. Each mL of the Standard Mercury Solution contains the equivalent of 1 µg of mercury.

Test Preparation— Unless otherwise directed in the individual monograph, use the quantity, in g, of the test substance calculated by the formula:

$$2.0 / L$$

in which L is the mercury limit, in ppm.

#### Method IIa

Standard Preparation— Pipet 2.0 mL of Standard Mercury Solution into a 100-mL beaker, and add 35 mL of water, 3 mL of sulfuric acid, and 1 mL of potassium permanganate solution. Cover the beaker with a watch glass, boil for a few seconds, and cool.

Test Preparation— Transfer the calculated amount of the test substance to a 100-mL beaker, and add 35 mL of water. Stir, and warm to assist solution, if necessary. Add 2 drops of phenolphthalein TS, and, as necessary, slowly neutralize with constant stirring, using 1 N sodium hydroxide or 1 N sulfuric acid. Add 3 mL of sulfuric acid and 1 mL of Potassium Permanganate Solution. Cover the beaker with a watch glass, boil for a few seconds, and cool.

Procedure— Assemble the Aeration Apparatus as shown in the accompanying diagram, with the aeration vessel and the trap empty, and the stopcock in the bypass position. Connect the apparatus to the absorption cell, and adjust the air or nitrogen flow rate so that, in the following procedure, maximum absorption and reproducibility are obtained without excessive foaming in the test solution. Obtain a smooth baseline reading at 253.6 nm, following the manufacturer's instructions for operating the instrument.

Treat the Standard Preparation and the Test Preparation similarly, as follows. Destroy the excess permanganate by adding Hydroxylamine Hydrochloride Solution, dropwise, until the solution is colorless. Immediately wash the solution into the aeration vessel with water, and dilute with water to 100 mL. Add 2 mL of Stannous Chloride Solution, and immediately



Connect the aeration vessel to the aeration apparatus. Turn the stopcock from the bypass position to the aerating position, and continue the aeration until the absorption peak has been passed and the recorder pen returns to the baseline. Disconnect the aeration vessel from the apparatus, and wash with water after each use. After correcting for any reagent blank, any absorbance produced by the Test Preparation does not exceed that produced by the Standard Preparation.

#### Method IIb

**Caution**—Some substances may react with explosive violence when digested with hydrogen peroxide. Exercise safety precautions at all times.

**Standard Preparation**—Pipet 2.0 mL of Standard Mercury Solution into a 125-mL conical flask, add 3 mL each of nitric acid and sulfuric acid, mix, and add an amount of 30 percent hydrogen peroxide equal to the total amount used in preparing the Test Preparation. Attach a suitable water-cooled condenser with a standard-taper joint to fit the flask, and reflux the mixture in a fume hood for 1 hour. Turn off the water circulating through the condenser, and heat until white fumes appear in the flask. Cool, and cautiously add 10 mL of water through the condenser, while swirling the flask. Again heat until white fumes appear, cool, and add an additional 15 mL of water. Remove the condenser, and rinse the sides of the flask to obtain a volume of 35 mL. Add 1 mL of Potassium Permanganate Solution, boil for a few seconds, and cool.

**Test Preparation**—Transfer the calculated amount of the test substance to a 125-mL conical flask. Add 5 mL each of nitric acid and sulfuric acid and a few glass beads. Attach a suitable water-cooled condenser with a standard-taper joint to fit the flask, and digest in a fume hood, preferably on a hot plate, and at a temperature not exceeding 120°, until charring begins. (If additional sulfuric acid is necessary to wet the specimen completely, add it carefully through the condenser, but do not allow the total volume added to exceed 10 mL.) After the test substance has been decomposed by the acid, cautiously add, dropwise through the condenser, 30 percent hydrogen peroxide, allowing the reaction to subside and again heating between drops (add the first few drops very slowly with sufficient mixing, in order to prevent a rapid reaction; discontinue heating if foaming becomes excessive). When the reaction has abated, heat cautiously, rotating the flask occasionally to prevent the specimen from caking on glass exposed to the heating unit. Maintain oxidizing conditions at all times during the digestion by adding small quantities of the hydrogen peroxide solution whenever the mixture turns brown or darkens. Continue the digestion until the organic matter is destroyed, and then reflux the mixture for 1 hour. Turn off the water circulating through the condenser, and heat until fumes of sulfur trioxide are copiously evolved and the solution becomes colorless or retains only a light straw color. Cool, and cautiously add 10 mL of water through the condenser, while swirling the flask. Again heat until white fumes appear.

Cool, and cautiously add 15 mL of water. Remove the condenser, and rinse the sides of the flask with a few mL of water to obtain a volume of 35 mL. Add 1 mL of Potassium Permanganate Solution, boil for a few seconds, and cool.

**Procedure**—Proceed as directed for Procedure under Method IIa.

**Auxiliary Information**—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Kahkashan Zaidi, Ph.D.</a> Senior Scientist 1-301-816-8269	(GC05) General Chapters 05

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#### 271 READILY CARBONIZABLE SUBSTANCES TEST

In tests for readily carbonizable substances, unless otherwise directed, add the specified quantity of the substance, finely powdered if in solid form, in small portions to the comparison container, which is made of colorless glass resistant to the action of sulfuric acid and contains the specified volume of [sulfuric acid TS](#) (see under Test Solutions).

Stir the mixture with a glass rod until solution is complete, allow the solution to stand for 15 minutes, unless otherwise directed, and compare the color of the solution with that of the specified matching fluid in a comparison container, which also is of colorless glass and has the same internal and cross-section dimensions, viewing the fluids transversely against a background of white porcelain or white glass.

When heat is directed in order to effect solution of the substance in the sulfuric acid TS, mix the sample and the acid in a test tube, heat as directed, and transfer the solution to the comparison container for matching with the designated Matching Fluid (see [Color and Achromicity](#) 631).

Special attention is directed to the importance of the concentration of sulfuric acid used in this test. The reagent of the required strength, i.e., 95.0 ± 0.5 percent of H<sub>2</sub>SO<sub>4</sub>, is designated as a "Test Solution."

**Auxiliary Information**—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Antonio Hernandez-Cardoso, B.S.</a> Scientist, Latin American Specialist 1-301-816-8308	(GC05) General Chapters 05

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#### 281 RESIDUE ON IGNITION

Portions of this general chapter have been harmonized with the corresponding texts of the European Pharmacopoeia and the Japanese Pharmacopoeia. The portions that are not harmonized are marked with symbols (◆◆). The harmonized texts of these pharmacopeias are therefore interchangeable, and the methods of the European Pharmacopoeia and/or the Japanese Pharmacopoeia may be used for demonstration of compliance instead of the present United States Pharmacopoeia general chapter. These pharmacopeias have undertaken not to make any unilateral change to this harmonized chapter.

The Residue on Ignition / Sulfated Ash test uses a procedure to measure the amount of residual substance not volatilized from a sample when the sample is ignited in the presence of sulfuric acid according to the procedure described below. This test is usually used for determining the content of inorganic impurities in an organic substance.

**Procedure**—Ignite a suitable crucible (for example, silica, platinum, quartz, or porcelain) at 600 ± 50° for 30 minutes, cool the crucible in a desiccator (silica gel or other suitable desiccant), and weigh it accurately. Weigh accurately ◆1 to 2 g of the substance, or ◆ the amount specified in the individual monograph, in the crucible.

Moisten the sample with a small amount (usually 1 mL) of sulfuric acid, then heat gently at a temperature as low as practicable until the sample is thoroughly charred. Cool; then, ◆unless otherwise directed in the individual monograph, ◆ moisten the residue with a small amount (usually 1 mL) of sulfuric acid; heat gently until white fumes are no longer evolved; and ignite at 600 ± 50°. ◆unless another temperature is specified in the individual monograph, ◆ until the residue is completely incinerated. Ensure that flames are not produced at any time during the procedure. Cool the crucible in a desiccator (silica gel or other suitable desiccant), weigh accurately, and calculate the percentage of residue.

Unless otherwise specified, if the amount of the residue so obtained exceeds the limit specified in the individual monograph, repeat the moistening with sulfuric acid, heating and igniting as before, using a 30-minute ignition period, until two consecutive weighings of the residue do not differ by more than 0.5 mg or until the percentage of residue complies with the limit in the individual monograph.

◆Conduct the ignition in a well-ventilated hood, but protected from air currents, and at as low a temperature as is possible to effect the complete combustion of the carbon. A muffle furnace may be used, if desired, and its use is recommended for the final ignition at 600 ± 50°.

Calibration of the muffle furnace may be carried out using an appropriate digital temperature meter and a working thermocouple probe calibrated against a standard thermocouple



traceable to the National Institute of Standards and Technology.

Verify the accuracy of the measuring and controlling circuitry of the muffle furnace by checking the positions in the furnace at the control set point temperature of intended use. Select positions that reflect the eventual method of use with respect to location of the specimen under test. The tolerance is  $\pm 25^\circ$  at each position measured.♦

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Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Horacio N. Pappa, Ph.D.</a> Senior Scientist and Latin American Liaison 1-301-816-8319	(GC05) General Chapters 05

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### 291 SELENIUM

Stock Solution— Dissolve 40.0 mg of metallic selenium in 100 mL of dilute nitric acid (1 in 2) in a 1000-mL volumetric flask, warming gently on a steam bath if necessary to effect solution, add water to volume, and mix. Pipet 5 mL of this solution into a 200-mL volumetric flask, add water to volume, and mix. Each mL of the resulting solution contains the equivalent of 1  $\mu$ g of selenium (Se).

Diaminonaphthalene Solution— Dissolve 100 mg of 2,3-diaminonaphthalene and 500 mg of hydroxylamine hydrochloride in 0.1 N hydrochloric acid to make 100 mL. Prepare this solution fresh on the day of use.

Standard Solution— Pipet 6 mL of Stock Solution into a 150-mL beaker, and add 25 mL of dilute nitric acid (1 in 30) and 25 mL of water.

Test Solution— Clean combustion of the test material is an important factor in conducting the test. For compounds that burn poorly and produce soot, the addition of magnesium oxide usually results in more thorough combustion and reduces soot formation. Where the need to add magnesium oxide has been identified, it is specified in the individual monograph.

Using a 1000-mL combustion flask and using 25 mL of dilute nitric acid (1 in 30) as the absorbing liquid, proceed as directed under [Oxygen Flask Combustion](#) (471), using a test specimen weighing 100 to 200 mg, unless directed otherwise in the individual monograph. Upon completion of the combustion, place a few mL of water in the cup, loosen the stopper, and rinse the stopper, the specimen holder, and the sides of the flask with about 10 mL of water. Transfer the solution with the aid of about 20 mL of water to a 150-mL beaker, and heat gently to the boiling temperature. Boil for 10 minutes, and allow the solution to cool to room temperature.

Procedure— Treat the Standard Solution, the Test Solution, and the reagent blank consisting of 25 mL of dilute nitric acid (1 in 30) and 25 mL of water, concomitantly and in parallel, as follows. Add ammonium hydroxide solution (1 in 2) to adjust to a pH of  $2.0 \pm 0.2$ . Dilute with water to 60 mL, and transfer to a low-actinic separator with the aid of 10 mL of water, adding the 10 mL of rinsings to the separator. Add 200 mg of hydroxylamine hydrochloride, swirl to dissolve, immediately add 5.0 mL of Diaminonaphthalene Solution, insert the stopper, and swirl to mix. Allow the solution to stand at room temperature for 100 minutes. Add 5.0 mL of cyclohexane, shake vigorously for 2 minutes, and allow the layers to separate. Discard the aqueous layer, and centrifuge the cyclohexane extract to remove any dispersed water. Determine the absorbances of the cyclohexane extracts of the Test Solution and the Standard Solution in a 1-cm cell at the wavelength of maximum absorbance at about 380 nm, with a suitable spectrophotometer, using the cyclohexane extract of the reagent blank as the blank, and compare the absorbances: the absorbance of the Test Solution is not greater than that of the Standard Solution where a 200-mg test specimen has been taken, or is not greater than one-half that of the Standard Solution where a 100-mg test specimen has been taken.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Kahkashan Zaidi, Ph.D.</a> Senior Scientist 1-301-816-8269	(GC05) General Chapters 05

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### 301 ACID-NEUTRALIZING CAPACITY

note—All tests shall be conducted at a temperature of  $37 \pm 3^\circ$ .

Standardization of pH Meter— Standardize a pH meter using the 0.05 m potassium biphthalate and 0.05 m potassium tetraoxalate standardizing buffers as described under [pH](#) (791).

Magnetic Stirrer— Transfer 100 mL of water to a 250-mL beaker containing a 40- x 10-mm (or other suitable size) magnetic stirring bar that is coated with solid perfluorocarbon and has a spin ring at its center. Adjust the power setting of the magnetic stirrer to produce a stirring rate of  $300 \pm 30$  rpm when the stirring bar is centered in the beaker, as determined by a suitable optical tachometer.

#### Test Preparation—

Powders— Transfer the accurately weighed portion of the substance specified in the individual monograph to a 250-mL beaker, add 70 mL of water, and mix on the Magnetic Stirrer for 1 minute.

Effervescent Solids— Transfer an accurately weighed quantity, equivalent to the minimum labeled dosage, to a 250-mL beaker, add 10 mL of water, and swirl the beaker gently while allowing the reaction to subside. Add another 10 mL of water, and swirl gently. Wash the walls of the beaker with 50 mL of water, and mix on the Magnetic Stirrer for 1 minute.

Suspensions and Other Liquids— Shake the container until the contents are uniform, and determine the density. Transfer an accurately weighed quantity of the uniform mixture, equivalent to the minimum labeled dosage, to a 250-mL beaker, add water to make a total volume of about 70 mL, and mix on the Magnetic Stirrer for 1 minute.

Lozenges— Accurately weigh not fewer than 20 lozenges, and determine the average weight. Select and weigh 2 lozenges, and transfer them to a 250-mL beaker containing 70 mL of water.

Nonchewable Tablets— Weigh not fewer than 20 tablets, and determine the average tablet weight. Grind the tablets to a fine powder, mix to obtain a uniform mixture, and transfer an accurately weighed quantity of it, equivalent to the minimum labeled dosage, to a 250-mL beaker. If wetting is desired, add not more than 5 mL of alcohol (neutralized to an apparent pH of 3.5), and mix to wet the specimen thoroughly. Add 70 mL of water, and mix on the Magnetic Stirrer for 1 minute.

Chewable Tablets— Prepare as directed for Nonchewable Tablets.

Tablets That Are Required To Be Chewed— Transfer 1 Tablet to a 250-mL beaker, add 50 mL of water, and mix on the Magnetic Stirrer for 1 minute.

Capsules— Weigh accurately not fewer than 20 capsules. Remove the capsule contents completely, with the aid of a cotton swab if necessary. Accurately weigh the empty capsules, and determine the average weight of the contents per capsule. Mix the combined capsule contents to obtain a uniform mixture, and proceed as directed for Nonchewable Tablets, beginning with "transfer an accurately weighed quantity of it."

Procedure for Powders, Effervescent Solids, Suspensions and Other Liquids, Lozenges, Nonchewable Tablets, Chewable Tablets, and Capsules— Pipet 30.0 mL of 1.0 N hydrochloric acid VS into the Test Preparation while continuing to stir with the Magnetic Stirrer. [note—Where the acid-neutralizing capacity of the specimen under test is greater than 25 mEq, use 60.0 mL of 1.0 N hydrochloric acid VS, and make the appropriate modifications in the calculation.] Stir for 15 minutes, accurately timed, after the addition of the acid, begin to titrate immediately, and in a period not to exceed an additional 5 minutes, titrate the excess hydrochloric acid with 0.5 N sodium hydroxide VS to attain a stable (for 10 to 15 seconds) pH of 3.5. Calculate the number of mEq of acid consumed by the formula:

$$\text{Total mEq} = (30 \times \text{NHCl}) - (\text{VNaOH} \times \text{NNaOH})$$

in which NHCl and NNaOH are the normalities of the hydrochloric acid VS and the sodium hydroxide VS, respectively; and VNaOH is the volume of sodium hydroxide VS used for titration. Express the result in terms of mEq of acid consumed per g of the substance tested.

Procedure for Tablets That Are Required To Be Chewed— Pipet 30.0 mL of 1.0 N hydrochloric acid VS into the Test Preparation while continuing to stir with the Magnetic Stirrer for 10 minutes, accurately timed, after the addition of the acid. Discontinue stirring briefly, and without delay remove any gum base from the beaker using a long needle. Promptly rinse the needle with 20 mL of water, collecting the washing in the beaker, and resume stirring for 5 minutes, accurately timed, then begin to titrate immediately, and in a period not to exceed an additional 5 minutes, titrate the excess hydrochloric acid with 0.5 N sodium hydroxide VS to attain a stable (for 10 to 15 seconds) pH of 3.5. Calculate the number of mEq of acid consumed by the Tablet tested by the formula:

$$\text{Total mEq} = (30 \times \text{NHCl}) - (\text{VNaOH} \times \text{NNaOH})$$

in which the terms are as defined above.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Kahkashan Zaidi, Ph.D.</a> Senior Scientist 1-301-816-8269	(GC05) General Chapters 05

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### 311 ALGINATES ASSAY

#### APPARATUS

The required apparatus (see Figure 1) contains a capillary metering valve, A, followed by a flowmeter, B, to control and monitor the flow of nitrogen through the system. Halogenated vinyl plastic tubing and a rubber fitting, C, are used to connect the flowmeter to a sidearm of a reaction flask, D. Flask D is a 250-ml round-bottom, boiling flask, resting in a suitable heating mantle, E. Flask D is provided with a 225-mm Hopkins coil reflux condenser, F. The condenser terminates in a U-shaped trap, G, which contains two 25-g bands of 20-mesh zinc, the bands being bounded and separated by three 3-inch plugs of glass wool. The trap terminates in an adapter, H, that by means of a halogenated vinyl plastic tubing and a twistcock connector, I, connects with a 250-ml gas washing bottle, J. The inlet (bubbling) tube extends almost to the bottom of the gas washing bottle, and it terminates in a fritted disk having a coarse porosity. The size of all glass joints is 24/40, except for the 45/50 joint of the gas washing bottle.

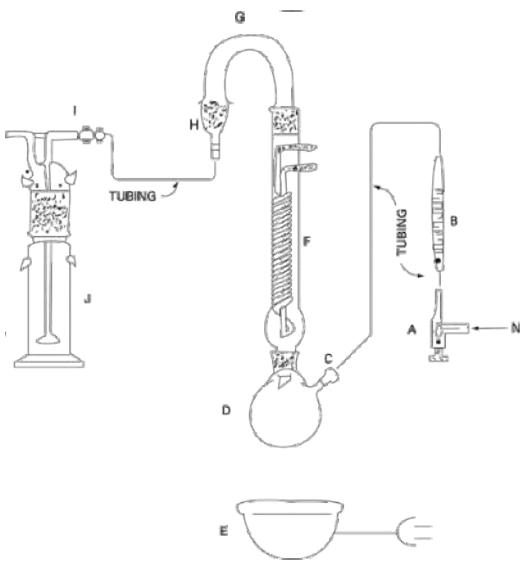


Fig. 1. Apparatus for Alginates Assay

#### SYSTEM SUITABILITY

Using d-glucuronolactone as the standard, proceed as directed for Procedure, but do not perform the preboiling steps. The system is suitable if the following criteria are met: (1) a blank determination results in a net titration value, C, between 0.02 and 0.06 mEq, calculated as follows:

$$\text{Ab} - \text{Bb}$$

in which Ab is the number of mEq of 0.25 N sodium hydroxide in the 25 mL used, and Bb is the number of mEq of 0.1 N hydrochloric acid used in the blank titration; and (2) the percentage of carbon dioxide, CO<sub>2</sub>, obtained from the standard is between 24.2% and 25.7%.

#### PROCEDURE

Unless otherwise directed in the individual monograph, transfer a specimen of about 250 mg, accurately weighed, into the reaction flask, D, add 50 mL of 0.1 N hydrochloric acid, insert several boiling chips, and connect the flask to the reflux condenser, F, using phosphoric acid as a lubricant. [note—Stopcock grease may be used for the other connections.] Connect

the nitrogen line to the sidearm of the flask, and adjust the flow of cooling water to about 2 L per minute.

[note—The following preboiling steps, outlined in this paragraph, are optional and need only be performed when the presence of inorganic carbonates is suspected.] Maintain the flow of nitrogen through the apparatus at 90 to 100 mL per minute. Raise the heating mantle, E, to the flask, heat the specimen to boiling, and boil gently for 2 minutes. Turn the heat off, lower the mantle, E, and allow to cool for about 10 minutes.

Connect the empty gas washing bottle assembly, J, and sweep the system with nitrogen at a rate of 90 to 100 mL per minute for 5 minutes. Reduce the nitrogen flow to 60 to 65 mL per minute, add 10 drops of butyl alcohol, 25.0 mL of 0.25 N sodium hydroxide VS, and 50 mL of distilled water into the bottle, rinsing down the inside of the gas washing bottle, and replace the cap. Detach the rubber fitting, C, from sidearm, and add 46 mL of hydrochloric acid through the sidearm of the boiling flask. Reattach the nitrogen line, raise the heating mantle, and heat the reaction mixture to boiling. After 2 hours of boiling, increase the nitrogen flow to 90 to 100 mL per minute, discontinue the heating, and lower the mantle. Allow to cool for 10 minutes. Disconnect, and disassemble the gas washing bottle. Using a directed stream of distilled water, thoroughly rinse all parts of the bubbling tube and cap, collecting the washings in the gas washing bottle. Use nitrogen to gently force all water out of the bubbling tube. To the bottle immediately add 10 mL of 10% barium chloride solution and a stirring bar. Insert a tight stopper, and stir gently for 1 minute. Allow to stand for at least 5 minutes. Add three drops of phenolphthalein TS, and titrate with 0.1 N hydrochloric acid VS.

Perform a blank determination (see Residual Titrations under [Titrimetry \(541\)](#)). Calculate the percentage of carbon dioxide, CO<sub>2</sub>, by the formula:

$$2200[(A - B) - C]/(1000W)(1 - D)$$

in which A is the number of mEq of 0.25 N sodium hydroxide in the 25 mL used; B is the number of mEq of 0.1 N hydrochloric acid used for the titration of the sample or the standard; C is the net titration value calculated in the blank determination; W is the weight, in g, of the sample or the standard taken; and D is the percentage expressed as a decimal (1 decimal place), obtained in the test for Loss on drying for the sample or for the standard.

\* This type of tubing is commonly referred to as Tygon tubing. This note is added for clarity and it does not constitute USP's endorsement of this product.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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### 331 AMPHETAMINE ASSAY

[USP Reference Standards \(11\)](#)—[USP Dextroamphetamine Sulfate RS](#).

Standard Preparation— Dissolve a suitable quantity of USP Dextroamphetamine Sulfate RS, accurately weighed, in 2 N sulfuric acid (saturated with chloroform), and dilute quantitatively with the same solvent to obtain a solution having a known concentration of about 0.5 mg of dextroamphetamine sulfate per mL.

Assay Preparation— Prepare as directed in the individual monograph.

Preparation of Chromatographic Column (see [Chromatography \(621\)](#))—Pack a pledge of fine glass wool in the base of a 25- × 300-mm chromatographic tube. Place 2 g of purified siliceous earth in a 100-mL beaker, add 1 mL of 0.1 N hydrochloric acid, and mix until a fluffy mixture is obtained. Transfer the mixture to the column, and tamp moderately to compress the material into a uniform mass. Transfer the Assay Preparation to the column, dry-rinse the beaker with 1 g of purified siliceous earth, and transfer to the column. Tamp a pledge of fine glass wool into place at the top of the column.

Procedure— Wash the column with 100 mL of chloroform previously saturated with water, and discard the washings. Place under the column, as a receiver, a 125-mL separator containing 10.0 mL of 2 N sulfuric acid previously saturated with chloroform. Pass through the column 35 mL of ammoniacal chloroform, prepared by equilibrating 2 mL of ammonium hydroxide and 100 mL of chloroform, and complete the elution with 70 mL of chloroform previously saturated with water. Remove the separator, shake vigorously for 1 minute, allow the layers to separate, discard the chloroform layer, and use the 10.0-mL acid solution of the sulfate salt of the amphetamine as the Assay Solution. Concomitantly determine the absorbance of the solution from the Standard Preparation and that of the Assay Solution in 1-cm cells at 280 nm and at the wavelength of maximum absorbance at about 257 nm, with a suitable spectrophotometer, using 2 N sulfuric acid previously saturated with chloroform as the blank. Record the absorbance of the solution from the Standard Preparation as AS and that of the Assay Solution as AU, and calculate as directed in the individual monograph.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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Reference Standards	Lili Wang, Technical Services Scientist 1-301-816-8129 <a href="mailto:RSTech@usp.org">RSTech@usp.org</a>	

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### 341 ANTIMICROBIAL AGENTS—CONTENT

An essential component of Injections preserved in multiple-dose containers is the agent or agents present to reduce the hazard of having introduced, in the course of removing some of the contents, accidental microbial contamination of the contents remaining. It is a Pharmacopeial requirement that the presence and amount added of such agent(s) be declared on the label of the container. The methods provided herein for the most commonly used agents are to be used to demonstrate that the declared agent is present but does not exceed the labeled amount by more than 20% of the labeled amount.

The concentration of an antimicrobial preservative added to a multiple-dose or single-dose parenteral, otic, nasal, and ophthalmic preparation may diminish during the shelf life of the product. Because it is recognized that the antimicrobial preservative concentration in a given preparation may decrease during the product's shelf life, the manufacturer shall determine the lowest level at which the preservative is effective, and the product should be so formulated as to assure that this level is exceeded throughout the product's shelf life. At the time of its manufacture, the product should contain the declared amount of antimicrobial preservative (within  $\pm 20\%$  to allow for manufacturing and analytical variations). The quantitative label statement of the preservative content is not intended to mean that the labeled quantity is retained during the shelf life of the product; rather, it is a statement of the amount added, within process limits, and which is not exceeded by more than 20%. An example of such a label statement is "\_\_\_\_(unit) added as preservative." [note—"\_\_\_\_(unit)" would be a number followed by the unit of measurement, e.g., 0.015 mg per mL or 0.1%.]

The most commonly used agents include the two mercurials, phenylmercuric nitrate and thimerosal and the four homologous esters of p-hydroxybenzoic acid, phenol, benzyl alcohol,



and chlorobutanol. The methods for the first two named are polarographic, while quantitative gas chromatography is employed in the determination of the other agents.

#### GENERAL GAS CHROMATOGRAPHIC METHOD

The general procedures set forth in the following paragraphs are applicable to the quantitative determination of benzyl alcohol, chlorobutanol, phenol, and the methyl, ethyl, propyl, and butyl esters of *p*-hydroxybenzoic acid, the latter being treated as a group, the individual members of which, if present, are capable of separate determination. Prepare the Internal

Standard Solution and the Standard Preparation for each agent as directed individually below. Unless otherwise directed below, prepare the Test Preparation from accurately measured portions of the Internal Standard Solution and the sample under test, of such size that the concentration of the agent and the composition of the solvent correspond closely to the concentration and composition of the Standard Preparation. Suggested operating parameters of the gas chromatograph apparatus are given in the accompanying table, the carrier gas being helium or nitrogen, and the detector being the flame-ionization type.

##### Suggested Operating Parameters of Gas Chromatograph Apparatus

Agent	Column Size		Column Packing Phases and Support	Flow Rate, mL per minute	Column Temperature
	Length	ID			
Benzyl Alcohol	1.8 m	3 mm	5% G16/S1A	50	140 <sup>v</sup>
Chlorobutanol	1.8 m	2 mm	5% G16/S1A	20	110 <sup>a</sup>
Phenol	1.2 m	3 mm	5% G16/S1A	50	145 <sup>a</sup>
Parabens	1.8 m	2 mm	5% G2/S1A	20	150 <sup>a</sup>

##### Benzyl Alcohol

Internal Standard Solution— Dissolve about 380 mg of phenol in 10 mL of methanol contained in a 200-mL volumetric flask. Add water to volume, and mix.

Standard Preparation— Dissolve about 180 mg of [USP Benzyl Alcohol RS](#), accurately weighed, in 20.0 mL of methanol contained in a 100-mL volumetric flask. Add Internal Standard Solution to volume, and mix.

Procedure— Separately inject equal volumes (about 5  $\mu$ L) of the Standard Preparation and the Test Preparation into the chromatograph, record the chromatograms with the apparatus adjusted to the parameters set forth in the accompanying table, and measure the areas under the peaks for benzyl alcohol and phenol. Calculate the content, in mg per mL, of benzyl alcohol ( $C_7H_8O$ ) in the specimen taken by the formula:

$$100(C/V)(p1 / p2)(P2 / P1)$$

in which C is the concentration, in mg per mL, of benzyl alcohol in the Standard Preparation; V is the volume, in mL, of the specimen under test used in preparing each 100 mL of the Test Preparation; p1 and p2 are the peak areas for benzyl alcohol and phenol, respectively, obtained from the Test Preparation; and P1 and P2 are the peak areas of benzyl alcohol and phenol, respectively, obtained from the Standard Preparation.

##### Chlorobutanol

Internal Standard Solution— Transfer about 140 mg of benzaldehyde to a 100-mL volumetric flask, add 10 mL of methanol, and swirl to dissolve. Dilute with water to volume, and mix.

Standard Preparation— Transfer about 125 mg of [USP Chlorobutanol RS](#), accurately weighed, to a 25-mL volumetric flask. Add 2 mL of methanol, swirl to dissolve, dilute with water to volume, and mix. Transfer 5.0 mL of this solution and 5.0 mL of Internal Standard Solution to a 25-mL flask, and mix to obtain a solution having a known concentration of about 2.5 mg of chlorobutanol per mL.

Test Preparation— Quantitatively dilute, if necessary, an accurately measured volume of the specimen under test with methanol to obtain a solution containing not more than about 5.0 mg of chlorobutanol per mL. Combine 3.0 mL of this solution with 3.0 mL of Internal Standard Solution, and mix.

Chromatographic System (see [Chromatography](#) 621)— [note—See accompanying table for column dimensions, column packing phase and support, flow rate, and column temperature.] The injection port temperature is maintained at 180<sup>o</sup>, and the detector temperature is maintained at 220<sup>o</sup>. Chromatograph the Standard Preparation, and record the peak responses as directed for Procedure: the relative retention times are about 0.8 for benzaldehyde and 1.0 for chlorobutanol; the resolution, R, between benzaldehyde and the chlorobutanol is not less than 2.0; and the relative standard deviation for replicate injections is not more than 2.0%.

Procedure— Separately inject equal volumes (about 1  $\mu$ L) of the Standard Preparation and the Test Preparation into the chromatograph, record the chromatograms, and measure the areas for the major peaks. Calculate the quantity, in mg, of chlorobutanol ( $C_4H_7Cl_3O$ ) in each mL of the specimen under test by the formula:

$$C(L/D)(RU / RS)$$

in which C is the concentration, in mg per mL, of chlorobutanol, calculated on the anhydrous basis, in the Standard Preparation; L is the labeled quantity, in mg, of chlorobutanol in each mL of the specimen under test; D is the concentration, in mg per mL, of chlorobutanol in the Test Preparation, based on the volume of specimen under test taken and the extent of dilution; and RU and RS are the ratios of the chlorobutanol peak to the benzaldehyde peak obtained from the Test Preparation and the Standard Preparation, respectively.

##### Phenol

Internal Standard Solution— Pipet 1 mL of [USP Benzyl Alcohol RS](#) into a 500-mL volumetric flask, add methanol to volume, and mix.

Standard Preparation— Dissolve about 75 mg of [USP Phenol RS](#), accurately weighed, in 7.5 mL of methanol contained in a 100-mL volumetric flask. Add 20.0 mL of Internal Standard Solution, then add water to volume, and mix.

Procedure— Separately inject equal volumes (about 3  $\mu$ L) of the Standard Preparation and the Test Preparation into the chromatograph, record the chromatograms with the apparatus adjusted to the parameters set forth in the accompanying table, and measure the areas under the peaks for phenol and benzyl alcohol. Calculate the content, in mg per mL, of phenol ( $C_6H_6O$ ) in each mL of the specimen taken by the formula:

$$100(C/V)(p1 / p2)(P2 / P1)$$

in which C is the concentration, in mg per mL, of phenol in the Standard Preparation; V is the volume, in mL, of the specimen under test used in preparing each 100 mL of the Test Preparation; p1 and p2 are the peak areas for phenol and benzyl alcohol, respectively, obtained from the Test Preparation; and P1 and P2 are the peak areas of phenol and benzyl alcohol, respectively, obtained from the Standard Preparation.

##### Methylparaben and Propylparaben

Internal Standard Solution— Place about 200 mg of benzophenone in a 250-mL volumetric flask, dilute with ether to volume, and mix.

Standard Preparation— Place 100 mg of [USP Methylparaben RS](#) and 10 mg of [USP Propylparaben RS](#), each accurately weighed, in a 200-mL volumetric flask, dilute with Internal Standard Solution to volume, and mix. Place 10 mL of this solution in a 25-mL conical flask, and proceed as directed for Test Preparation, beginning with “Add 3 mL of pyridine.”

Test Preparation— Pipet 10 mL of the specimen under test and 10 mL of the Internal Standard Solution into a small separator. Shake vigorously, allow the layers to separate, draw off the aqueous layer into a second separator, and transfer the ether layer into a small flask through a funnel containing anhydrous sodium sulfate. Extract the aqueous layer with two 10-mL portions of ether, also filtering the extracts through the anhydrous sodium sulfate. Evaporate the combined extracts under a current of dry air until the volume is reduced to about 10 mL, then transfer the residue to a 25-mL conical flask. Add 3 mL of pyridine, complete the evaporation of the ether, and boil on a hot plate until the volume is reduced to about 1 mL.

Cool, and add 1 mL of a suitable silylation agent, such as bis(trimethylsilyl)trifluoracetamide, bis(trimethylsilyl)acetamide, or a mixture of hexamethyldisilazane and trimethylchlorosilane [2:1 or 3:1 (v/v)]. Mix, and allow to stand for not less than 15 minutes.

Procedure— Separately inject equal volumes (2  $\mu$ L) of the silylated solution from the Standard Preparation and the Test Preparation into the chromatograph, record the chromatograms with the apparatus adjusted to the parameters set forth in the accompanying table, and measure the areas under the peaks for methylparaben, propylparaben, and



benzophenone. Calculate the content, in  $\mu\text{g}$  per mL, of methylparaben (C<sub>8</sub>H<sub>8</sub>O<sub>3</sub>) in the sample under test by the formula:

$$10(\text{CM} / \text{V})(\text{p1} / \text{p3})(\text{P3} / \text{P1})$$

in which CM is the concentration, in  $\mu\text{g}$  per mL, of methylparaben in the Standard Preparation; V is the volume, in mL, of the specimen taken; p1 and p3 are the peak areas for methylparaben and benzophenone, respectively, obtained from the Test Preparation; and P1 and P3 are the peak areas of methylparaben and benzophenone, respectively, obtained from the Standard Preparation. Similarly, calculate the content, in  $\mu\text{g}$  per mL, of propylparaben (C<sub>10</sub>H<sub>12</sub>O<sub>3</sub>) in the specimen under test by the formula:

$$10(\text{CP} / \text{V})(\text{p2} / \text{p3})(\text{P3} / \text{P2})$$

in which CP is the concentration, in  $\mu\text{g}$  per mL, of propylparaben in the Standard Preparation; V is the volume, in mL, of the specimen taken; p2 and p3 are the peak areas for propylparaben and benzophenone, respectively, obtained from the Test Preparation; and P2 and P3 are the peak areas of propylparaben and benzophenone, respectively, obtained from the Standard Preparation.

Ethylparaben and butylparaben may be determined in a similar manner.

#### POLAROGRAPHIC METHOD

##### Phenylmercuric Nitrate

Standard Preparation— Dissolve about 100 mg of phenylmercuric nitrate, accurately weighed, in sodium hydroxide solution (1 in 250) contained in a 1000-mL volumetric flask, warming if necessary to effect solution, add the sodium hydroxide solution to volume, and mix. Pipet 10 mL of this solution into a 25-mL volumetric flask, and proceed as directed under Test Preparation, beginning with "add 2 mL of potassium nitrate solution (1 in 100)."

Test Preparation— Pipet 10 mL of the specimen under test into a 25-mL volumetric flask, add 2 mL of potassium nitrate solution (1 in 100) and 10 mL of pH 9.2 alkaline borate buffer (see under Buffer Solutions in the section [Reagents, Indicators, and Solutions](#)), and adjust to a pH of 9.2, if necessary, by the addition of 2 N nitric acid. Add 1.5 mL of freshly prepared gelatin solution (1 in 1000), then add the pH 9.2 alkaline borate buffer to volume, and mix.

Procedure— Pipet a portion of the Test Preparation into the polarographic cell, and deaerate by bubbling nitrogen through the solution for 15 minutes. Insert the dropping mercury electrode of a suitable polarograph (see [Polarography](#) 801), and record the polarogram from  $-0.6$  to  $-1.5$  volts versus the saturated calomel electrode. Determine the diffusion current of the Test Preparation, (id)U, as the difference between the residual current and the limiting current. Similarly and concomitantly determine the diffusion current, (id)S, of the Standard Preparation. Calculate the quantity, in  $\mu\text{g}$ , of phenylmercuric nitrate (C<sub>6</sub>H<sub>5</sub>HgNO<sub>3</sub>) in each mL of the specimen taken by the formula:

$$2.5\text{C}[(\text{id})\text{U} / (\text{id})\text{S}]$$

in which C is the concentration, in  $\mu\text{g}$  per mL, of phenylmercuric nitrate in the Standard Preparation.

##### Thimerosal

Standard Preparation— On the day of use, place about 25 mg of [USP Thimerosal RS](#), accurately weighed, in a 250-mL volumetric flask, add water to volume, and mix. Protect from light. Pipet 15 mL of this solution into a 25-mL volumetric flask, add 1.5 mL of gelatin solution (1 in 1000), then add potassium nitrate solution (1 in 100) to volume, and mix.

Test Preparation— Pipet 15 mL of the test specimen into a 25-mL volumetric flask, add 1.5 mL of gelatin solution (1 in 1000), add potassium nitrate solution (1 in 100) to volume, and mix.

Procedure— Transfer a portion of the Test Preparation to a polarographic cell, and deaerate by bubbling nitrogen through the solution for 15 minutes. Insert the dropping mercury electrode of a suitable polarograph (see [Polarography](#) 801), and record the polarogram from  $-0.2$  to  $-1.4$  volts versus the saturated calomel electrode. Determine the diffusion current, (id)U, as the difference between the residual current and the limiting current. Similarly and concomitantly determine the diffusion current, (id)S, of the Standard Preparation.

Calculate the quantity, in  $\mu\text{g}$ , of thimerosal (C<sub>6</sub>H<sub>9</sub>HgNaO<sub>2</sub>S) in each mL of the test specimen taken by the formula:

$$1.667\text{C}[(\text{id})\text{U} / (\text{id})\text{S}]$$

in which C is the concentration, in  $\mu\text{g}$  per mL, of thimerosal in the Standard Preparation; and the other terms are as defined therein.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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#### 345 ASSAY FOR CITRIC ACID/CITRATE AND PHOSPHATE

The following ion chromatographic general procedure is provided for the determination of citric acid/citrate and phosphate in compendial articles, when specified in the individual monographs. Identification tests for citrate and phosphate are provided separately under USP general chapter [Identification Tests—General](#) 191. The procedure for preparation of the Standard Preparations used for the assay depends on whether or not citrate and phosphate are being assayed concomitantly, as indicated below.

[USP Reference Standards](#) 11—[USP Citric Acid RS](#).

Mobile Phase— Transfer an appropriate volume of water (resistivity not less than 18 megohm-cm) to a suitable container, and degas with helium for not less than 20 minutes. Add an appropriate volume of 50% (w/w) carbonate-free sodium hydroxide or potassium hydroxide to obtain a 20 mM sodium hydroxide or potassium hydroxide solution. Alternatively, a 20 mM sodium hydroxide or potassium hydroxide eluant can be generated electrolytically using an automatic eluant generator. [note—Protect the Mobile Phase from atmospheric carbon dioxide.]

Standard Preparations— Use Standard Preparation 1 for an assay for citric acid/citrate only. Use Standard Preparation 2 when a concomitant assay for citrate and phosphate is intended.

Standard Preparation 1—Dissolve [USP Citric Acid RS](#) in freshly prepared 1 mM sodium hydroxide to obtain a solution having a known concentration of about 20  $\mu\text{g}$  per mL of citrate (C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>).

Standard Preparation 2—Dissolve [USP Citric Acid RS](#) and monobasic sodium phosphate in freshly prepared 1 mM sodium hydroxide to obtain a solution having known concentrations of about 20  $\mu\text{g}$  per mL and 12  $\mu\text{g}$  per mL of citrate and phosphate (PO<sub>4</sub>), respectively.

Assay Preparation for Citric Acid/Citrate Assay— Unless stated otherwise in the monograph, dissolve an appropriate quantity of a solid dosage form in freshly prepared 1 mM sodium hydroxide to obtain a solution containing about 20  $\mu\text{g}$  per mL of citrate. If the dosage form is a liquid formulation, dilute with water, and add a freshly prepared sodium hydroxide solution to obtain a solution containing about 20  $\mu\text{g}$  per mL of citrate in 1 mM sodium hydroxide.

Assay Preparation for Phosphate Assay— Unless stated otherwise in the monograph, dissolve an appropriate quantity of a solid dosage form in freshly prepared 1 mM sodium

hydroxide to obtain a solution containing about 12  $\mu\text{g}$  per mL of phosphate. If the dosage form is a liquid formulation, dilute with water, and add a freshly prepared sodium hydroxide solution to obtain a solution containing about 12  $\mu\text{g}$  per mL of phosphate in 1 mM sodium hydroxide.

Chromatographic System (see [Chromatography 621](#))—The liquid chromatograph is equipped with a suitable anion trap column; a 4-mm  $\times$  50-mm guard column and a 4-mm  $\times$  250-mm analytical column, both packed with L61 packing; and an electrochemical detector with suppressed conductivity detection using either a micromembrane anion autosuppressor or a suitable chemical suppression system. All columns are maintained at a temperature of 30° and eluted at a flow rate of 2 mL per minute. [note—An anion trap column designed to remove trace anion contaminants in the Mobile Phase should be added to the column assembly before the injector.] Chromatograph Standard Preparation 1 or Standard Preparation 2, as appropriate, and record the peak area responses as directed for Procedure: the tailing factor is not more than 2.0; and the relative standard deviation of the peak areas for citrate (and phosphate where appropriate), for six replicate injections of Standard Preparation 1 or Standard Preparation 2, is not more than 1.5%.

Procedure— Separately inject 10  $\mu\text{L}$  each of the appropriate Standard Preparation and the Assay Preparation into the chromatograph, record the chromatograms, and measure the peak areas for citrate and phosphate, as appropriate. Determine the concentrations of citrate or phosphate in the portion of Assay Preparation taken by the formula:

$$\text{CS (rU / rS)}$$

in which CS is the concentration of citrate or phosphate, in  $\mu\text{g}$  per mL, in the appropriate Standard Preparation; and rU and rS are the peak areas of citrate or phosphate obtained from the Assay Preparation and the Standard Preparation, respectively.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

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### 351 ASSAY FOR STEROIDS

The following procedure is applicable for determination of those Pharmacopeial steroids that possess reducing functional groups such as  $\text{C}=\text{O}$ -ketols.

Standard Preparation— Dissolve in alcohol a suitable quantity of the USP Reference Standard specified in the individual monograph, previously dried under the conditions specified in the individual monograph and accurately weighed, and dilute quantitatively and stepwise with alcohol to obtain a solution having a concentration of about 10  $\mu\text{g}$  per mL. Pipet 20 mL of this solution into a glass-stoppered, 50-mL conical flask.

Assay Preparation— Prepare as directed in the individual monograph.

Procedure— To each of the two flasks containing the Assay Preparation and the Standard Preparation, respectively, and to a similar flask containing 20.0 mL of alcohol to serve as the blank, add 2.0 mL of a solution prepared by dissolving 50 mg of blue tetrazolium in 10 mL of methanol, and mix. Then to each flask add 2.0 mL of a mixture of alcohol and tetramethylammonium hydroxide TS (9:1), mix, and allow to stand in the dark for 90 minutes. Without delay, concomitantly determine the absorbances of the solutions from the Assay Preparation and the Standard Preparation at about 525 nm, with a suitable spectrophotometer, against the blank. Calculate the result by the formula given in the individual monograph, in which C is the concentration, in  $\mu\text{g}$  per mL, of the Reference Standard in the Standard Preparation; and AU and AS are the absorbances of the solutions from the Assay Preparation and the Standard Preparation, respectively.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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### 361 BARBITURATE ASSAY

Internal Standard, Internal Standard Solution, Standard Preparation, and Assay Preparation— Prepare as directed in the individual monograph.

Chromatographic System— Under typical conditions, the gas chromatograph is equipped with a flame-ionization detector and contains a 4-mm  $\times$  0.9-m glass column packed with 3% liquid phase G10 on support 80- to 100-mesh S1A. The column is maintained at a temperature of  $200 \pm 10^\circ$ , and the injection port and detector are maintained at about  $225^\circ$ , the column temperature being varied within the designated tolerance, as necessary, to meet System Suitability specifications and provide suitable retention times. Use a suitable carrier gas, such as dry nitrogen, at an appropriate flow rate, such as 60 to 80 mL per minute. Use on-column injection. [note—If the instrument is not equipped for on-column injection, use an injection port lined with glass that has been washed successively with chromic acid cleansing solution, water, methanol, chloroform, a 1 in 10 solution of trimethylchlorosilane in chloroform, and chloroform.]

System Suitability (see [Chromatography 621](#))— Chromatograph five replicate injections of the Standard Preparation, and record peak responses as directed for Procedure: the relative standard deviation for the ratio RS is not more than 1.5%. In a suitable chromatogram, the resolution, R, between the barbituric acid and the Internal Standard is not less than the value given in the individual monograph, and the tailing factor, T, for each of the two peaks is not more than 2.0.

Procedure— Inject a suitable portion (about 5  $\mu\text{L}$ ) of the Standard Preparation into a suitable gas chromatograph, and record the chromatogram. Similarly inject a suitable portion of the Assay Preparation, and record the chromatogram. Calculate the content of the barbituric acid or barbituric acid in the assay specimen by the formula given in the individual monograph, in which RU is the ratio of the peak response of the barbituric acid to that of the Internal Standard obtained for the Assay Preparation; QS is the ratio of the weight of the barbituric acid to that of the Internal Standard in the Standard Preparation; Ci is the concentration, in mg per mL, of Internal Standard in the Internal Standard Solution; and RS is the ratio of the peak response of the barbituric acid to that of the Internal Standard in the Standard Preparation.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.



Topic/Question	Contact	Expert Committee
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## 371 COBALAMIN RADIOTRACER ASSAY

All radioactive determinations required by this method should be made with a suitable counting assembly over a period of time optimal for the particular counting assembly used. All procedures should be performed in replicate to obtain the greatest accuracy.

USP Reference Standards (11) — [USP Cyanocobalamin RS](#).

**Cyanocobalamin Tracer Reagent**— Dilute an accurately measured volume of a solution of radioactive cyanocobalamin\* with water to yield a solution having a radioactivity between 500 and 5000 counts per minute per mL. Add 1 drop of cresol per L of solution prepared, and store in a refrigerator.

**Standardization**— Prepare a solution of a weighed quantity of [USP Cyanocobalamin RS](#) in water to contain 20 to 50 µg per mL. Perform the entire assay on a 10.0-mL portion of this solution, proceeding as directed under Assay Preparation, beginning with "Add water to make a measured volume."

**Cresol—Carbon Tetrachloride Solution**— Mix equal volumes of carbon tetrachloride and freshly distilled cresol.

**Phosphate—Cyanide Solution**— Dissolve 100 mg of potassium cyanide in 1000 mL of a saturated solution of dibasic sodium phosphate, and mix.

**Butanol-Benzalkonium Chloride Solution**— Dilute benzalkonium chloride solution (17 in 100) with water (3:1), and mix with 36 volumes of butyl alcohol.

**Alumina-Resin Column**— Place a pledge of glass wool in the bottom of a constricted glass tube such as a 50-mL buret. With the tube held in an upright position, add a volume of a slurry of ion-exchange resin (see in the section [Reagents, Indicators, and Solutions](#)), in water, sufficient to give a column of settled resin 7 cm in height. When the solid has settled somewhat, allow the water to drain so that there is only 1 cm of liquid above the resin column, and tamp the resin lightly. Then add a volume of a slurry of anhydrous alumina (not acid-washed) in water sufficient to increase the height of the settled column to 10 cm, and allow the water to drain to about 1 cm from the top of the alumina. Add a pledge of glass wool, and wash the column, using a total of 50 mL of water, and again drain to within 1 cm of the top of the column. Prepare a fresh column for each determination.

**Assay Preparation**— Transfer to a beaker a weighed quantity or measured volume of the preparation to be assayed, equivalent in vitamin B12 activity to that of 200 to 500 µg of cyanocobalamin. Add water to make a measured volume of not less than 25 mL, then add 5.0 mL of Cyanocobalamin Tracer Reagent. Add, while working under a hood, 5 mg of sodium nitrite and 2 mg of potassium cyanide for each mL of the resulting solution. Adjust the solution with diluted hydrochloric acid to a pH of approximately 4, and heat on a steam bath for 15 minutes. Cool, and adjust the solution with 1 N sodium hydroxide to a pH between 7.6 and 8.0. Centrifuge or filter to remove any undissolved solids.

**Procedure**— Transfer the Assay Preparation to a 250-mL centrifuge bottle, add 10 mL of Cresol—Carbon Tetrachloride Solution, suitably close the bottle with a glass, polyethylene, or foil-wrapped rubber stopper, shake vigorously for 2 to 5 minutes, and centrifuge. Remove and save the lower, solvent layer. Repeat the extraction using a 5-mL portion of Cresol—Carbon Tetrachloride Solution, and combine the lower, solvent-layer extracts in a centrifuge bottle or separator of 50- to 100-mL capacity.

Wash the combined extracts with successive 10-mL portions of 5 N sulfuric acid until the last washing is practically colorless (two washings usually suffice). During each washing, shake for 2 to 5 minutes, allow the layers to separate, centrifuge, if necessary, and discard the acid layer. Wash further with two successive 10-mL portions of Phosphate—Cyanide Solution. Finally, wash with 10 mL of water. Discard all of the washings.

To the washed extract add 30 mL of a mixture of Butanol-Benzalkonium Chloride Solution and carbon tetrachloride (2:1). Extract with two 5-mL portions of water, each time shaking vigorously for 1 minute, centrifuging, and removing and saving the upper, aqueous layer.

Pass the combined aqueous extracts through the Alumina-Resin Column at a rate of about 1 mL per minute, maintaining a 1-cm layer of liquid on the head of the column by adding water as needed. Discard as much of the forerun as is colorless (usually about 5 mL), and collect the colored eluate (usually about 10 mL) in a 50-mL centrifuge tube or separator containing 500 µL of diluted acetic acid. Extract the eluate by shaking for 2 to 5 minutes with 5 mL of Cresol—Carbon Tetrachloride Solution, and discard the upper, aqueous layer. To the extract add 5.0 mL of water, 5 mL of carbon tetrachloride, and 10 mL of butyl alcohol. Shake, allow to separate until the upper layer is clear, and remove the upper, aqueous layer.

Determine the absorbances of the aqueous extract, in a 1-cm cell, at 361 nm and 550 nm, with a suitable spectrophotometer, using a tungsten light source. Make the 361-nm reading using a filter capable of reducing stray light. Calculate the ratio A361/A550: the purity of the aqueous extract is acceptable if the ratio is between 3.10 and 3.40. If a ratio outside this range is observed, purify the aqueous extract by repeating the extraction cycle, proceeding as directed in the foregoing paragraph.

If an acceptable absorbance ratio is observed in the aqueous extract, determine the radioactivity, in counts per minute, using a suitable counter over a period optimal for the particular counting assembly used. Average the results, and correct the average for the observed background radioactivity determined over two or more 30-minute periods.

**Calculation**— Calculate the cobalamin content, expressed in µg of cyanocobalamin, of the portion taken for assay by the formula:

$$R(CS / CU)(AU / AS)$$

in which R is the quantity, in µg, of cyanocobalamin in the portion of the standard solution taken; CS and CU are the corrected average radioactivity values, expressed in counts per minute per mL, of the standard and assay solutions, respectively; and AU and AS are the absorbances determined at 361 nm of the assay and standard solutions, respectively.

\* A solution of cyanocobalamin made radioactive by the incorporation of 60Co is available from Merck and Co., Inc., Rahway, NJ 07065.

**Auxiliary Information**— Please [check for your question in the FAQs](#) before contacting USP.

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## 381 ELASTOMERIC CLOSURES FOR INJECTIONS



Change to read:

## INTRODUCTION

Elastomeric closures for containers used in the types of preparations defined in the general test chapter [Injections](#) (1) are made of materials obtained by vulcanization (cross-linking) polymerization, polyaddition, or polycondensation of macromolecular organic substances (elastomers). Closure formulations contain natural or synthetic elastomers and inorganic and organic additives to aid or control vulcanization, impart physical and chemical properties or color, or stabilize the closure formulation.

- This chapter applies to closures used for long-term storage of preparations defined in the general test chapter [Injections](#) (1). Such closures are typically used as part of a vial, bottle, or pre-fill syringe package system. • (RB 1-May-2009)

This chapter applies to closures formulated with natural or synthetic elastomeric substances. This chapter does not apply to closures made from silicone elastomer; however, it does apply to closures treated with silicone (e.g., Dimethicone, NF). When performing the tests in this chapter, it is not required that closures be treated with silicone, although there is no restriction prohibiting the use of siliconized closures.

- This chapter also applies to closures coated with other lubricious materials (e.g., materials chemically or mechanically bonded to the closure) that are not intended to, and in fact do not provide, a barrier to the base elastomer. When performing the tests, closures with lubricious non-barrier coatings are to be tested in their coated state.

The following comments relate solely to closures laminated or coated with materials intended to provide, or in fact function as, a barrier to the base elastomer (e.g., PTFE or lacquer coatings). It is not permissible to use a barrier material in an attempt to change a closure that does not meet compendial requirements to one that does conform. Therefore, all Physicochemical Tests apply to the base formula of such closures, as well as to the coated or laminated closure. To obtain Physicochemical Tests results, the tests are to be performed on uncoated or non-laminated closures of the same elastomeric compound, as well as to the laminated or coated closure. The Functionality Tests apply to and are to be performed using the laminated or coated elastomeric closure. Biological Tests apply to the lamination or coating material, as well as to the base formula. Biological Tests may be performed on the laminated or coated closure, or they may be performed on the laminate/coating material and the uncoated or non-laminated closures of the same elastomeric compound. In the latter case, the results are to be reported separately. The base formula used for physicochemical or biological tests intended to support the compendial compliance of a barrier-coated closure should be similar to the corresponding coated closure in configuration and size.

For all [Elastomeric Closures for Injection](#) (381) tests performed on any closure type, it is important to document the closure being tested, including a full description of the elastomer, and any lubrication, coating, laminations, or treatments applied. • (RB 1-May-2009)

This chapter states test limits for Type I and Type II elastomeric closures. Type I closures are those used for aqueous preparations. Type II closures are typically intended for nonaqueous preparations and are those which, having properties optimized for special uses, may not meet all requirements listed for Type I closures because of physical configuration, material of construction, or both. If a closure fails to meet one or more of the Type I test requirements, but still meets the Type II requirements for the test(s), the closure is assigned a final classification of Type II.

This chapter is intended as an initial screen to identify elastomeric closures that might be appropriate for use with injectable preparations on the basis of their biological compatibility, their aqueous extract physicochemical properties, and their functionality. All elastomeric closures suitable for use with injectable preparations comply with either Type I or Type II test limits. However, this specification is not intended to serve as the sole evaluation criteria for the selection of such closures.

The following closure evaluation requirements are beyond the scope of this chapter:

- The establishment of closure identification tests and specifications
- The verification of closure-product physicochemical compatibility
- The identification and safety determination of closure leachables found in the packaged product
- The verification of packaged product closure functionality under actual storage and use conditions

The manufacturer of the injectable product (the end user) must obtain from the closure supplier an assurance that the composition of the closure does not vary and that it is the same as that of the closure used during compatibility testing. When the supplier informs the end user of changes in the composition, compatibility testing must be repeated, totally or partly, depending on the nature of the changes. Closures must be properly stored, cleaned for removal of environmental contaminants and endotoxins, and sterilized prior to use in packaging injectable products.

## CHARACTERISTICS

Elastomeric closures are translucent or opaque and have no characteristic color, the latter depending on the additives used. They are homogeneous and practically free from flash and adventitious materials (e.g., fibers, foreign particles, and waste rubber.)

## IDENTIFICATION

Closures are made of a wide variety of elastomeric materials and optional polymeric coatings. For this reason, it is beyond the scope of this chapter to specify identification tests that encompass all possible closure presentations. However, it is the responsibility of the closure supplier and the injectable product manufacturer (the end user) to verify the closure elastomeric formulation and any coating or laminate materials used according to suitable identification tests. Examples of some of the analytical test methodologies that may be used include specific gravity, percentage of ash analysis, sulfur content determination, FTIR-ATR test, thin-layer chromatography of an extract, UV absorption spectrophotometry of an extract, or IR absorption spectrophotometry of a pyrolyzate.

Change to read:

## TEST PROCEDURES

Elastomeric closures shall conform to biological, physicochemical, and functionality requirements both as they are shipped by the closure supplier to the injectable product manufacturer (the end user), and in their final ready-to-use state by the end user.

For those elastomeric closures processed by the supplier prior to distribution to the end user, the supplier shall demonstrate compendial conformance of closures exposed to such processing and/or sterilization steps. Similarly, if elastomeric closures received by the end user are subsequently processed or sterilized, the end user is responsible for demonstrating the continued conformance of closures to compendial requirements subsequent to such processing and/or sterilization conditions (i.e., in their ready-to-use state). This is especially important if closures shall be exposed to processes or conditions that may significantly impact the biological, physicochemical, or functionality characteristics of the closure (e.g., gamma irradiation).

For closures that are normally lubricated •with silicone• (RB 1-May-2009) prior to use, it is permissible to perform physicochemical testing on non-lubricated closures, in order to avoid potential method interference and/or difficulties in interpreting test results. •For closures supplied with other lubricious non-barrier coatings, all tests are to be performed using the coated closure.

For closures coated or laminated with coatings intended to provide a barrier function (e.g., PTFE or lacquer coatings), physicochemical compendial tests apply to the uncoated base elastomer, as well as to the coated closure. In this case, suppliers are responsible for demonstrating physicochemical compendial compliance of the coated closure, as well as of the uncoated closure, processed or treated in a manner simulating conditions typically followed by the supplier for such coated closures prior to shipment to the end user. The uncoated closure subject to physicochemical tests should be similar to the corresponding coated closure in size and configuration. End users of coated closures are also responsible for demonstrating the continued physicochemical compendial conformance of the coated closure, processed or treated in a manner simulating conditions typically employed by the end user prior to use. • (RB 1-May-2009)

In all cases, it is appropriate to document all conditions of closure processing, pretreatment, sterilization or lubrication when reporting test results.



• [Table 1](#) summarizes the testing requirements of closures, and the responsibilities of the supplier and the end user.

Table 1

Closure Types (As Supplied or Used)	Test Requirements		
	Physicochemical Tests	Functionality Tests	Biological Tests
Closure with or without Silicone Coating	• Tests are to be performed.	• Tests are to be performed.	• Tests are to be performed.
	• Silicone use is optional.	• Silicone use is optional.	• Silicone use is optional.
	• Responsibility: supplier and end user	• Responsibility: supplier and end user	• Responsibility: supplier and end user
Closures with Lubricious Coating (Non- Barrier Material; Not Silicone)	• Tests are to be performed on coated closures.	• Tests are to be performed on coated closures.	• Tests are to be performed on coated closures.
	• Responsibility: supplier and end user	• Responsibility: supplier and end user	• Responsibility: supplier and end user
Closures with Barrier Coating	• Tests are to be performed on coated closures.	• Tests are to be performed on coated closures.	• Tests are to be performed on coated closures.
	• Responsibility: supplier and end user	• Responsibility: supplier and end user OR: • Tests are to be performed on uncoated closures (base formula) and the lamine/coating material (report results separately).	• Responsibility: supplier and end user
	AND: • Tests are to be performed on uncoated closures (base formula).		
	• Responsibility: supplier		• Responsibility: supplier and end user

• (RB 1-May-2009)

#### BIOLOGICAL TESTS

Two stages of testing are indicated. The first stage is the performance of an in vitro test procedure as described in general test chapter [Biological Reactivity Tests, In Vitro](#) (87). Materials that do not meet the requirements of the in vitro test are subjected to the second stage of testing, which is the performance of the in vivo tests, Systemic Injection Test and Intracutaneous Test, according to the procedures set forth in the general test chapter [Biological Reactivity Tests, In Vivo](#) (88). Materials that meet the requirements of the in vitro test are not required to undergo in vivo testing.

Type I and Type II closures must both conform to the requirements of either the in vitro or the in vivo biological reactivity tests. [note—Also see the general information chapter [The Biocompatibility of Material Used in Drug Containers, Medical Devices, and Implants](#) (1031).]

#### PHYSICOCHEMICAL TESTS

##### Preparation of Solution S

Place whole, uncut closures corresponding to a surface area of  $100 \pm 10 \text{ cm}^2$  into a suitable glass container. Cover the closures with 200 mL of Purified Water or Water for Injection. If it is not possible to achieve the prescribed closure surface area ( $100 \pm 10 \text{ cm}^2$ ) using uncut closures, select the number of closures that will most closely approximate  $100 \text{ cm}^2$ , and adjust the volume of water used to the equivalent of 2 mL per each 1  $\text{cm}^2$  of actual closure surface area used. Boil for 5 minutes, and rinse five times with cold Purified Water or Water for Injection

Place the washed closures into a Type I glass wide-necked flask (see [Containers—Glass](#) (660)), add the same quantity of Purified Water or Water for Injection initially added to the closures, and weigh. Cover the mouth of the flask with a Type I glass beaker. Heat in an autoclave so that a temperature of  $121 \pm 2^\circ\text{C}$  is reached within 20 to 30 minutes, and maintain this temperature for 30 minutes. Cool to room temperature over a period of about 30 minutes. Add Purified Water or Water for Injection to bring it up to the original mass. Shake, and immediately decant and collect the solution. [note—This solution must be shaken before being used in each of the tests.]

##### Preparation of Blank

Prepare a blank solution similarly, using 200 mL of Purified Water or Water for Injection omitting the closures.

##### Appearance of Solution (Turbidity/Opalescence and Color)

##### Determination of Turbidity (Opalescence)

note—The determination of turbidity may be performed by visual comparison (Procedure A), or instrumentally using a suitable ratio turbidimeter (Procedure B). For a discussion of turbidimetry, see [Spectrophotometry and Light-Scattering](#) (851). Instrumental assessment of clarity provides a more discriminatory test that does not depend on the visual acuity of the analyst.

Hydrazine Sulfate Solution—Dissolve 1.0 g of hydrazine sulfate, in water and dilute with water to 100.0 mL. Allow to stand for 4 to 6 hours.

Hexamethylenetetramine Solution—Dissolve 2.5 g of hexamethylenetetramine in 25.0 mL of water in a 100 mL glass—stoppered flask.

Opalescence Stock Suspension—Add 25.0 mL of Hydrazine Sulfate Solution to the Hexamethylenetetramine Solution in the flask. Mix, and allow to stand for 24 hours. This suspension is stable for 2 months, provided it is stored in a glass container free from surface defects. The suspension must not adhere to the glass and must be well mixed before use.

Opalescence Standard Suspension—Prepare a suspension by diluting 15.0 mL of the Opalescence Stock Suspension with water to 1000.0 mL. Opalescence Standard Suspension is stable for about 24 hours after preparation.

Reference Suspensions—Prepare according to [Table 2](#). Mix and shake before use. [note—Stabilized formazin suspensions that can be used to prepare stable, diluted turbidity standards are available commercially and may be used after comparison with the standards prepared as described.]

Table 2

	Reference Suspension A	Reference Suspension B	Reference Suspension C	Reference Suspension D
Standard of Opalescence	5.0 mL	10.0 mL	30.0 mL	50.0 mL
Water	95.0 mL	90.0 mL	70.0 mL	50.0 mL
Nephelometric turbidity units	3 NTU	6 NTU	18 NTU	30 NTU

Procedure A: Visual Comparison—Use identical test tubes made of colorless, transparent, neutral glass with a flat base and an internal diameter of 15 to 25 mm. Fill one tube to a depth of 40 mm with Solution S, one tube to the same depth with water, and four others to the same depth with Reference Suspensions A, B, C, and D. Compare the solutions in diffuse daylight 5 minutes after preparation of the Reference Suspensions, viewing vertically against a black background. The light conditions shall be such that Reference Suspension

A can be readily distinguished from water and that Reference Suspension B can be readily distinguished from Reference Suspension A.

REQUIREMENT— Solution S is not more opalescent than Reference Suspension B for Type I closures, and not more opalescent than Reference Suspension C for Type II closures.

Solution S is considered clear if its clarity is the same as that of water when examined as described above, or if its opalescence is not more pronounced than that of Reference Suspension A (refer to [Table 3](#)).

Procedure B: Instrumental Comparison— Measure the turbidity of the Reference Suspensions in a suitable calibrated turbidimeter (see [Spectrophotometry and Light Scattering](#) (851)). The blank should be run and the results corrected for the blank. Reference Suspensions A, B, C, and D represent 3, 6, 18 and 30 Nephelometric Turbidity Units (NTU), respectively. Measure the turbidity of Solution S using the calibrated turbidimeter.

REQUIREMENT— The turbidity of Solution S is not greater than that for Reference Suspension B (6 NTU FTU) for Type I closures, and is not greater than that for Reference Suspension C (18 NTU FTU) for Type II closures (refer to [Table 3](#)).

Table 3

	Comparison Method	
Opalescence Requirements	Procedure A (Visual)	Procedure B (Instrumental)
Type I closures	no more opalescent than Suspension B	no more than 6 NTU
Type II closures	no more opalescent than Suspension C	no more than 18 NTU

#### Determination of Color

Color Standard— Prepare a solution by diluting 3.0 mL of Matching Fluid O (see [Color and Achromicity](#) (631)) with 97.0 mL of diluted hydrochloric acid.

Procedure— Use identical tubes made of colorless, transparent, neutral glass with a flat base and an internal diameter of 15 to 25 mm. Fill one tube to a depth of 40 mm with Solution S, and the second with Color Standard. Compare the liquids in diffuse daylight, viewing vertically against a white background.

Requirement— Solution S is not more intensely colored than the Color Standard.

#### Acidity or Alkalinity

Bromothymol Blue Solution— Dissolve 50 mg of bromothymol blue in a mixture of 4 mL of 0.02 M sodium hydroxide and 20 mL of alcohol. Dilute with water to 100 mL.

Procedure— To 20 mL of Solution S add 0.1 mL of Bromothymol Blue Solution. If the solution is yellow, titrate with 0.01 N sodium hydroxide until a blue endpoint is reached. If the solution is blue, titrate with 0.01 N hydrochloric acid until a yellow endpoint is reached. If the solution is green, it is neutral and no titration is required.

Blank Correction— Test 20 mL of Blank similarly. Correct the results obtained for Solution S by subtracting or adding the volume of titrant required for the Blank, as appropriate. ([Reference Titrimetry](#) (541)).

Requirement— Not more than 0.3 mL of 0.01 N sodium hydroxide produces a blue color, or not more than 0.8 mL of 0.01 N hydrochloric acid produces a yellow color, or no titration is required.

#### Absorbance

Procedure— [note—Perform this test within 5 hours of preparing Solution S.] Filter Solution S through a 0.45-μm pore size filter, discarding the first few mL of filtrate. Measure the absorbance of the filtrate at wavelengths between 220 and 360 nm in a 1-cm cell using the blank in a matched cell in the reference beam. If dilution of the filtrate is required before measurement of the absorbance, correct the test results for the dilution.

Requirement— The absorbances at these wavelengths do not exceed 0.2 for Type I closures or 4.0 for Type II closures.

#### Reducing Substances

Procedure— [note—Perform this test within 4 hours of preparing Solution S.] To 20.0 mL of Solution S add 1 mL of diluted sulfuric acid and 20.0 mL of 0.002 M potassium permanganate. Boil for 3 minutes. Cool, add 1 g of potassium iodide, and titrate immediately with 0.01 M sodium thiosulfate, using 0.25 mL of starch solution TS as the indicator. Perform a titration using 20.0 mL of blank and note the difference in volume of 0.01 M sodium thiosulfate required.

Requirement— The difference between the titration volumes is not greater than 3.0 mL for Type I closures and not greater than 7.0 mL for Type II closures.

#### Heavy Metals

Procedure— Proceed as directed for Method 1 under [Heavy Metals](#) (231). Prepare the Test Preparation using 10.0 mL of Solution S.

Requirement— Solution S contains not more than 2 ppm of heavy metals as lead.

#### Extractable Zinc

Test Solution— Prepare a Test Solution by diluting 10.0 mL of Solution S to 100 mL with 0.1 N hydrochloric acid. Prepare a test blank similarly, using the Blank for Solution S.

Zinc Standard Solution— Prepare a solution (10 ppm Zn) by dissolving zinc sulfate in 0.1 N hydrochloric acid.

Reference Solutions— Prepare not fewer than 3 Reference Solutions by diluting the Zinc Standard Solution with 0.1 N hydrochloric acid. The concentrations of zinc in these Reference Solutions are to span the expected limit of the Test Solution.

Procedure— Use a suitable atomic absorption spectrophotometer (see [Spectrophotometry and Light Scattering](#) (851)) equipped with a zinc hollow-cathode lamp and an air-acetylene flame. An alternative procedure such as an appropriately validated inductively coupled plasma analysis (ICP) may be used.

Test each of the Reference Solutions at the zinc emission line of 213.9 nm at least 3 times. Record the steady readings. Rinse the apparatus with the test blank solution each time, to ensure that the reading returns to initial blank value. Prepare a calibration curve from the mean of the readings obtained for each Reference Solution. Record the absorbance of the Test Solution. Determine the ppm zinc concentration of the Test Solution using the calibration curve.

Requirement— Solution S contains not more than 5 ppm of extractable zinc.

#### Ammonium

Alkaline Potassium Tetraiodomercurate Solution— Prepare a 100 mL solution containing 11 g of potassium iodide and 15 g of mercuric iodide in water. Immediately before use, mix 1 volume of this solution with an equal volume of a 250 g per L solution of sodium hydroxide.

Test Solution— Dilute 5 mL of Solution S to 14 mL with water. Make alkaline if necessary by adding 1 N sodium hydroxide, and dilute with water to 15 mL. Add 0.3 mL of Alkaline Potassium Tetraiodomercurate Solution, and close the container.

Ammonium Standard Solution— Prepare a solution of ammonium chloride in water (1 ppm NH<sub>4</sub>). Mix 10 mL of the 1 ppm ammonium chloride solution with 5 mL water and 0.3 mL of Alkaline Potassium Tetraiodomercurate Solution. Close the container.

Requirement— After 5 minutes, any yellow color in the Test Solution is no darker than the Ammonium Standard Solution (no more than 2 ppm of NH<sub>4</sub> in Solution S).

#### Volatile Sulfides

Procedure— Place closures, cut if necessary, with a total surface area of  $20 \pm 2 \text{ cm}^2$  in a 100-mL flask, and add 50 mL of a 20 g per L citric acid solution. In the same manner and at the same time, prepare a control solution in a separate 100-mL flask by dissolving 0.154 mg of sodium sulfide in 50 mL of a 20 g per L citric acid solution. Place a piece of lead acetate paper over the mouth of each flask, and hold the paper in position by placing over it an inverted weighing bottle. Heat the flasks in an autoclave at  $121 \pm 2^\circ$  for 30 minutes.



Requirement— Any black stain on the paper produced by Solution S is not more intense than that produced by the control solution.

#### FUNCTIONALITY TESTS

NOTE—Samples treated as described for preparation of Solution S and air dried should be used for Functionality Tests of Penetrability, Fragmentation, and Self-Sealing Capacity. Functionality Tests are performed on closures intended to be pierced by a hypodermic needle. The Self-Sealing Capacity test is required only for closures intended for multiple-dose containers. The needle specified for each test is a lubricated long bevel (bevel angle  $12 \pm 2^\circ$ ) hypodermic needle<sup>1</sup>.

##### Penetrability

Procedure— Fill 10 suitable vials to the nominal volume with water, fit the closures to be examined, and secure with a cap. Using a new hypodermic needle as described above for each closure, pierce the closure with the needle perpendicular to the surface.

Requirement— The force for piercing is no greater than 10 N (1 kgf) for each closure, determined with an accuracy of  $\pm 0.25$  N (25 gf).

##### Fragmentation

Closures for Liquid Preparations— Fill 12 clean vials with water to 4 mL less than the nominal capacity. Fit the closures to be examined, secure with a cap, and allow to stand for 16 hours.

Closures for Dry Preparations— Fit closures to be examined into 12 clean vials, and secure each with a cap.

Procedure— Using a hypodermic needle as described above fitted to a clean syringe, inject into each vial 1 mL of water while removing 1 mL of air. Repeat this procedure 4 times for each closure, piercing each time at a different site. Use a new needle for each closure, checking that it is not blunted during the test. Filter the total volume of liquid in all the vials through a single filter with a nominal pore size no greater than 0.5  $\mu\text{m}$ . Count the rubber fragments on the surface of the filter visible to the naked eye.

Requirement— There are no more than 5 fragments visible. This limit is based on the assumption that fragments with a diameter  $>50 \mu\text{m}$  are visible to the naked eye. In case of doubt or dispute, the particles are examined microscopically to verify their nature and size.

##### Self-Sealing Capacity

Procedure— Fill 10 suitable vials with water to the nominal volume. Fit the closures that are to be examined, and cap. Using a new hypodermic needle as described above for each closure, pierce each closure 10 times, piercing each time at a different site. Immerse the 10 vials in a solution of 0.1% (1 g per L) methylene blue, and reduce the external pressure by 27 kPa for 10 minutes. Restore to atmospheric pressure, and leave the vials immersed for 30 minutes. Rinse the outside of the vials.

Requirement— None of the vials contain any trace of blue solution.

1 Refer to ISO 7864, Sterile hypodermic needles for single use with an external diameter of 0.8 mm (21 Gauge).

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Desmond G. Hunt, Ph.D.</a> Scientist 1-301-816-8341	(PPI05) Parenteral Products-Industrial 05

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391 EPINEPHRINE ASSAY

[USP Reference Standards](#) 11 — [USP Epinephrine Bitartrate RS](#).

Ferro-citrate Solution— On the day needed, dissolve 1.5 g of ferrous sulfate in 200 mL of water to which have been added 1.0 mL of dilute hydrochloric acid (1 in 12) and 1.0 g of sodium bisulfite. Dissolve 500 mg of sodium citrate in 10 mL of this solution, and mix.

Buffer Solution— In a 50-mL volumetric flask mix 4.2 g of sodium bicarbonate, 5.0 g of potassium bicarbonate, and 18 mL of water (not all of the solids will dissolve at this stage). To another 18 mL of water add 3.75 g of aminoacetic acid and 1.7 mL of 6 N ammonium hydroxide, mix to dissolve, and transfer this solution to the 50-mL volumetric flask containing the other mixture. Dilute with water to volume, and mix until solution is complete.

Standard Preparation— Transfer about 18 mg of [USP Epinephrine Bitartrate RS](#), accurately weighed, to a 100-mL volumetric flask with the aid of 20 mL of sodium bisulfite solution (1 in 50), dilute with water to volume, and mix. Transfer 5.0 mL of this solution to a 50-mL volumetric flask, dilute with sodium bisulfite solution (1 in 500) to volume, and mix. [note—Make the final dilution when the assay is carried out.] The concentration of [USP Epinephrine Bitartrate RS](#) in the Standard Preparation is about 18  $\mu\text{g}$  per mL.

Assay Preparation— Transfer to a 50-mL volumetric flask an accurately measured volume of the injection under assay, equivalent to about 500  $\mu\text{g}$  of epinephrine, dilute with sodium bisulfite solution (1 in 500) to volume, if necessary, and mix. [note—The final concentration of sodium bisulfite is in the range of 1 to 3 mg per mL, any bisulfite present in the injection under assay being taken into consideration.]

Procedure— Into three 50-mL glass-stoppered conical flasks transfer, separately, 20.0-mL aliquots of the Standard Preparation, the Assay Preparation, and sodium bisulfite solution (1 in 500) to provide the blank. To each flask add 200  $\mu\text{L}$  of Ferro-citrate Solution and 2.0 mL of Buffer Solution, mix, and allow the solutions to stand for 30 minutes. Determine the absorbances of the solutions in 5-cm cells at the wavelength of maximum absorbance at about 530 nm, with a suitable spectrophotometer, using the blank to set the instrument.

Calculate the quantity, in mg, of epinephrine ( $\text{C}_9\text{H}_{13}\text{NO}_3$ ) in each mL of the injection taken by the formula:

$$(183.21 / 333.30)(0.05C / V)(AU / AS)$$

in which 183.21 and 333.30 are the molecular weights of epinephrine and epinephrine bitartrate, respectively; C is the concentration, in  $\mu\text{g}$  per mL, of [USP Epinephrine Bitartrate RS](#) in the Standard Preparation; and V is the volume, in mL, of injection taken.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Kahkashan Zaidi, Ph.D.</a> Senior Scientist 1-301-816-8269	(AER05) Aerosols05
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## 401 FATS AND FIXED OILS

The following definitions and general procedures apply to fats, fixed oils, waxes, resins, balsams, and similar substances.

## PREPARATION OF SPECIMEN

If a specimen of oil shows turbidity owing to separated stearin, warm the container in a water bath at 50° until the oil is clear, or if the oil does not become clear on warming, pass it through dry filter paper in a funnel contained in a hot-water jacket. Mix thoroughly, and weigh at one time as many portions as are needed for the various determinations, using preferably a bottle having a pipet dropper, or a weighing buret. Keep the specimen melted, if solid at room temperature, until the desired portions of specimen are withdrawn.

## SPECIFIC GRAVITY

Determine the specific gravity of a fat or oil as directed under [Specific Gravity \(841\)](#).

## MELTING TEMPERATURE

Determine the melting temperature as directed for substances of Class II (see [Melting Range or Temperature \(741\)](#)).

## ACID VALUE (FREE FATTY ACIDS)

The acidity of fats and fixed oils in this Pharmacopeia may be expressed as the number of mL of 0.1 N alkali required to neutralize the free acids in 10.0 g of substance. Acidity is frequently expressed as the Acid Value, which is the number of mg of potassium hydroxide required to neutralize the free acids in 1.0 g of the substance. Unless otherwise directed in the individual monograph, use Method I.

## Method I

Procedure— Unless otherwise directed, dissolve about 10.0 g of the substance, accurately weighed, in 50 mL of a mixture of equal volumes of alcohol and ether (which has been neutralized to phenolphthalein with 0.1 N potassium hydroxide or 0.1 N sodium hydroxide, unless otherwise specified) contained in a flask. If the test specimen does not dissolve in the cold solvent, connect the flask with a suitable condenser and warm slowly, with frequent shaking, until the specimen dissolves. Add 1 mL of phenolphthalein TS, and titrate with 0.1 N potassium hydroxide VS or 0.1 N sodium hydroxide VS until the solution remains faintly pink after shaking for 30 seconds. Calculate either the Acid Value or the volume of 0.1 N alkali required to neutralize 10.0 g of specimen (free fatty acids), whichever is appropriate. Calculate the Acid Value by the formula:

$$56.11V \times N/W$$

in which 56.11 is the molecular weight of potassium hydroxide; V is the volume, in mL; N is the normality of the potassium hydroxide solution or the sodium hydroxide solution; and W is the weight, in g, of the sample taken.

If the volume of 0.1 N potassium hydroxide VS or 0.1 N sodium hydroxide VS required for the titration is less than 2 mL, a more dilute titrant may be used, or the sample size may be adjusted accordingly. The results may be expressed in terms of the volume of titrant used or in terms of the equivalent volume of 0.1 N potassium hydroxide or 0.1 N sodium hydroxide.

If the oil has been saturated with carbon dioxide for the purpose of preservation, gently reflux the alcohol-ether solution for 10 minutes before titration. The oil may be freed from carbon dioxide also by exposing it in a shallow dish in a vacuum desiccator for 24 hours before weighing the test specimens.

## Method II

Procedure— Prepare 125 mL of a solvent mixture consisting of equal volumes of isopropyl alcohol and toluene. Before use, add 2 mL of a 1% solution of phenolphthalein in isopropyl alcohol to the 125-mL mixture, and neutralize with alkali to a faint but permanent pink color. Weigh accurately the appropriate amount of well-mixed liquid sample indicated in the table below, and dissolve it in the neutralized solvent mixture. If the test specimen does not dissolve in the cold solvent, connect the flask with a suitable condenser and warm slowly, with frequent shaking, until the specimen dissolves. Shake vigorously while titrating with 0.1 N potassium hydroxide VS or 0.1 N sodium hydroxide VS to the first permanent pink of the same intensity as that of the neutralized solvent before mixing with the sample. Calculate the Acid Value as indicated in Method I.

Acid Value	Sample Weight (g)
0–1	20
1–4	10
4–15	2.5
15–74.9	0.5
≥75.0	0.1

## ESTER VALUE

The Ester Value is the number of mg of potassium hydroxide required to saponify the esters in 1.0 g of the substance. If the Saponification Value and the Acid Value have been determined, the difference between these two represents the Ester Value.

Procedure— Place 1.5 g to 2 g of the substance in a tared, 250-mL flask, weigh accurately, add 20 mL to 30 mL of neutralized alcohol, and shake. Add 1 mL of phenolphthalein TS, and titrate with 0.5 N alcoholic potassium hydroxide VS until the free acid is neutralized. Add 25.0 mL of 0.5 N alcoholic potassium hydroxide VS, and proceed as directed under Saponification Value, beginning with "Heat the flask" and omitting the further addition of phenolphthalein TS. The difference between the volumes, in mL, of 0.5 N hydrochloric acid consumed in the actual test and in the blank test, multiplied by 28.05 and divided by the weight in g of the specimen taken, is the Ester Value.

## HYDROXYL VALUE

The Hydroxyl Value is the number of mg of potassium hydroxide equivalent to the hydroxyl content of 1.0 g of the substance.

Pyridine–Acetic Anhydride Reagent— Just before use, mix 3 volumes of freshly opened or freshly distilled pyridine with 1 volume of freshly opened or freshly distilled acetic anhydride.

Procedure— Transfer a quantity of the substance, determined by reference to the [accompanying table](#) and accurately weighed, to a glass-stoppered, 250-mL conical flask, and add 5.0 mL of Pyridine–Acetic Anhydride Reagent. Transfer 5.0 mL of Pyridine–Acetic Anhydride Reagent to a second glass-stoppered, 250-mL conical flask to provide the reagent blank. Fit both flasks with suitable glass-jointed reflux condensers, heat on a steam bath for 1 hour, add 10 mL of water through each condenser, and heat on the steam bath for 10 minutes more. Cool, and to each add 25 mL of butyl alcohol, previously neutralized to phenolphthalein TS with 0.5 N alcoholic potassium hydroxide, by pouring 15 mL through each condenser and, after removing the condensers, washing the sides of both flasks with the remaining 10-mL portions. To each flask add 1 mL of phenolphthalein TS, and titrate with 0.5 N alcoholic potassium hydroxide VS, recording the volume, in mL, consumed by the residual acid in the test solution as T and that consumed by the blank as B. In a 125-mL conical flask, mix about 10 g of the substance, accurately weighed, with 10 mL of freshly distilled pyridine, previously neutralized to phenolphthalein TS, add 1 mL of phenolphthalein TS, and titrate with 0.5 N alcoholic potassium hydroxide VS, recording the volume, in mL, consumed by the free acid in the test specimen as A, or use the Acid Value to obtain A. Calculate the Hydroxyl Value taken by the formula:



$$(56.11N / W)[B + (WA / C) - T]$$

in which W and C are the weights, in g, of the substances taken for the acetylation and for the free acid determination, respectively; N is the exact normality of the alcoholic potassium hydroxide; and 56.11 is the molecular weight of potassium hydroxide.

Hydroxyl Value Range	Weight of Test Specimen, g
0 to 20	10
20 to 50	5
50 to 100	3
100 to 150	2
150 to 200	1.5
200 to 250	1.25
250 to 300	1.0
300 to 350	0.75

#### IODINE VALUE

The Iodine Value represents the number of g of iodine absorbed, under the prescribed conditions, by 100 g of the substance. Unless otherwise specified in the individual monograph, determine the Iodine Value by Method I.

##### Method I (Hanus Method)

Procedure— Transfer an accurately weighed quantity of sample, as determined from the accompanying table, into a 250-mL iodine flask, dissolve it in 10 mL of chloroform, add 25.0 mL of iodobromide TS, insert the stopper in the vessel securely, and allow it to stand for 30 minutes protected from light, with occasional shaking. Then add, in the order named, 30 mL of [potassium iodide TS](#) and 100 mL of water, and titrate the liberated iodine with 0.1 N sodium thiosulfate VS, shaking thoroughly after each addition of thiosulfate. When the iodine color becomes quite pale, add 3 mL of starch TS, and continue the titration with 0.1 N sodium thiosulfate VS until the blue color is discharged. Perform a blank test at the same time with the same quantities of the same reagents and in the same manner (see [Residual Titrations](#) 541). Calculate the Iodine Value from the formula:

$$[126.9(VB - VS)N] / 10W$$

in which 126.9 is the atomic weight of iodine; VB and VS are the volumes, in mL, of 0.1 N sodium thiosulfate VS consumed by the blank test and the actual test, respectively; N is the exact normality of the sodium thiosulfate VS; and W is the weight, in g, of the substance taken for the test. [note—If more than half of the iodobromide TS is absorbed by the portion of the substance taken, repeat the determination, using a smaller portion of the substance under examination.]

##### Sample Weights

Iodine value expected	Weight in g, $\pm 0.001$
<5	3.000
5–20	1.000
21–50	0.400
51–100	0.200
101–150	0.130
151–200	0.100

##### Method II

Potassium Iodide Solution— Dissolve 10.0 g of potassium iodide in water to make 100 mL. Store in light-resistant containers.

Starch Indicator Solution— Mix 1 g of soluble starch with sufficient cold water to make a thin paste. Add, while stirring, to 100 mL of boiling water. Mix, and cool. Use only the clear solution.

Procedure— Melt the sample, if it is not already liquid. [note—The temperature during melting should not exceed the melting point of the sample by more than  $10^{\circ}$ .] Pass through two pieces of filter paper to remove any solid impurities and the last traces of moisture. The filtration may be performed in an air oven at  $100^{\circ}$  but should be completed within 5 minutes  $\pm$  30 seconds. The sample must be absolutely dry. All glassware must be absolutely clean and completely dry. After filtration, allow the filtered sample to achieve a temperature of  $68^{\circ}$  to  $71 \pm 1^{\circ}$  before weighing the sample. Once the sample has achieved a temperature of  $68^{\circ}$  to  $71 \pm 1^{\circ}$ , immediately weigh the sample into a 500-mL iodine flask, using the weights and weighing accuracy noted in the accompanying table. [note—The weight of the substance must be such that there will be an excess of [iodochloride TS](#) of 50% to 60% of the amount added, that is, 100% to 150% of the amount absorbed.] Add 15 mL of a fresh mixture of cyclohexane and glacial acetic acid (1:1), and swirl to dissolve the sample. Add 25.0 mL of iodochloride TS, insert the stopper securely in the flask, and swirl to mix. Allow it to stand at  $25 \pm 5^{\circ}$ , protected from light, with occasional shaking, for 1.0 or 2.0 hours, depending on the Iodine Value (IV) of the sample: IV less than 150, 1.0 hour; IV equal to or greater than 150, 2.0 hours. Then, within 3 minutes after the indicated reaction time, add, in the order named, 20 mL of Potassium Iodide Solution and 150 mL of recently boiled and cooled water, and mix. Within 30 minutes, titrate the liberated iodine with 0.1 N sodium thiosulfate VS, while stirring by mechanical means after each addition of thiosulfate. When the yellow iodine color has almost disappeared, add 1 to 2 mL of Starch Indicator Solution, and continue the titration with 0.1 N sodium thiosulfate VS until the blue color is discharged. Perform a blank test at the same time with the same quantities of the same reagents and in the same manner (see [Residual Titrations](#) 541). The difference between the volumes, in mL, of 0.1 N sodium thiosulfate consumed by the blank test and the actual test, multiplied by 1.269 and divided by the weight, in g, of the sample taken, is the Iodine Value.

#### PEROXIDE VALUE

The Peroxide Value is the number that expresses, in milliequivalents of active oxygen, the quantity of peroxide contained in 1000 g of the substance. [note—This test must be performed promptly after sampling to avoid oxidation of the test specimen.]

Procedure— Unless otherwise directed, place about 5 g of the substance, accurately weighed, in a 250-mL conical flask fitted with a ground-glass stopper. Add 30 mL of a mixture of glacial acetic acid and chloroform (3:2), shake to dissolve, and add 0.5 mL of saturated potassium iodide solution. Shake for exactly 1 minute, and add 30 mL of water. Titrate with 0.01 N sodium thiosulfate VS, adding the titrant slowly with continuous shaking, until the yellow color is almost discharged. Add 5 mL of starch TS, and continue the titration, shaking vigorously, until the blue color is discharged. Perform a blank determination under the same conditions. [note—The volume of titrant used in the blank determination must not exceed 0.1 mL.] The difference between the volumes, in mL, of 0.01 N sodium thiosulfate consumed in the actual test and in the blank test, multiplied by 10 and divided by the weight, in g, of the specimen taken, is the Peroxide Value.

#### SAPONIFICATION VALUE

The Saponification Value is the number of mg of potassium hydroxide required to neutralize the free acids and saponify the esters contained in 1.0 g of the substance.

Procedure— Place 1.5 g to 2 g of the substance in a tared, 250-mL flask, weigh accurately, and add to it 25.0 mL of 0.5 N alcoholic potassium hydroxide. Heat the flask on a steam bath, under a suitable condenser to maintain reflux for 30 minutes, frequently rotating the contents. Then add 1 mL of phenolphthalein TS, and titrate the excess potassium hydroxide



with 0.5 N hydrochloric acid VS. Perform a blank determination under the same conditions (see Residual Titrations under [Titrimetry \(541\)](#)). The titration also can be carried out potentiometrically. The difference between the volumes, in mL, of 0.5 N hydrochloric acid consumed in the actual test and in the blank test, multiplied by 56.1 and the exact normality of the 0.5 N hydrochloric acid VS, and divided by the weight in g of specimen taken, is the Saponification Value.

If the oil has been saturated with carbon dioxide for the purpose of preservation, expose it in a shallow dish in a vacuum desiccator for 24 hours before weighing the test specimens.

#### UNSAPONIFIABLE MATTER

The term "Unsaponifiable Matter" in oils or fats, refers to those substances that are not saponifiable by alkali hydroxides but are soluble in the ordinary fat solvents, and to products of saponification that are soluble in such solvents.

Procedure— Transfer about 5.0 g of the oil or fat, accurately weighed, to a 250-mL conical flask, add 50 mL of an alcoholic potassium hydroxide solution prepared by dissolving 12 g of potassium hydroxide in 10 mL of water and diluting this solution with alcohol to 100 mL, and heat the flask on a steam bath under a suitable condenser to maintain reflux for 1 hour, swirling frequently. Cool to a temperature below 25°, and transfer the contents of the flask to a separator having a polytetrafluoroethylene stopcock, rinsing the flask with two 50-mL portions of water that are added to the separator (do not use grease on stopcock). Extract with three 100-mL portions of ether, combining the ether extracts in another separator containing 40 mL of water. Gently rotate or shake the separator for a few minutes. [note—Violent agitation may result in the formation of a difficult-to-separate emulsion.] Allow the mixture to separate, and discard the lower aqueous phase. Wash the ether extract with two additional 40-mL portions of water, and discard the lower aqueous phase. Wash the ether extract successively with a 40-mL portion of potassium hydroxide solution (3 in 100) and a 40-mL portion of water. Repeat this potassium hydroxide solution-water wash sequence three times. Wash the ether extract with 40-mL portions of water until the last washing is not reddened by the addition of 2 drops of phenolphthalein TS. Transfer the ether extract to a tared flask, and rinse the separator with 10 mL of ether, adding the rinsings to the flask. Evaporate the ether on a steam bath, and add 6 mL of acetone to the residue. Remove the acetone in a current of air, and dry the residue at 105° until successive weighings differ by not more than 1 mg. Calculate the percentage of unsaponifiable matter in the portion of oil or fat taken by the formula:

$$100(\text{WR} / \text{WS})$$

in which WR is the weight, in g, of the residue; and WS is the weight, in g, of the oil or fat taken for the test.

Dissolve the residue in 20 mL of alcohol, previously neutralized to the phenolphthalein endpoint, add phenolphthalein TS, and titrate with 0.1 N alcoholic sodium hydroxide VS to the first appearance of a faint pink color that persists for not less than 30 seconds. If the volume of 0.1 N alcoholic sodium hydroxide required is greater than 0.2 mL, the separation of the layers was incomplete; the residue weighed cannot be considered as "unsaponifiable matter," and the test must be repeated.

#### SOLIDIFICATION TEMPERATURE OF FATTY ACIDS

Preparation of the Fatty Acids— Heat 75 mL of glycerin–potassium hydroxide solution (made by dissolving 25 g of potassium hydroxide in 100 mL of glycerin) in an 800-mL beaker to 150°, and add 50 mL of the clarified fat, melted if necessary. Heat the mixture for 15 minutes with frequent stirring, but do not allow the temperature to rise above 150°. Saponification is complete when the mixture is homogeneous, with no particles clinging to the beaker at the meniscus. Pour the contents of the beaker into 500 mL of nearly boiling water in an 800-mL beaker or casserole, add slowly 50 mL of dilute sulfuric acid (made by adding water and sulfuric acid (3:1)), and heat the solution, with frequent stirring, until the fatty acids separate cleanly as a transparent layer. Wash the acids with boiling water until free from sulfuric acid, collect them in a small beaker, place on a steam bath until the water has settled and the fatty acids are clear, filter into a dry beaker while hot, and dry at 105° for 20 minutes. Place the warm fatty acids in a suitable container, and cool in an ice bath until they congeal.

Test for Complete Saponification— Place 3 mL of the dry acids in a test tube, and add 15 mL of alcohol. Heat the solution to boiling, and add an equal volume of 6 N ammonium hydroxide. A clear solution results.

Procedure— Using an apparatus similar to the "Congealing Temperature Apparatus" specified therein, proceed as directed for Procedure under [Congealing Temperature \(651\)](#), reading "solidification temperature" for "congealing point" (the terms are synonymous). The average of not less than four consecutive readings of the highest point to which the temperature rises is the solidification temperature of the fatty acids.

#### FATTY ACID COMPOSITION

Standard Solution— Prepare an ester mixture of known composition containing the esters required in the individual monograph. This Standard Solution may contain other components. [note—Ester mixtures are available commercially from Nu-Chek-Prep, Inc., P.O. Box 295, Elysian, MN 56028. Typical Nu-Chek-Prep ester mixtures useful in this test include Nu-Chek

17A and Nu-Chek 19A.] Nu-Chek mixture 17A has the following composition:

Percentage	Fatty Acid Ester	Carbon-chain Length	No. of Double Bonds
1.0	methyl myristate	14	0
4.0	methyl palmitate	16	0
3.0	methyl stearate	18	0
3.0	methyl arachidate	20	0
3.0	methyl behenate	22	0
3.0	methyl lignocerate	24	0
45.0	methyl oleate	18	1
15.0	methyl linoleate	18	2
3.0	methyl linolenate	18	3
20.0	methyl erucate	22	1

Nu-Chek mixture 19A has the following composition:

Percentage	Fatty Acid Ester	Carbon-chain Length	No. of Double Bonds
7.0	methyl caprylate	8	0
5.0	methyl caprate	10	0
48.0	methyl laurate	12	0
15.0	methyl myristate	14	0
7.0	methyl palmitate	16	0
3.0	methyl stearate	18	0
12.0	methyl oleate	18	1
3.0	methyl linoleate	18	2

Test Solution— [note—If fatty acids containing more than 2 double bonds are present in the test specimen, remove air from the flask by purging it with nitrogen for a few minutes.] Transfer about 100 mg of the test specimen to a 50-mL conical flask fitted with a suitable water-cooled reflux condenser and a magnetic stir bar. Add 4 mL of 0.5 N methanolic sodium



oxide solution, and reflux until fat globules disappear (usually 5 to 10 minutes). Add 5 mL of a solution prepared by dissolving 14 g of boron trifluoride in methanol to make 100 mL. Swirl to mix, and reflux for 2 minutes. Add 4 mL of chromatographic n-heptane through the condenser, and reflux for 1 minute. Cool, remove the condenser, add about 15 mL of saturated sodium chloride solution, shake, and allow the layers to separate. Pass the n-heptane layer through 0.1 g of anhydrous sodium sulfate (previously washed with chromatographic n-heptane) into a suitable flask. Transfer 1.0 mL of this solution to a 10-mL volumetric flask, dilute with chromatographic n-heptane to volume, and mix.

**System Suitability Solution**— Transfer about 20 mg each of stearic acid, palmitic acid and oleic acid to a 25-mL conical flask fitted with a suitable water-cooled reflux condenser and a magnetic stir bar, and proceed as directed for **Test Solution**, beginning with “Add 5.0 mL of a solution prepared by dissolving.”

**Chromatographic System** (see [Chromatography \(621\)](#))— The gas chromatograph is equipped with a flame-ionization detector, maintained at a temperature of about 260°, a splitless injection system, and a 0.53-mm × 30-m fused-silica capillary column bonded with a 1.0-μm layer of phase G16. The chromatograph is programmed to maintain the column temperature at 70° for about 2 minutes after injection, then to increase the temperature at the rate of 5° per minute to 240°, and finally to maintain this temperature for 5 minutes. The injection port temperature is maintained at about 220°. The carrier gas is helium with a linear velocity of about 50 cm per second.

Chromatograph the **System Suitability Solution**, and record the peak responses as directed for **Procedure**: the relative retention times are about 0.87 for methyl palmitate, 0.99 for methyl stearate, and 1.0 for methyl oleate; the resolution, R, between methyl stearate and methyl oleate is not less than 1.5; and the relative standard deviation of the peak area responses for the palmitate and stearate peaks for replicate injections is not more than 6.0%. The relative standard deviation of the peak area response ratio of the palmitate to stearate peaks from these replicate injections is not more than 1.0%.

**Procedure**— Separately inject equal volumes (about 1 μL) of the **Standard Solution** and the **Test Solution** into the chromatograph, record the chromatograms, identify the fatty acid ester peaks in the chromatogram of the **Test Solution** by comparing the retention times of these peaks with those obtained in the chromatogram of the **Standard Solution**, and measure the peak areas for all of the fatty acid ester peaks in the chromatogram obtained from the **Test Solution**. Calculate the percentage of each fatty acid component in the test specimen by the formula:

$$100(A/B)$$

in which A is the area of the peak response obtained for each individual fatty acid ester component; and B is the sum of the peak areas of all of the peaks, excluding the solvent peak, in the chromatogram obtained from the **Test Solution**.

#### WATER AND SEDIMENT IN FIXED OILS

**Apparatus**— The preferred centrifuge has a diameter of swing (d = distance from tip to tip of whirling tubes) of 38 to 43 cm and is operated at a speed of about 1500 rpm. If a centrifuge of different dimensions is used, calculate the desired rate of revolution by the formula:

$$\text{rpm} = 1500 \sqrt{40.6/d}$$

The centrifuge tubes are pear-shaped, and are shaped to accept closures. The total capacity of each tube is about 125 mL. The graduations are clear and distinct, reading upward from the bottom of the tube according to the scale shown in the [accompanying table](#).

Volume (mL)	Scale Division (mL)
0 to 3	0.1
3 to 5	0.5
5 to 10	1.0
10 to 25	5.0
25 to 50	25.0
50 to 100	50.0

**Procedure**— Place 50.0 mL of benzene in each of two centrifuge tubes, and to each tube add 50.0 mL of the oil, warmed if necessary to re-incorporate separated stearin, and mixed thoroughly at 25°. Insert the stopper tightly into the tubes, and shake them vigorously until the contents are mixed thoroughly, then immerse the tubes in a water bath at 50° for 10 minutes. Centrifuge for 10 minutes. Read the combined volume of water and sediment at the bottom of each tube. Centrifuge repeatedly for 10-minute periods until the combined volume of water and sediment remains constant for 3 consecutive readings. The sum of the volumes of combined water and sediment in the two tubes represents the percentage, by volume, of water and sediment in the oil.

#### ANISIDINE VALUE

The anisidine value is defined as 100 times the optical density measured in a 1-cm cell of a solution containing 1 g of the substance to be examined in 100 mL of a mixture of solvents and reagents according to the method described below. [note—Carry out the operations as rapidly as possible, avoiding exposure to actinic light.]

**Test Solution A**— Dissolve 0.500 g of the substance to be examined in isooctane, and dilute with the same solvent to 25.0 mL.

**Test Solution B**— To 5.0 mL of **Test Solution A** add 1.0 mL of a 2.5 g per L solution of p-anisidine in glacial acetic acid, shake, and store protected from light.

**Standard Solution**— To 5.0 mL of isooctane add 1.0 mL of a 2.5 g per L solution of p-anisidine in glacial acetic acid, shake, and store protected from light.

**Procedure**— Measure the absorbance of **Test Solution A** at 350 nm using isooctane as the blank. Measure the absorbance of **Test Solution B** at 350 nm exactly 10 minutes after its preparation, using the **Standard Solution** as the compensation liquid. Calculate the Anisidine Value from the expression:

$$\frac{25(1.2A_s - A_b)}{m}$$

in which As is the absorbance of **Test Solution B** at 350 nm; Ab is the absorbance of **Test Solution A** at 350 nm; and m is the weight, in g, of the substance to be examined in **Test Solution A**.

#### TOTAL OXIDATION VALUE (TOTOX)

Total Oxidation Value is defined by the formula:

$$2PV + AV$$

in which PV is the Peroxide Value, and AV is the Anisidine Value.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Hong Wang, Ph.D.</a> Scientist 1-301-816-8351	(EGC05) Excipient General Chapters

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Pharmacopeial Forum: Volume No. 34(3) Page 736

## 411 FOLIC ACID ASSAY

The following procedure is provided for the estimation of folic acid as an ingredient of Pharmacopeial preparations containing other active constituents.

[USP Reference Standards](#) 11 — [USP Folic Acid RS](#).

Mobile Phase— Place 2.0 g of monobasic potassium phosphate in a 1-liter volumetric flask, and dissolve in about 650 mL of water. Add 12.0 mL of a 1 in 4 solution of tetrabutylammonium hydroxide in methanol, 7.0 mL of 3 N phosphoric acid, and 240 mL of methanol. Cool to room temperature, adjust with either 3 N phosphoric acid or 6 N ammonium hydroxide to a pH of 7.0, dilute with water to volume, and mix. Pass through a 0.45- $\mu$ m filter, and recheck the pH before use. [note—The methanol-to-water ratio may be varied by up to 3 percent and the pH may be increased up to 7.15 to achieve better separation.]

Diluting Solvent— Prepare as directed under Mobile Phase. Adjust to a pH of 7.0, and bubble nitrogen through the solution for 30 minutes before use.

Internal Standard Solution— Dissolve about 25 mg of methylparaben in 2.0 mL of methanol, dilute with Diluting Solvent to 50 mL, and mix.

Standard Folic Acid Solution— Transfer about 12 mg of [USP Folic Acid RS](#), accurately weighed, to a low-actinic, 50-mL volumetric flask, dissolve in 2 mL of ammonium hydroxide, dilute with Diluting Solvent to volume, and mix.

Standard Preparation— Transfer 2.0 mL of Standard Folic Acid Solution to a low-actinic, 25-mL volumetric flask, add 2.0 mL of Internal Standard Solution, add Diluting Solvent to volume, and mix.

Assay Preparation— Transfer an accurately weighed or measured portion of the preparation to be assayed, containing about 1 mg of folic acid, to a low-actinic, 50-mL volumetric flask, add 4.0 mL of Internal Standard Solution, add Diluting Solvent to volume, and mix.

Chromatographic System (see [Chromatography](#) 621)— The liquid chromatograph is equipped with a 280-nm detector and a 15-cm  $\times$  3.9-mm column that contains packing L1. The flow rate is about 1.0 mL per minute. Chromatograph the Standard Preparation, and record the peak responses as directed for Procedure: there is baseline separation of folic acid and methylparaben.

Procedure— Separately inject equal volumes (about 10  $\mu$ L) of Standard Preparation and Assay Preparation into the chromatograph, record the chromatograms, and measure the responses for the major peaks. The relative retention times are about 0.8 for folic acid and 1.0 for methylparaben. Calculate the quantity, in  $\mu$ g, of C19H19N7O6 in the portion of the preparation taken by the formula:

$$50C(RU / RS)$$

in which C is the concentration, in  $\mu$ g per mL, of USP Folic Acid RS in the Standard Preparation; and RU and RS are the ratios of the response of the folic acid peak to that of the methylparaben peak obtained from the Assay Preparation and the Standard Preparation, respectively.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Curtis Phinney</a> 1-301-816-8540	(DSN05) Dietary Supplements - Non-Botanicals
Reference Standards	Lili Wang, Technical Services Scientist 1-301-816-8129 <a href="mailto:RSTech@usp.org">RSTech@usp.org</a>	

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Pharmacopeial Forum: Volume No. 28(3) Page 727

## 425 IODOMETRIC ASSAY—ANTIBIOTICS

The following method is provided for the assay of most of the Pharmacopeial penicillin antibiotic drugs and their dosage forms, for which iodometric titration is particularly suitable.

Standard Preparation— Dissolve in the solvent specified in the table of Solvents and Final Concentrations a suitable quantity of the USP Reference Standard specified in the individual monograph, previously dried under the conditions specified in the individual monograph and accurately weighed, and dilute quantitatively and stepwise with the same solvent to obtain a solution having a known concentration of about that specified in the table. Pipet 2.0 mL of this solution into each of two 125-mL glass-stoppered conical flasks.

## Solvents and Final Concentrations

Antibiotic	Solvent*	Final Concentration
Amoxicillin	Water	1.0 mg per mL
Ampicillin	Water	1.25 mg per mL
Ampicillin Sodium	Buffer No. 1	1.25 mg per mL
Cloxacillin Sodium	Water	1.25 mg per mL
Cyclacillin	Water	1.0 mg per mL
Dicloxacillin Sodium	Buffer No. 1	1.25 mg per mL

Penicillin Sodium	Buffer No. 1	1.25 mg per mL
Nafcillin Sodium	Buffer No. 1	1.25 mg per mL
Oxacillin Sodium	Buffer No. 1	1.25 mg per mL
Penicillin G Potassium	Buffer No. 1	2,000 units per mL
Penicillin G Sodium	Buffer No. 1	2,000 units per mL
Penicillin V Potassium	Buffer No. 1	2,000 units per mL
Phenethicillin Potassium	Buffer No. 1	2,000 units per mL

\* Unless otherwise noted, the Buffers are the potassium phosphate buffers defined in the section Media and Diluents under [Antibiotics—Microbial Assays](#) 81, except that sterilization is not required before use.

**Assay Preparation**— Unless otherwise specified in the individual monograph, dissolve in the solvent specified in the table of Solvents and Final Concentrations a suitable quantity, accurately weighed, of the specimen under test, and dilute quantitatively with the same solvent to obtain a solution having a known final concentration of about that specified in the table. Pipet 2 mL of this solution into each of two 125-mL glass-stoppered conical flasks.

#### Procedure—

**Inactivation and Titration**— To 2.0 mL of the Standard Preparation and of the Assay Preparation, in respective flasks, add 2.0 mL of 1.0 N sodium hydroxide, mix by swirling, and allow to stand for 15 minutes. To each flask add 2.0 mL of 1.2 N hydrochloric acid, add 10.0 mL of 0.01 N iodine VS, immediately insert the stopper, and allow to stand for 15 minutes. Titrate with 0.01 N sodium thiosulfate VS. As the endpoint is approached, add 1 drop of [starch iodide paste TS](#), and continue the titration to the discharge of the blue color.

**Blank Determination**— To a flask containing 2.0 mL of the Standard Preparation add 10.0 mL of 0.01 N iodine VS. If the Standard Preparation contains amoxicillin or ampicillin, immediately add 0.1 mL of 1.2 N hydrochloric acid. Immediately titrate with 0.01 N sodium thiosulfate VS. As the endpoint is approached, add 1 drop of [starch iodide paste TS](#), and continue the titration to the discharge of the blue color. Similarly treat a flask containing 2.0 mL of the Assay Preparation.

**Calculations**— Calculate the microgram (or unit) equivalent (F) of each mL of 0.01 N sodium thiosulfate consumed by the Standard Preparation by the formula:

$$(2CP) / (B - I)$$

in which C is the concentration, in mg per mL, of Reference Standard in the Standard Preparation, P is the potency, in  $\mu$ g (or units) per mg, of the Reference Standard, B is the volume, in mL, of 0.01 N sodium thiosulfate consumed in the Blank determination, and I is the volume, in mL, of 0.01 N sodium thiosulfate consumed in the Inactivation and titration. Calculate the potency of the specimen under test by the formula given in the individual monograph.

**Auxiliary Information**— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Ahalya Wise, M.S.</a> Scientist 1-301-816-8161	(MDANT05) Monograph Development-Antibiotics

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#### 429 LIGHT DIFFRACTION MEASUREMENT OF PARTICLE SIZE

Light diffraction is one of the most widely used techniques for measuring the size of a wide range of particles from very fine to very coarse. The method is popular because it is quick and easy to use, flexible, and it can be adapted to measure samples presented in various physical forms. The method depends on the analysis of the diffraction pattern produced when particles are exposed to a collimated beam of light. As the patterns are characteristic of the particle size, mathematical analysis can produce an accurate, repeatable picture of the size distribution.

This chapter provides guidance on the measurement of size distributions of particles in any phase system (e.g., powders, sprays, aerosols, suspensions, emulsions, and gas bubbles in liquids) through analysis of their angular light-scattering patterns. Traditionally, the measurement of particle size using laser diffraction has been limited to particles in the range 0.1  $\mu$ m to 3 mm. Due to recent advances in optics and lens and equipment design and construction, newer instruments are routinely capable of exceeding this range (e.g., 0.1  $\mu$ m to 8 mm). It is the responsibility of the user to demonstrate the applicability of the instrument for its intended use and to validate any method prior to its adoption for routine use.

For nonspherical particles, an equivalent-sphere size distribution is obtained because the technique uses the assumption of spherical particles in its optical model. The resulting particle size distribution may be different from those obtained by methods based on other physical principles (such as sedimentation or sieving). The laser diffraction technique cannot distinguish between scattering by single particles and scattering by clusters of primary particles forming an agglomerate or an aggregate. If the presence of aggregates is suspected, this can be investigated using other techniques such as microscopy.

The laser diffraction technique is based on the phenomenon that particles scatter light in all directions with an intensity pattern that is dependent on particle size. All present instruments assume a spherical shape for the particles. Historically, the early laser diffraction instruments used only scattering at small angles and, thus, has been known by the following names: Fraunhofer diffraction, (near-) forward light scattering, and low-angle laser light scattering (LALLS).

However, the technique since has been broadened to include light scattering in a wider angular range by application of the Mie theory, in addition to the Fraunhofer approximation and anomalous diffraction.

The measurement of particle size is an important aspect in the characterization of raw materials and drug formulations. To monitor production and particle stability, efficient and rapid methods for particle sizing are needed. The electronic measurement of samples offers a convenient solution to this problem. However, the electronic measurement will often have to be accompanied by microscopical investigation to determine the type of particles being investigated. Modern drug formulations may also be particulate formulations where the particle size will be below 100  $\mu$ m, and reliable and reproducible methods are needed for the quality control of these drug products.

#### PRINCIPLE

A representative sample, dispersed at an adequate concentration in a suitable liquid or gas, is passed through the beam of a monochromatic light source, usually from a laser. The light scattered by the particles at various angles is measured by a multi-element detector, and numerical values relating to the scattering pattern are then recorded for subsequent analysis. These numerical scattering values are then transformed, using an appropriate optical model and mathematical procedure, to yield the proportion of total volume to a discrete number of size classes forming a volumetric particle size distribution (e.g., x50 describes a particle diameter corresponding to 50% of the cumulative undersize distribution).

#### APPARATUS

A typical setup for a laser diffraction instrument is shown in [Figure 1](#).

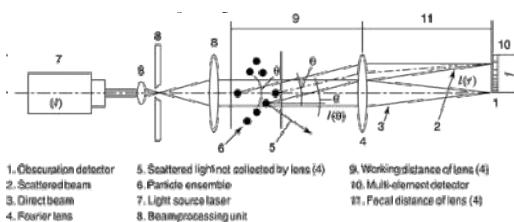


Fig. 1. Typical setup for a laser diffraction instrument.

A representative sample, dispersed at an adequate concentration, is passed through the light beam in a measuring zone by a transporting fluid (gas or liquid); this measuring zone should be within the working distance of the lens used. In some cases the particle stream in a process is illuminated directly by the laser beam for measurement, as in the case of sprays, aerosols, and air bubbles in liquids. In other cases (such as emulsions, pastes, and powders), representative samples can be dispersed in suitable liquids. Often dispersants (such as wetting agents or stabilizers) or mechanical forces (such as agitation or ultrasonication), or both, are applied to deagglomerate particles and to stabilize the dispersion. For these liquid dispersions, a recirculating system is most commonly used, consisting of an optical measurement cell, a dispersion bath (usually equipped with stirrer and ultrasonic elements), a pump, and tubing.

Dry powders can also be converted into aerosols through the use of dry powder dispersers, which apply mechanical forces for deagglomeration. In this case, a dosing device feeds the disperser with a constant mass flow of sample. The disperser uses the energy of compressed gas or the differential pressure to a vacuum to disperse the particles. It outputs an aerosol that is blown through the measuring zone, usually into the inlet of a vacuum pipe that collects the particles.

There are two positions in which the particles can enter the laser beam. In the conventional case, the particles enter the parallel beam before and within the working distance of the collecting lens. In the so-called reversed Fourier optics case, the particles enter behind the collecting lens, and thus in a converging beam.

The advantage of the conventional setup is that a reasonable path length for the sample is allowed within the working distance of the lens. The second setup allows only small path lengths but enables measurement of scattered light at larger angles, which is useful when submicron particles are present.

The interaction of the incident light beam with the dispersed particles results in a scattering pattern with different light intensities at various angles. The total angular intensity distribution, consisting of both direct and scattered light, is then focused by a positive lens or an assembly of lenses onto a multi-element detector. The lens(es) provide(s) for a scattering pattern which, within limits, is not dependent upon the location of the particles in the light beam. Thus the continuous angular intensity distribution is converted into a discrete spatial intensity distribution on a set of detector elements.

It is assumed that the recorded scattering pattern of the particle ensemble is identical to the sum of the patterns from all individual single scattering particles presented in random relative positions. Note that only a limited angular range of scattered light is collected by the lens(es), and thus by the detector.

#### PREPARATION OF THE SAMPLE

Prepare a representative sample of suitable volume for the measurement by an adequate sample splitting technique.

Sprays, aerosols, and gas bubbles in liquid should be measured directly, provided that their concentration is at an appropriate level, since sampling or dilution is generally impossible without altering the particle size distribution.

The dispersion procedure is adjusted to the purpose of the measurement: for example, whether agglomerates should be detected or broken down to primary particles.

For the development of a method, it is necessary to check that comminution of the primary particles does not occur, and conversely that a good dispersion of the agglomerates has been achieved. The dry particles may be examined microscopically before and after the addition and dispersion of aliquots of the dispersing liquid to determine if the particle size has changed, and if the agglomerates are dispersed but the primary particles are not fractured. The effect of the dispersion process can usually be checked by changing the dispersing energy and monitoring the change of the size distribution. The measured size distribution does not change significantly if the sample is well dispersed and the particles are neither fragile nor soluble. Microscopy may also be used to investigate primary particle comminution and adequacy of dispersion.

#### Dispersion Liquids

A variety of liquids are available for the dispersion of powders and must have the following characteristics:

- be transparent at the laser wavelength and free from air bubbles or other particles,
- be compatible with the materials used in the instrument (O-ring, tubing, etc.),
- not dissolve or alter the size of the particulate materials,
- favor easy and stable dispersion of the particulate material,
- have suitable viscosity to enable calculation,
- have a refractive index that differs from that of the material (for the Mie calculation), and
- not be hazardous to health and meet safety requirements.

A low-foaming surfactant and dispersant may be used to facilitate the wetting of the particles and to stabilize the dispersion. A preliminary check on the dispersion quality can be made by visual or microscopic inspection of the suspension.

If very small samples are required, it is also possible to take fractional samples out of a well-mixed sample paste if the material is neither fragile nor soluble. The consistency of the paste then prevents segregation errors. The pastes are formed by adding dispersant to the sample dropwise, while mixing it with a spatula. As long as the mixture forms lumps, single drops should be added while continuing the mixing after each drop. A good consistency for the paste is one like honey or toothpaste. If the paste becomes too fluid by mistake, it cannot be used, and a new preparation is initiated.

Alternatively, a concentrated suspension may be prepared. While stirring this concentrated suspension, a small aliquot is removed and transferred to the optical measurement cell containing the blank dispersing medium. Care must be taken to ensure the complete transfer of the sample and that settling of the larger particles does not occur.

Where a dispersant that does not dissolve the particles cannot be found, it may be possible to use a prefiltered, saturated solution of the sample in the dispersing solvent as the dispersant. Such a saturated solution may be produced by stirring an excess of sample in the dispersing solvent for several hours. For weak acids and weak bases, buffering of the dispersing solvent at low or high pH, respectively, can assist in identifying a suitable dispersant. The saturated medium is filtered using a membrane filter to remove any undissolved sample before use. This approach is not suitable if the sample forms a more viscous concentrated solution.

#### Dispersion Gases

For dry dispersion and spray applications, a compressed gas is sometimes used. If used, it is essential that it is free from oil, water, and particles. To achieve this, a dryer with a filter is required. Any vacuum unit is located away from the measurement zone, so that the output of the hot air does not reach the measuring zone. Avoid drafts in order to avoid unstable particulate streams.

#### Concentration

The particle concentration in the dispersion should be above a minimum level, which for many instruments will correspond to about 5% obscuration, in order to produce an acceptable signal-to-noise ratio in the detector. Likewise, it should be below a maximum level in order to avoid multiple scattering (for example, 35% above 20  $\mu\text{m}$  and 15% below 20  $\mu\text{m}$ ).

The optimum concentration is influenced by the laser beam width, the path length of the measurement zone, the optical properties of the particles, and the sensitivity of the detector elements. In view of the above, perform measurements at different particle concentrations in order to decide on the optimum concentration range that achieves the required



obscuration for any typical sample of material.

## MEASUREMENT

### Setting Up the Instrument and Blank Measurement

After selection of the appropriate particle size range and proper alignment of the optical part of the instrument, a blank measurement is performed in which a particle-free dispersion medium is used.

### Measurement of the Scattering of Dispersed Sample(s)

Generally, a large number of detector scans or sweeps at short time intervals is used (typically some 2 seconds or 1000 sweeps). For each detector element an average signal is calculated, sometimes together with its standard deviation. Data are stored in the computer memory. The magnitude of the signal from each detector element depends upon the detection area, the light intensity, and the quantum efficiency. The coordinates (size and position) of the detector elements, together with the focal distance of the lens, determine the region of scattering angles for each element. These factors are factory-determined and stored in the computer.

Most instruments also measure the intensity of the central laser beam. The difference between a dispersed sample and a blank experiment is given as an obscuration value, which is indicative of the total amount of scattering light and the particle concentration.

### Selection of an Appropriate Optical Model

Most often either the Fraunhofer approximation or the Mie theory is used, though other approximations are sometimes applied for calculation of the scattering matrix. Below approximately 25  $\mu\text{m}$ , the differences between the optical models become more significant. In this size range, the proper application of the Mie theory (assuming accurate real and imaginary refractive index values) provides the greatest accuracy. When using the Mie theory, the refractive indices of particulate and medium, or their ratio, are entered into the instrument to allow calculation of the model matrix. Often, small values of the imaginary part of the refractive index (about 0.01–0.1) are applied to cope with the surface roughness of the particles.<sup>4</sup> In order to obtain traceable results, it is essential that the refractive index values used are reported.

### Conversion of Scattering Pattern into Particle Size Distribution

This deconvolution step is the inverse of the calculation of a scattering pattern for a given particle size distribution. The fact that rapidly measured data always contain some random and systematic errors may cause erroneous size distribution results. Several mathematical procedures have been developed for use in the different instruments available. They allow for some weighting of deviations between measured and calculated scattering patterns (such as least squares), some constraints (such as non-negativity for amounts of particles), or (or in combination with) some smoothing of the size distribution curve. A new procedure uses the observed fluctuations of the detector signals to introduce proper weighting of these data and to calculate confidence intervals for the particle size distribution.

The algorithms used are specific to each make and model of equipment and are proprietary. The differences in the algorithms between different instruments can give rise to differences in the particle size statistics. For this reason, when reporting the particle size distribution and statistics for a given material, also report the cell type, sample state and preparation, together with the make and model of the equipment.

### Replicates

The required precision of the method is dependent on the characteristics of the material (milled versus not milled, robust versus fragile), and also on the requirements of the application (formulation type and technique). Appropriate measurement conditions are established experimentally, in relation to the desired precision. In general, at least three different representative samples from the same batch are measured. The repeatability of the particle size distribution parameter is as follows: for any central value of the distribution, for example the median ( $x_{50}$ ), the coefficient of variation is less than 10%. For values away from the center of the distribution, for example  $x_{10}$  and  $x_{90}$ , the coefficient of variation cannot exceed 15%. Below 10  $\mu\text{m}$ , these maximum values are doubled.

### System Suitability

The system suitability test is used to verify that the precision and accuracy are adequate for the analysis to be done. The test is based on the concept that the equipment, electronics, and analytical operations constitute an integral system that can be evaluated as such. This can be done by measuring at regular time intervals a control material of known size distribution. In general, unless otherwise specified in the individual monograph, the mean values of three measurements must deviate from the established value by less than 10% for  $x_{50}$ , and by less than 15% for  $x_{10}$  and  $x_{90}$ . Below 10  $\mu\text{m}$ , these maximum values are doubled.

### Reporting of Results

The distribution statistics are usually reported by the instrument data system. Most common parameters are calculated from the cumulative distribution by interpolation. Percentile sizes,  $x_m$ , represent the particle size in relation to which  $m$  percent of the distribution is smaller. (The notation  $d_m$  is also used and is equivalent to  $x_m$ .)  $Q_y$  represents the percent smaller than  $y$  microns. Mean sizes, such as  $D_{4,3}$ , the arithmetic volume mean diameter, can also be calculated by representing the distribution as a collection of spherical particles with diameters of the size band midpoints. Unless otherwise stated, parameters are calculated on the volume or mass basis.

## QUALIFICATION

### Calibration

Laser diffraction systems are based on the direct measurement of the diffraction pattern of particles, but with idealized properties of the particles. Thus, calibration in the strict sense is not required. However, it is still necessary and desirable to confirm the correct operation of the instrument with a qualification procedure.

### Accuracy and Repeatability

Primarily, qualification validation can be made with any certified or standard reference material, acceptable for use in the particular industry. Here, the total measurement procedure is being examined, including sampling, sample dispersion, sample transport through the measuring zone, measurement, and the deconvolution procedure. It is essential that the total operational procedure is adequately described in full detail.

Certified or standard reference materials consisting of a known distribution having a range of spherical particles over one decade of size are preferred. They are certified to mass percentage by an absolute technique, if available, and used in conjunction with an agreed, detailed operational procedure. It is essential that the real and imaginary part of the complex refractive index are precisely specified for the material if the Mie theory is applied in data analysis.

The response of a laser diffraction instrument is considered adequate if the mean value of  $x_{50}$  obtained from at least three independent measurements does not exceed the certified range of values of the certified or standard reference material by more than 3%. The mean values for  $x_{10}$  and  $x_{90}$  must not exceed the certified range of values by more than 5%. For repeatability, the coefficient of variation must be less than 3% for  $x_{50}$  and less than 5% for  $x_{10}$  and  $x_{90}$ . Below 10  $\mu\text{m}$ , these maximum values are doubled.

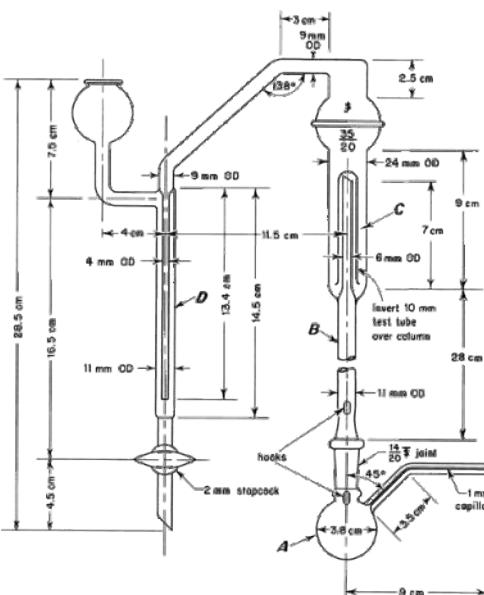
Although the use of spherical materials is preferable, nonspherical ones may also be used. Preferably, these have certified or typical values coming from laser diffraction analyses according to an agreed, detailed operational procedure. If the reference values come from methods other than laser diffraction, a significant bias may result. The reason for this bias is that the different principles applied in the various methods may lead to different responses to the particles, and thus to different equivalent-sphere diameters for the same nonspherical particle.

1 Small differences in the assumed complex refractive index may cause significant differences in the resulting particle size distributions.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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Apparatus— The apparatus for methoxy determination is shown diagrammatically in the [accompanying figure](#).



#### Apparatus for Methoxy Determination

The boiling flask, A, is fitted with a capillary side-arm for the introduction of carbon dioxide or nitrogen and is connected to a column, B, which serves to separate aqueous hydriodic acid from the more volatile methyl iodide. The methyl iodide passes through water in a scrubber trap, C, and is finally absorbed in the bromine–acetic acid solution in absorption tube D. The carbon dioxide or nitrogen is introduced through a pressure-regulating device and connected to the apparatus by a small capillary containing a small cotton pledge. [note— Avoid the use of organic solvents in cleaning this apparatus, since traces remaining may interfere with the determination. This test is used also for ethoxy determination with an 80-minute reaction time and a titrant equivalent of 0.751 mg of (OC<sub>2</sub>H<sub>5</sub>).]

For greater convenience in use and cleaning, a ground-glass ball joint connects the two upright columns of the apparatus. The top of the scrubber C consists of a 35/20 ball joint, the upper half of which is connected to the side-arm leading into tube D. This permits taking the apparatus apart and facilitates adding the water to the trap. Also, it allows access to the loose inverted (10-mm) test tube that serves as the trap over the inner tube of the scrubber C.

#### Reagents—

bromine-acetic acid solution— Dissolve 100 g of potassium acetate in 1000 mL of a solution consisting of 900 mL of glacial acetic acid and 100 mL of acetic anhydride. On the day of use, to 145 mL of this solution add 5 mL of bromine.

hydriodic acid— A colorless, or nearly colorless, constant-boiling reagent solution, prepared for this purpose, is available commercially. If not obtained commercially, it may be prepared by distilling hydriodic acid over red phosphorus, passing carbon dioxide or nitrogen through the apparatus during the distillation. Use the constant-boiling mixture (between 55% and 58% of HI) distilling between 126° and 127°, which is colorless or nearly colorless. [Caution—Exercise safety precautions when distilling Hydriodic Acid. ] Place the acid in small, amber, glass-stoppered bottles previously flushed with carbon dioxide, or nitrogen, seal with paraffin, and store in a cool, dark place.

Procedure— Prepare the apparatus by disconnecting the ball joint and pouring water into trap C until it is half-full. Connect the two parts, using a minimal amount of a suitable silicone grease to seal the ball joint. Add 7 mL of Bromine-Acetic Acid Solution to absorption tube D. Weigh the sample in a tared gelatin capsule, and add it to the boiling flask along with a few boiling chips or pieces of porous plate. Finally add 6 mL of Hydriodic Acid and attach the flask to the column, using a minimal amount of a suitable silicone grease to seal the junction. Bubble the carbon dioxide or nitrogen through the apparatus at the rate of 2 bubbles per second, place the boiling flask in an oil bath or heating mantle heated to 150°, and continue the reaction for 40 minutes for methoxy determination, or 80 minutes for ethoxy determination. Drain the contents of the absorption tube into a 500-mL conical flask containing 10 mL of sodium acetate solution (1 in 4). Rinse the tube with water, adding the rinsings to the flask, and finally dilute with water to about 125 mL. Add formic acid, dropwise, with swirling, until the reddish brown color of the bromine is discharged, then add 3 additional drops. A total of 12 to 15 drops usually is required. Allow to stand for 3 minutes, and add 15 mL of diluted sulfuric acid and 3 g of potassium iodide, and titrate immediately with 0.1 N sodium thiosulfate VS, using 3 mL of starch TS as the indicator. Perform a blank determination, including also a gelatin capsule, and make any necessary correction. Each mL of 0.1 N sodium thiosulfate is equivalent to 0.517 mg of (OCH<sub>3</sub>).

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	Hong Wang, Ph.D. Scientist 1-301-816-8351	(EGC05) Excipient General Chapters



## Chemical Method

note—Determine from the labeling if the vitamin in the assay specimen is niacin or niacinamide, and use the corresponding standard preparation (either Standard Niacin Preparation or Standard Niacinamide Preparation) as directed in the Procedure.

Cyanogen Bromide Solution— Dissolve 5 g of cyanogen bromide in water to make 50 mL. [Caution—Prepare this solution under a hood, as cyanogen bromide volatilizes at room temperature, and the vapor is highly irritating and poisonous.]

Sulfanilic Acid Solution— To 2.5 g of sulfanilic acid add 15 mL of water and 3 mL of 6 N ammonium hydroxide. Mix, add, with stirring, more 6 N ammonium hydroxide, if necessary, until the acid dissolves, adjust the solution with 3 N hydrochloric acid to a pH of about 4.5, using [bromocresol green RS](#) as an external indicator, and dilute with water to 25 mL.

Standard Niacin Stock Solution— Transfer 25.0 mg of [USP Niacin RS](#) to a 500-mL volumetric flask, dissolve in alcohol solution (1 in 4), dilute with alcohol solution (1 in 4) to volume, and mix. Store in a refrigerator. Each mL of this solution contains 50 µg of [USP Niacin RS](#).

Standard Niacin Preparation— Transfer 10.0 mL of Standard Niacin Stock Solution to a 100-mL volumetric flask, dilute with water to volume, and mix. Each mL of this solution contains 5 µg of [USP Niacin RS](#).

Standard Niacinamide Stock Solution— Transfer 50.0 mg of [USP Niacinamide RS](#) to a 500-mL volumetric flask, dissolve in alcohol solution (1 in 4), dilute with alcohol solution (1 in 4) to volume, and mix. Store in a refrigerator. Each mL of this solution contains 100 µg of [USP Niacinamide RS](#).

Standard Niacinamide Preparation— Transfer 10.0 mL of Standard Niacinamide Stock Solution to a 100-mL volumetric flask, dilute with water to volume, and mix. Each mL of this solution contains 10 µg of [USP Niacinamide RS](#).

## Assay Preparation— Prepare as directed in the individual monograph.

Procedure— Pipet into four marked tubes the quantities of the appropriate Standard Preparation, the Assay Preparation, the ammonia dilution, and water indicated in the accompanying table. Then add the other constituents, respectively, as listed in the table, according to the directions given herein.

## Reaction Mixtures for Niacin or Niacinamide Assay—Chemical Method

Constituent	Tube 1, mL	Tube 2, mL	Tube 3, mL	Tube 4, mL
Standard Preparation	1.0	1.0	—	—
Assay Preparation	—	—	1.0	1.0
Ammonia Dilution (ammonium hydroxide, diluted to 1 in 50)	0.5	0.5	0.5	0.5
Water	6.5	1.5	6.5	1.5
Cyanogen Bromide Solution	—	5.0	—	5.0
Sulfanilic Acid Solution	2.0	2.0	2.0	2.0
Hydrochloric Acid	1 drop	—	1 drop	—

To Tube 1 add the Sulfanilic Acid Solution, shake well, add the hydrochloric acid, mix, place in a suitable spectrophotometer, and adjust to zero absorbance at 450 nm. To Tube 2 add the Cyanogen Bromide Solution, mix, and 30 seconds, accurately timed, after completion of the addition of the cyanogen bromide, add the Sulfanilic Acid Solution, with swirling. Close the tube, place it in the spectrophotometer, and after 2 minutes measure its absorbance at 450 nm against Tube 1 as a blank, designating the absorbance as AS. Repeat the procedure with Tubes 3 (as blank) and 4, designating the absorbance of Tube 4 as AU. Calculate the quantity of niacin or niacinamide in the sample as directed in the individual monograph.

## Microbiological Method

Test Solution of Material to be Assayed— Place the prescribed amount of the material to be assayed in a flask of suitable size, and proceed by one of the methods given below. The concentrations of the sulfuric acid and sodium hydroxide solutions used are not stated in each instance because these concentrations may be varied depending upon the amount of material taken for assay, volume of test solution, and buffering effect of material.

(a) For Dry or Semidry Materials that Contain No Appreciable Amount of Basic Substances—Add a volume of dilute sulfuric acid (1 in 35) equal, in mL, to not less than 10 times the dry weight of the material, in g, but the resulting solution shall contain not more than 5.0 mg of niacin in each mL. If the material is not readily soluble, comminute it so that it may be evenly dispersed in the liquid, then agitate vigorously, and wash down the sides of the flask with dilute sulfuric acid (1 in 35).

Heat the mixture in an autoclave at 121° to 123° for 30 minutes, and cool. If lumping occurs, agitate the mixture until the particles are evenly dispersed. Adjust the mixture with sodium hydroxide solution to a pH of 6.8, dilute with water to make a final measured volume that has a concentration of niacin equivalent to that of Standard Niacin Solution, and filter.

(b) For Dry or Semidry Materials that Contain Appreciable Amounts of Basic Substances—Add sufficient sulfuric acid solution to bring the pH of the mixture to between 5.0 and 6.0. Add such an amount of water that the total volume of liquid shall be equal in mL to not less than ten times the dry weight of the assay specimen, in g, but the resulting solution shall contain not more than 5.0 mg of niacin in each mL. Then add the equivalent of 10 mL of dilute sulfuric acid (2 in 7) for each 100 mL of liquid, and proceed as directed under (a), beginning with the second paragraph.

(c) For Liquid Materials—Adjust the material with either sulfuric acid solution or sodium hydroxide solution to a pH of 5.0 to 6.0. Add such an amount of water that the total volume of liquid shall be equal, in mL, to not less than 10 times the volume of the specimen, in mL, but the resulting solution shall contain not more than 5.0 mg of niacin in each mL. Then add the equivalent of 10 mL of dilute sulfuric acid (2 in 7) for each mL of liquid, and proceed as directed under (a), beginning with the second paragraph.

Standard Niacin Stock Solution I— Transfer 50.0 mg of [USP Niacin RS](#) to a 500-mL volumetric flask, dissolve in alcohol, dilute with alcohol to volume, and mix. Store in a refrigerator. Each mL of this solution contains 100 µg of [USP Niacin RS](#).

Standard Niacin Stock Solution II— To 100.0 mL of Standard Niacin Stock Solution I add water to make 1000.0 mL. Store under toluene in a refrigerator. Each mL of this solution contains 10 µg of [USP Niacin RS](#).

Standard Niacin Solution— Dilute a suitable volume of Standard Niacin Stock Solution II with water to such a measured volume so that after incubation as described in the Assay Procedure the transmittance of the 5.0-mL level of Standard Niacin Solution is equivalent to that of a dried cell weight of not less than 1.25 mg, when the inoculated blank is set at 100 percent transmittance. This concentration is usually between 10 ng and 40 ng of niacin per mL. Prepare a fresh Standard Niacin Solution for each assay.

## Basal Medium Stock Solution—

Acid-hydrolyzed Casein Solution	25 mL
Cystine-Tryptophan Solution	25 mL
Dextrose Anhydrous	10 g
Sodium Acetate Anhydrous	5 g
Adenine-Guanine-Uracil Solution	5 mL
Riboflavin-Thiamine Hydrochloride-Biotin Solution	5 mL
Aminobenzoic Acid-Calcium Pantothenate-Pyridoxine Hydrochloride Solution	5 mL



Salt Solution A	5 mL
Salt Solution B	5 mL

Dissolve the anhydrous dextrose and sodium acetate in the solutions previously mixed, and adjust with 1 N sodium hydroxide to a pH of 6.8. Finally, add water to make 250 mL.

Acid-Hydrolyzed Casein Solution— Mix 100 g of vitamin-free casein with 500 mL of constant-boiling hydrochloric acid [approximately 20 percent (w/w) HCl], and reflux the mixture for 24 hours. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redisolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ( $\pm 0.1$ ), and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 hour, and filter. Repeat the treatment with activated charcoal if the filtrate does not appear straw-colored to colorless. Store under toluene in a refrigerator. Filter the solution if a precipitate forms upon storage.

Cystine-Tryptophan Solution— Suspend 4.0 g of L-cystine and 1.0 g of L-tryptophan (or 2.0 g of dl-tryptophan) in 700 to 800 mL of water, heat to 70° to 80°, and add the 20 percent (w/w) hydrochloric acid, dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a refrigerator at a temperature not below 10°.

Adenine-Guanine-Uracil Solution— Dissolve 100 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 5.0 mL of the 20 percent (w/w) hydrochloric acid, cool, and add water to make 100 mL. Store under toluene in a refrigerator.

Riboflavin-Thiamine Hydrochloride-Biotin Solution— Prepare a solution containing, in each mL, 20  $\mu$ g of riboflavin, 10  $\mu$ g of thiamine hydrochloride, and 0.04  $\mu$ g of biotin by dissolving crystalline riboflavin, crystalline thiamine hydrochloride, and crystalline biotin (free acid) in dilute glacial acetic acid (1 in 850). Store, protected from light, under toluene in a refrigerator.

Aminobenzoic Acid-Calcium Pantothenate-Pyridoxine Hydrochloride Solution— Prepare a solution of neutral 25 percent alcohol having a concentration of 10  $\mu$ g of aminobenzoic acid, 20  $\mu$ g of calcium pantothenate, and 40  $\mu$ g of pyridoxine hydrochloride per mL. Store in a refrigerator.

Salt Solution A— Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid, and store under toluene.

Salt Solution B— Dissolve 10 g of magnesium sulfate, 500 mg of sodium chloride, 500 mg of ferrous sulfate, and 500 mg of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and store under toluene.

Stock Culture of Lactobacillus plantarum— Dissolve 2.0 g of water-soluble yeast extract in 100 mL of water, add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture with stirring, on a steam bath, until the agar dissolves. Add approximately 10-mL portions of the hot solution to test tubes, plug the tubes with cotton, sterilize for 15 minutes in an autoclave at 121° to 123°, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of Lactobacillus plantarum,\* incubating for 16 to 24 hours at any selected temperature between 30° and 37°, but held constant to within  $\pm 0.5$ °, and finally store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for inoculum if the culture is more than 1 week old.

Culture Medium— To each of a series of test tubes containing 5.0 mL of the Basal Medium Stock Solution add 5.0 mL of water containing 1.0  $\mu$ g of niacin. Plug the tubes with cotton, sterilize for 15 minutes in an autoclave at 121° to 123°, and cool.

Inoculum— Make a transfer of cells from the stock culture of Lactobacillus plantarum to a sterile tube containing 10 mL of culture medium. Incubate this culture for 16 to 24 hours at any selected temperature between 30° and 37°, but held constant to within  $\pm 0.5$ °. The cell suspension so obtained is the inoculum.

Calibration of Spectrophotometer— Add aseptically 1 mL of Inoculum to approximately 300 mL of Culture Medium containing 1 mL of Standard Niacin Solution. Incubate the inoculated medium for the same period and at the same temperature to be employed in the Assay Procedure.

Following the incubation period, centrifuge and wash the cells three times with approximately 50-mL portions of [saline TS](#), and then resuspend the cells in about 25 mL of the saline solution.

Dry to constant weight a 10-mL portion, accurately measured, using a steam bath and completing the drying in vacuum at 100°, and calculate the dry weight of the cells, in mg per mL, corrected for the amount of sodium chloride present.

Dilute a second portion, accurately measured, of the saline cell suspension with the saline solution so that each mL contains a known quantity of cells equivalent to 500  $\mu$ g on a dried basis. To test tubes add, in triplicate, 0.5 mL, 1.0 mL, 1.5 mL, 2.0 mL, 2.5 mL, 3.0 mL, 4.0 mL, and 5.0 mL, respectively, of this diluted cell suspension and 5.0 mL of Basal Medium

Stock Solution, and make the volume in each tube to 10.0 mL with saline solution. Using as the blanks three similar tubes containing no cell suspension, measure the light transmittance of each tube under the same conditions to be employed in the assay. Plot the observations as the ordinate on cross-section paper against the cell content, expressed as mg of dry weight, as the abscissa.

Repeat this procedure at least twice for the spectrophotometer to be used in the assay. Draw the composite curve best representing the three or more individual curves relating transmittance to cell density for the spectrophotometer under the conditions of the assay.

Assay Procedure— Prepare standard niacin tubes as follows. To test tubes add, in duplicate, 0.0 mL, 0.5 mL, 1.0 mL, 1.5 mL, 2.0 mL, 2.5 mL, 3.0 mL, 3.5 mL, 4.0 mL, 4.5 mL, and 5.0 mL, respectively, of Standard Niacin Solution. To each tube add 5.0 mL of Basal Medium Stock Solution and water to make 10.0 mL.

Prepare tubes containing the material to be assayed as follows. To test tubes add, in duplicate, 1.0 mL, 2.0 mL, 3.0 mL, and 4.0 mL, respectively, of the test solution of the material to be assayed. To each tube add 5.0 mL of Basal Medium Stock Solution and water to make 10.0 mL. After mixing, plug the tubes with cotton or cover with caps, and sterilize in an autoclave at 121° to 123°. (Overheating the assay tubes may produce unsatisfactory results.) Cool, aseptically inoculate each tube with 1 drop of Inoculum, and incubate for 16 to 24 hours at any selected temperature between 30° and 37°, but held constant to within  $\pm 0.5$ °. Contamination of the assay tubes with any foreign organism invalidates the assay.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, to which 1 drop of a suitable antifoam agent solution may be added, and transfer to an optical container. After agitating its contents, place the container in a spectrophotometer that has been set at a specific wavelength between 540 nm and 660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 seconds or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. If this transmittance reading corresponds to a dried cell weight greater than 600  $\mu$ g per tube, or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

Then with the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. Disregard the results of the assay if the difference between the transmittance observed at the highest level of the standard and that of the inoculated blank is less than the difference corresponding to a dried cell weight of 1.25 mg per tube.

Calculation— Prepare a standard curve of the niacin standard transmittances for each level of Standard Niacin Solution plotted against  $\mu$ g of niacin contained in the respective tubes. From this standard curve, determine by interpolation the niacin content of the test solution in each tube. Disregard transmittance values equivalent to less than 0.5 mL or more than 4.5 mL of Standard Niacin Solution. The niacin content of the test material is calculated from the average values obtained from not less than six tubes that do not vary by more than  $\pm 10$  percent from the average. If the transmittance values of less than six tubes containing the test solution are within the range of the 0.5- to 4.5-mL levels of the niacin standard tubes, the data are insufficient to permit calculation of the concentration of niacin in the test material. Transmittance values of inoculated blank exceeding readings corresponding to dried cell weights of more than 600  $\mu$ g per tube indicate the presence of an excessive amount of niacin in the Basal Medium Stock Solution and invalidate the assay.

Multiply the values obtained by 0.992 if the results are to be expressed as niacinamide.

\* Pure cultures of Lactobacillus plantarum may be obtained, as number 8014, from the American Type Culture Collection, P.O. Box 1549, Manassas, VA 20108.



Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Curtis Phinney</a> 1-301-816-8540	(DSN05) Dietary Supplements - Non-Botanicals
Reference Standards	Lili Wang, Technical Services Scientist 1-301-816-8129 <a href="mailto:RSTech@usp.org">RSTech@usp.org</a>	

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**451 NITRITE TITRATION**

The following general method is provided for the determination of most of the Pharmacopeial sulfonamide drugs and their dosage forms, as well as of other Pharmacopeial drugs for which nitrite titration is particularly suitable.

[USP Reference Standards](#) [11](#)—[USP Sulfanilamide RS](#).

**Procedure**— Accurately weigh about 500 mg in the case of a sulfonamide, or otherwise the quantity specified in the individual monograph, and transfer to a suitable open vessel. Add 20 mL of hydrochloric acid and 50 mL of water, stir until dissolved, cool to about 15°, and slowly titrate with 0.1 M sodium nitrite VS that previously has been standardized against [USP Sulfanilamide RS](#).

Determine the endpoint electrometrically, using suitable electrodes (platinum-calomel or platinum-platinum). Place the buret tip below the surface of the solution to eliminate air oxidation of the sodium nitrite, and stir the solution gently, using a magnetic stirrer, without pulling a vortex of air under the surface, maintaining the temperature at about 15°. The titration may be carried out manually, or by means of an automatic titrator. In performing it manually, add the titrant until the titration is within 1 mL of the endpoint, and then add it in 0.1-mL portions, allowing not less than 1 minute between additions. (The instrument needle deflects and then returns to approximately its original position until the endpoint is reached.)

The weight, in mg, of the substance to which each mL of 0.1 M sodium nitrite VS is equivalent is as stated in the individual monograph.

For the assay of Tablets of the sulfonamides or other drugs, reduce not less than 20 tablets to a fine powder, weigh accurately a portion of the powder, equivalent to about 500 mg if a sulfonamide, or the quantity of drug specified in the individual monograph, and proceed as directed in the foregoing, beginning with "transfer to a suitable open vessel."

For the assay of Injections and other liquid forms where the nitrite titration is specified, pipet a portion, equivalent to about 500 mg if a sulfonamide, or the quantity of drug specified in the individual monograph, into a suitable open vessel, and proceed as directed in the foregoing, beginning with "Add 20 mL of hydrochloric acid."

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Antonio Hernandez-Cardoso, B.S.</a> Scientist, Latin American Specialist 1-301-816-8308	(GC05) General Chapters 05
Reference Standards	Lili Wang, Technical Services Scientist 1-301-816-8129 <a href="mailto:RSTech@usp.org">RSTech@usp.org</a>	

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**461 NITROGEN DETERMINATION**

Some alkaloids and other nitrogen-containing organic compounds fail to yield all of their nitrogen upon digestion with sulfuric acid; therefore these methods cannot be used for the determination of nitrogen in all organic compounds.

**METHOD I**

**Nitrates and Nitrites Absent**— Place about 1 g of the substance, accurately weighed, in a 500-mL Kjeldahl flask of hard borosilicate glass. The material to be tested, if solid or semisolid, may be wrapped in a sheet of nitrogen-free filter paper for convenience in transferring it to the flask. Add 10 g of powdered potassium sulfate or anhydrous sodium sulfate, 500 mg of powdered cupric sulfate, and 20 mL of sulfuric acid. Incline the flask at an angle of about 45°, and gently heat the mixture, keeping the temperature below the boiling point until frothing has ceased. Increase the heat until the acid boils briskly, and continue the heating until the solution has been clear green in color or almost colorless for 30 minutes. Allow to cool, add 150 mL of water, mix the contents of the flask, and again cool. Add cautiously 100 mL of sodium hydroxide solution (2 in 5), in such manner as to cause the solution to flow down the inner side of the flask to form a layer under the acid solution. Immediately add a few pieces of granulated zinc, and without delay connect the flask to a Kjeldahl connecting bulb (trap), previously attached to a condenser, the delivery tube from which dips beneath the surface of 100 mL of boric acid solution (1 in 25) contained in a conical flask or a wide-mouth bottle of about 500-mL capacity. Mix the contents of the Kjeldahl flask by gentle rotation, and distill until about four-fifths of the contents of the flask has distilled over. Titrate with 0.5 N sulfuric acid VS, determining the endpoint potentiometrically. Perform a blank determination, and make any necessary correction. Each mL of 0.5 N sulfuric acid VS is equivalent to 7.003 mg of nitrogen.

When the nitrogen content of the substance is known to be low, the 0.5 N sulfuric acid VS may be replaced by 0.1 N sulfuric acid VS. Each mL of 0.1 N sulfuric acid VS is equivalent to 1.401 mg of nitrogen.

**Nitrates and Nitrites Present**— Place a quantity of the substance, accurately weighed, corresponding to about 150 mg of nitrogen, in a 500-mL Kjeldahl flask of hard borosilicate glass, and add 25 mL of sulfuric acid in which 1 g of salicylic acid previously has been dissolved. Mix the contents of the flask, and allow the mixture to stand for 30 minutes with frequent shaking. To the mixture add 5 g of powdered sodium thiosulfate, again mix, then add 500 mg of powdered cupric sulfate, and proceed as directed under Nitrates and Nitrites Absent, beginning with "Incline the flask at an angle of about 45°."

When the nitrogen content of the substance is known to exceed 10%, 500 mg to 1 g of benzoic acid may be added, prior to digestion, to facilitate the decomposition of the substance.

**METHOD II**

**Apparatus**— Select an appropriate 300-mL Kjeldahl flask, from which the nitrogen is first liberated by acid digestion and then transferred quantitatively to the titration vessel by steam distillation.

**Procedure**— Place an accurately weighed or measured quantity of the material, equivalent to 2 to 3 mg of nitrogen, in the digestion flask of the apparatus. Add 1 g of a powdered mixture of potassium sulfate and cupric sulfate (10:1), and wash down any adhering material from the neck of the flask with a fine jet of water. Add 7 mL of sulfuric acid, allowing it to rinse down the wall of the flask, then, while swirling the flask, add 1 mL of 30 percent hydrogen peroxide cautiously down the side of the flask. (Do not add hydrogen peroxide during the digestion.)

Heat the flask over a free flame or an electric heater until the solution has a clear blue color and the sides of the flask are free from carbonaceous material. Cautiously add to the



gation mixture 70 mL of water, cool the solution, and arrange for steam distillation. Add through a funnel 30 mL of sodium hydroxide solution (2 in 5) in such manner as to cause the solution to flow down the inner side of the flask to form a layer under the acid solution, rinse the funnel with 10 mL of water, tightly close the apparatus, and begin the distillation with steam immediately. Receive the distillate in 15 mL of boric acid solution (1 in 25), to which has been added 3 drops of methyl red-methylene blue TS and sufficient water to cover the end of the condensing tube. Continue the distillation until the distillate measures 80 to 100 mL. Remove the absorption flask, rinse the end of the condensing tube with a small quantity of water, and titrate the distillate with 0.01 N sulfuric acid VS. Perform a blank determination, and make any necessary correction. Each mL of 0.01 N sulfuric acid VS is equivalent to 140.1  $\mu$ g of nitrogen.

When a quantity of material containing more than 2 to 3 mg of nitrogen is taken, 0.02 N or 0.1 N sulfuric acid may be employed, provided that at least 15 mL is required for the titration. If the total dry weight of material taken is greater than 100 mg, increase proportionately the quantities of sulfuric acid and sodium hydroxide.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Antonio Hernandez-Cardoso, B.S.</a> Scientist, Latin American Specialist 1-301-816-8308	(GC05) General Chapters 05

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## 466 ORDINARY IMPURITIES

This test, where called for in the individual monograph, is provided to evaluate the presence of ordinary impurities in official articles. Ordinary impurities are defined as those species in drug substances and/or drug products that have no significant, undesirable biological activity in the amounts present. These impurities may arise out of the synthesis, preparation, or degradation of compendial articles. In certain instances, impurities that pose a potential health risk may be detected. Because these impurities would not be individually identified by the strict use of this General Chapter, a separate evaluation may be necessary to ensure that the detected impurities fit the requirements set forth in the definition of Ordinary Impurities. Selections of tests and assays allow for anticipated amounts of impurities that are unobjectionable for the customary use of the article.

**Reporting and Specifications**— The value of 2.0%, unless otherwise specified in the individual monograph, was selected as the general limit for the total amount of ordinary impurities in monographs where documentation did not support adoption of other values.

Where a monograph sets limits on concomitant components and/or specified impurities/degradation products, these species are not to be included in the estimation of ordinary impurities unless so stated in the individual monograph. Concomitant components are defined as species characteristic of many drug substances that are not considered to be impurities in the Pharmacopeial sense. Examples of concomitant components are geometric and optical isomers (or racemates) and antibiotics that are mixtures. Any component that can be considered a toxic impurity because of significant undesirable biological effect is not considered to be a concomitant component.

**Methodology**— Unless otherwise specified in an individual monograph, estimation of the amount and number of ordinary impurities is made by relative methods rather than by strict comparison to individual Reference Standards. Nonspecific detection of ordinary impurities is also consistent with this classification.

Typical evaluation methods used for ordinary impurities are thin-layer chromatographic (TLC) techniques. See [Chromatography](#) (621) for a general discussion of the thin-layer chromatographic technique. Tests for related substances or chromatographic purity may also be used to evaluate the presence of ordinary impurities. Other methods (e.g., HPLC, HPTLC, etc.) may also be used with adequate justification as an alternate method. Unless otherwise specified in the individual monograph, use the following method.

**Test Solution**— Prepare, in the solvent specified in the monograph, a solution of the substance under test having an accurately known final concentration of about 10 mg per mL. [note—Heat or sonication may be used to dissolve the drug substance where use of such does not adversely affect the compound.]

**Standard Solutions**— Prepare, in the solvent specified in the monograph, solutions of the USP Reference Standard or designated substance having accurately known concentrations of 0.01 mg per mL, 0.05 mg per mL, 0.1 mg per mL, and 0.2 mg per mL. [note—Heat or sonication may be used to dissolve the drug substance where use of such does not adversely affect the compound.]

**Procedure**— Use a thin-layer chromatographic plate coated with a 0.25-mm layer of chromatographic silica gel mixture, and the Eluant specified in the monograph. Apply equal volumes (20  $\mu$ L) of the Test Solution and Standard Solutions to the plate, using a stream of nitrogen to dry the spots.

Allow the chromatogram to develop in a pre-equilibrated chamber until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the chamber, and air-dry. View the plate using the visualization technique(s) specified. Locate any spots other than the principal spot in the chromatogram of the Test Solution, and determine their relative intensities by comparison with the chromatograms of the appropriate Standard Solutions. See discussion above with regard to reporting and specifying total ordinary impurities.

### key for visualization techniques

(1) Use UV light at 254 nm and at about 366 nm.

(2) Use Iodoplatinate TS.

(3) Solution A—Mix 850 mg of bismuth subnitrate with 40 mL of water and 10 mL of glacial acetic acid.

**Solution B**—Dissolve 8 g of potassium iodide in 20 mL of water. Mix A and B together to obtain a Stock Solution which can be stored for several months in a dark bottle. Mix 10 mL of the Stock Solution with 20 mL of glacial acetic acid, and dilute with water to make 100 mL, to prepare the spray reagent.

(4) Ninhydrin Spray—Dissolve 200 mg of ninhydrin in 100 mL of alcohol. Heat the plate after spraying.

(5) Acid Spray—In an ice bath, add slowly and cautiously, with stirring, 10 mL of sulfuric acid to 90 mL of alcohol. Spray the plate, and heat until charred.

(6) Acid-Dichromate Spray—Add sufficient potassium dichromate to 100 mL of sulfuric acid to make a saturated solution. Spray the plate, and heat until charred.

(7) Vanillin—Dissolve 1 g of vanillin in 100 mL of sulfuric acid.

(8) Chloramine T-Trichloroacetic Acid—Mix 10 mL of a 3% aqueous solution of chloramine T with 40 mL of a 25% alcoholic solution of trichloroacetic acid. Prepare immediately before use.

(9) Folin-C—Add 10 g of sodium tungstate and 2.5 g of sodium molybdate to 70 mL of water, add 5 mL of 85% phosphoric acid and 10 mL of 36% hydrochloric acid, and reflux this solution for 10 hours.

(10) KMnO<sub>4</sub>—Dissolve 100 mg of Potassium Permanganate in 100 mL of water.

(11) DAB—Mix 1 g of p-dimethylaminobenzaldehyde in 100 mL of 0.6 N hydrochloric acid.

(12) DAC—Mix 100 mg of p-dimethylaminocinnamaldehyde in 100 mL of 1 N hydrochloric acid.

(13) Ferricyanide—Mix equal volumes of a 1% ferric chloride solution and a 1% potassium ferricyanide solution. Use immediately.

(14) Fast Blue B—Reagent A—Dissolve 500 mg of Fast Blue B Salt in 100 mL of water.

Reagent B—0.1 N sodium hydroxide.

Spray first with A, then with B.



(15) Alkaline Ferric Cyanide—Dilute 1.5 mL of a 1% potassium ferricyanide solution with water to 20 mL, and add 10 mL of 15% sodium hydroxide solution.

(16) Iodine Spray—Prepare a 0.5% solution of iodine in chloroform.

(17) Expose the plate for 10 minutes to iodine vapors in a pre-equilibrated closed chamber, on the bottom of which there are iodine crystals.

(18) Solution A—Dissolve 0.5 g of potassium iodide in 50 mL of water.

Solution B—Prepare a solution of 0.5 g of soluble starch in 50 mL of hot water.

Just prior to use, mix equal volumes of Solution A and Solution B.

(19) PTSS—Dissolve 20 g of p-toluenesulfonic acid in 100 mL of alcohol, spray the plate, dry for 15 minutes at 110°, and view under UV light at 366 nm.

(20) o-Tolidine Spray—Dissolve 160 mg of o-tolidine in 30 mL of glacial acetic acid, dilute with water to make 500 mL, add 1 g of potassium iodide, and mix until the potassium iodide has dissolved.

(21) Mix 3 mL of chloroplatinic acid solution (1 in 10) with 97 mL of water, followed by the addition of 100 mL of potassium iodide solution (6 in 100) to prepare the spray reagent.

(22) Iodine–Methanol Spray—Prepare a mixture of [iodine TS](#) and methanol (1:1).

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Antonio Hernandez-Cardoso, B.S.</a> Scientist, Latin American Specialist 1-301-816-8308	(GC05) General Chapters 05

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## 467 RESIDUAL SOLVENTS

### INTRODUCTION

This general chapter applies to existing drug substances, excipients, and products. All substances and products are subject to relevant control of solvents likely to be present in a substance or product.

Where the limits to be applied comply with those given below, tests for residual solvents are not generally mentioned in specific monographs because the solvents employed may vary from one manufacturer to another.

The objective of this general chapter is to provide acceptable amounts of residual solvents in pharmaceuticals for the safety of the patient. The general chapter recommends the use of less toxic solvents and describes levels considered to be toxicologically acceptable for some residual solvents.

For pharmacopeial purposes, residual solvents in pharmaceuticals are defined as organic volatile chemicals that are used or produced in the manufacture of drug substances or excipients, or in the preparation of drug products. The residual solvents are not completely removed by practical manufacturing techniques. Appropriate selection of the solvent for the synthesis of a drug substance or an excipient may enhance the yield, or determine characteristics such as crystal form, purity, and solubility. Therefore, the solvent may sometimes be a critical element in the synthetic process. This general chapter does not address solvents deliberately used as excipients nor does it address solvates. However, the content of solvents in such products should be evaluated and justified.

Because residual solvents do not provide therapeutic benefit, they should be removed, to the extent possible, to meet ingredient and product specifications, good manufacturing practices, or other quality-based requirements. Drug products should contain no higher levels of residual solvents than can be supported by safety data. Solvents that are known to cause unacceptable toxicities (Class 1, [Table 1](#)) should be avoided in the production of drug substances, excipients, or drug products unless their use can be strongly justified in a risk-benefit assessment. Solvents associated with less severe toxicity (Class 2, [Table 2](#)) should be limited in order to protect patients from potential adverse effects. Ideally, less toxic solvents (Class 3, [Table 3](#)) should be used where practical. The complete list of solvents included in this general chapter is given in Appendix 1. These tables and the list are not exhaustive. For the purposes of this Pharmacopeia, when a manufacturer has received approval from a competent regulatory authority for the use of a new solvent not currently listed in this general chapter, it is the responsibility of that manufacturer to notify the USP regarding the identity of this solvent, the approved residual solvent limit in the article, and the appropriate test procedure for this residual solvent in the article. The USP will then address this topic in the individual monograph. When a new solvent has been approved through the ICH process, this new solvent will be added to the appropriate list in this general chapter. At that time consideration will be given for removal of the specific solvent test requirement in the individual monograph.

Testing of drug substances, excipients, and drug products for residual solvents should be performed when production or purification processes are known to result in the presence of such residual solvents. It is only necessary to test for residual solvents that are used or produced in the manufacture or purification of drug substances, excipients, or products.

Although manufacturers may choose to test the drug product, a cumulative procedure may be used to calculate the residual solvent levels in the drug product from the levels in the ingredients used to produce the drug product. If the calculation results in a level equal to or below that provided in this general chapter, no testing of the drug product for residual solvents need be considered. If, however, the calculated level is above the recommended level, the drug product should be tested to ascertain whether the formulation process has reduced the relevant solvent level to within the acceptable amount. A drug product should also be tested if a residual solvent is used during its manufacture.

For the purposes of this Pharmacopeia, when a manufacturer has received approval from a competent regulatory authority for a higher level of residual solvent, it is the responsibility of that manufacturer to notify the USP regarding the identity of this solvent and the approved residual solvent limit in the article. The USP will then address this topic in the individual monograph.

See Appendix 2 for additional background information related to residual solvents.

### CLASSIFICATION OF RESIDUAL SOLVENTS BY RISK ASSESSMENT

The term "tolerable daily intake" (TDI) is used by the International Program on Chemical Safety (IPCS) to describe exposure limits of toxic chemicals and the term "acceptable daily intake" (ADI) is used by the World Health Organization (WHO) and other national and international health authorities and institutes. The term "permitted daily exposure" (PDE) is defined as a pharmaceutically acceptable intake of residual solvents to avoid confusion of differing values for ADIs of the same substance.

Residual solvents assessed in this general chapter are listed in Appendix 1 by common names and structures. They were evaluated for their possible risk to human health and placed into one of three classes as follows:

Residual Solvent Class	Assessment
Class 1	Solvents to be avoided Known human carcinogens Strongly suspected human carcinogens Environmental hazards



Class 2	Solvents to be limited
	Nongenotoxic animal carcinogens or possible causative agents of other irreversible toxicity, such as neurotoxicity or teratogenicity.
	Solvents suspected of other significant but reversible toxicities.
Class 3	Solvents with low toxic potential
	Solvents with low toxic potential to humans; no health-based exposure limit is needed. [note—Class 3 residual solvents have PDEs of 50 mg or more per day.]

\* For residual solvents with PDEs of more than 50 mg per day, see the discussion in the section Class 3 under Limits of Residual Solvents.

#### METHODS FOR ESTABLISHING EXPOSURE LIMITS

The method used to establish permitted daily exposures for residual solvents is presented in Appendix 3.

For articles that are designated "for veterinary use only", higher levels for the PDE and concentration limit may be justified in exceptional cases based upon the actual daily dose, actual target species, and relevant toxicological data and considering consumer safety impact. For the purpose of this Pharmacopeia, when a manufacturer has received approval from a competent regulatory authority for a higher limit, it is the responsibility of that manufacturer to notify the USP regarding the approved residual solvent limit in the article and the justification. The USP will then address this topic in the individual monograph.

#### OPTIONS FOR DESCRIBING LIMITS OF CLASS 2 RESIDUAL SOLVENTS

Two options are available when setting limits for Class 2 residual solvents.

##### Option 1

The concentration limits in ppm stated in [Table 2](#) are used. They were calculated using equation (1) below by assuming a product weight of 10 g administered daily.

$$\text{Concentration (ppm)} = (1000 \mu\text{g}/\text{mg} \times \text{PDE})/\text{dose}$$

Here, PDE is given in terms of mg per day, and dose is given in g per day.

These limits are considered acceptable for all drug substances, excipients, and drug products. Therefore, this option may be applied if the daily dose is not known or fixed. If all drug substances and excipients in a formulation meet the limits given in Option 1, these components may be used in any proportion. No further calculation is necessary provided the daily dose does not exceed 10 g. Products that are administered in doses greater than 10 g per day are to be considered under Option 2.

##### Option 2

It is not necessary for each component of the drug product to comply with the limits given in Option 1. The PDE in terms of mg per day as stated in [Table 2](#) can be used with the known maximum daily dose and equation (1) above to determine the concentration of residual solvent allowed in a drug product. Such limits are considered acceptable provided that it has been demonstrated that the residual solvent has been reduced to the practical minimum. The limits should be realistic in relation to analytical precision, manufacturing capability, and reasonable variation in the manufacturing process. The limits should also reflect contemporary manufacturing standards.

Option 2 may be applied by adding the amounts of a residual solvent present in each of the components of the drug product. The sum of the amounts of solvent per day should be less than that given by the PDE.

Consider an example of the application of Option 1 and Option 2 to acetonitrile concentration in a drug product. The permitted daily exposure to acetonitrile is 4.1 mg per day; thus, the Option 1 limit is 410 ppm. The maximum administered daily weight of a drug product is 5.0 g, and the drug product contains two excipients. The composition of the drug product and the calculated maximum content of residual acetonitrile are given in the following table.

Component	Amount in Formulation (g)	Acetonitrile Content (ppm)	Daily Exposure (mg)
Drug substance	0.3	800	0.24
Excipient 1	0.9	400	0.36
Excipient 2	3.8	800	3.04
Drug product	5.0	728	3.64

Excipient 1 meets the Option 1 limit, but the drug substance, excipient 2, and drug product do not meet the Option 1 limit. Nevertheless, the drug product meets the Option 2 limit of 4.1 mg per day and thus conforms to the acceptance criteria in this General Chapter.

Consider another example using acetonitrile as the residual solvent. The maximum administered daily weight of a drug product is 5.0 g, and the drug product contains two excipients. The composition of the drug product and the calculated maximum content of residual acetonitrile are given in the following table.

Component	Amount in Formulation (g)	Acetonitrile Content (ppm)	Daily Exposure (mg)
Drug substance	0.3	800	0.24
Excipient 1	0.9	2000	1.80
Excipient 2	3.8	800	3.04
Drug product	5.0	1016	5.08

In this example, the drug product meets neither the Option 1 nor the Option 2 limit according to this summation. The manufacturer could test the drug product to determine if the formulation process reduced the level of acetonitrile. If the level of acetonitrile was not reduced to the allowed limit during formulation, the product fails to meet the solvent limits as described in this chapter and the manufacturer of the drug product should take other steps to reduce the amount of acetonitrile in the drug product. In some instances the manufacturer may have received approval from a competent regulatory authority for such a higher level of residual solvent. If this is the case, it is the responsibility of that manufacturer to notify the USP regarding the identity of this solvent and the approved residual solvent limit in the article. The USP will then address this topic in the individual monograph.

#### ANALYTICAL PROCEDURES

Residual solvents are typically determined using chromatographic techniques such as gas chromatography. Compendial methods for testing for residual solvent content are described under the Identification, Control, and Quantification of Residual Solvents section of this general chapter. The General Notices discuss the use of other methods in special circumstances (see Procedures in Tests and Assays). If Class 3 solvents are present, a nonspecific method such as loss on drying may be used.



#### REPORTING LEVELS OF RESIDUAL SOLVENTS

Manufacturers of pharmaceutical products need certain information about the content of residual solvents in drug substances or excipients in order to meet the criteria of this general chapter. The following statements are given as acceptable examples of the information that could be provided from a supplier of drug substances or excipients to a pharmaceutical manufacturer. The supplier might choose one of the following as appropriate:

- Only Class 3 solvents are likely to be present. Loss on drying is less than 0.5%.
- Only Class 2 solvents X, Y, ... are likely to be present. All are below the Option 1 limit. (Here the supplier would name the Class 2 solvents represented by X, Y, ...)
- Only Class 2 solvents X, Y, ... and Class 3 solvents are likely to be present. Residual Class 2 solvents are below the Option 1 limit and residual Class 3 solvents are below 0.5%.

The phrase "likely to be present" as used in the above examples refers to the solvent used or produced in the final manufacturing step and to solvents that are used or produced in earlier manufacturing steps and not removed consistently by a validated process.

If Class 1 solvents are likely to be present, they should be identified and quantified. If solvents of Class 2 or 3 are present at greater than their Option 1 limits or 0.5%, respectively, they should be identified and quantified.

#### LIMITS OF RESIDUAL SOLVENTS

##### Ethylene Oxide

[note—The test for ethylene oxide is conducted only where specified in the individual monograph.] The standard solution parameters and the procedure for determination are described in the individual monograph. Unless otherwise specified in the individual monograph, the limit is 10 µg per g.

##### Class 1 (solvents to be avoided)

Class 1 residual solvents ([Table 1](#)) should not be employed in the manufacture of drug substances, excipients, and drug products because of the unacceptable toxicities or deleterious environmental effects of these residual solvents. However, if their use in order to produce a medicinal product with a significant therapeutic advance is unavoidable, their levels should be restricted as shown in [Table 1](#), unless otherwise stated in the individual monograph. The solvent 1,1,1-trichloroethane is included in [Table 1](#) because it is an environmental hazard.

The stated limit of 1500 ppm is based on a review of safety data.

When Class 1 residual solvents are used or produced in the manufacture or purification of a drug substance, excipient, or drug product and are not removed by the process, these solvents should be identified and quantified. The procedures described in the Identification, Control, and Quantification of Residual Solvents section of this general chapter are to be applied wherever possible. Otherwise an appropriate validated procedure is to be employed.

Table 1. Class 1 Residual Solvents  
(solvents that should be avoided)

Solvent	Concentration Limit (ppm)	Concern
Benzene	2	Carcinogen
Carbon tetrachloride	4	Toxic and environmental hazard
1,2-Dichloroethane	5	Toxic
1,1-Dichloroethene	8	Toxic
1,1,1-Trichloroethane	1500	Environmental hazard

##### Class 2

Class 2 residual solvents ([Table 2](#)) should be limited in drug substances, excipients, and drug products because of the inherent toxicities of the residual solvents. PDEs are given to the nearest 0.1 mg per day, and concentrations are given to the nearest 10 ppm. The stated values do not reflect the necessary analytical precision of the determination procedure. Precision should be determined as part of the procedure validation.

If Class 2 residual solvents are present at greater than their Option 1 limits, they should be identified and quantified. The procedures described in the Identification, Control, and Quantification of Residual Solvents section of this general chapter are to be applied wherever possible. Otherwise an appropriate validated procedure is to be employed.

[note—The following Class 2 residual solvents are not readily detected by the headspace injection conditions described in the Identification, Control, and Quantification of Residual Solvents section of this general chapter: formamide, 2-ethoxyethanol, 2-methoxyethanol, ethylene glycol, N-methylpyrrolidone, and sulfolane. Other appropriate validated procedures are to be employed for the quantification of these residual solvents. Such procedures shall be submitted to the USP for review and possible inclusion in the relevant individual monograph. In addition, USP Residual Solvent Class 2—Mixture C RS can be used to develop an alternative procedure.]

Table 2. Class 2 Residual Solvents

Solvent	PDE (mg/day)	Concentration Limit (ppm)
Acetonitrile	4.1	410
Chlorobenzene	3.6	360
Chloroform	0.6	60
Cyclohexane	38.8	3880
1,2-Dichloroethene	18.7	1870
1,2-Dimethoxyethane	1.0	100
N,N-Dimethylacetamide	10.9	1090
N,N-Dimethylformamide	8.8	880
1,4-Dioxane	3.8	380
2-Ethoxyethanol	1.6	160
Ethylene glycol	6.2	620
Formamide	2.2	220
Hexane	2.9	290
Methanol	30.0	3000
2-Methoxyethanol	0.5	50
Methylbutylketone	0.5	50
Methylcyclohexane	11.8	1180
Methylene chloride	6.0	600



N-Methylpyrrolidone	5.3	530
Nitromethane	0.5	50
Pyridine	2.0	200
Sulfolane	1.6	160
Tetrahydrofuran	7.2	720
Tetralin	1.0	100
Toluene	8.9	890
Trichloroethylene	0.8	80
Xylene <sup>*</sup>	21.7	2170

\* Usually 60% m-xylene, 14% p-xylene, 9% o-xylene with 17% ethyl benzene

### Class 3

Class 3 residual solvents (Table 3) may be regarded as less toxic and of lower risk to human health than Class 1 and Class 2 residual solvents. Class 3 includes no solvent known as a human health hazard at levels normally accepted in pharmaceuticals. However, there are no long-term toxicity or carcinogenicity studies for many of the residual solvents in Class 3.

Available data indicate that they are less toxic in acute or short-term studies and negative in genotoxicity studies.

It is considered that amounts of these residual solvents of 50 mg per day or less (corresponding to 5000 ppm or 0.5% under Option 1) would be acceptable without justification. Higher amounts may also be acceptable provided they are realistic in relation to manufacturing capability and good manufacturing practice. For the purposes of this Pharmacopeia, when a manufacturer has received approval from a competent regulatory authority for such a higher level of residual solvent, it is the responsibility of that manufacturer to notify the USP regarding the identity of this solvent and the approved residual solvent limit in the article. The USP will then address this topic in the individual monograph. If a Class 3 solvent limit in an individual monograph is greater than 50 mg per day, that residual solvent should be identified and quantified. The procedures described in the Identification, Control, and Quantification of Residual Solvents section of this General Chapter, with appropriate modifications to the standard solutions, are to be applied wherever possible. Otherwise an appropriate validated procedure is to be employed.

Table 3. Class 3 Residual Solvents  
(limited by GMP or other quality-based requirements in drug substances, excipients, and drug products)

Acetic acid	Heptane
Acetone	Isobutyl acetate
Anisole	Isopropyl acetate
1-Butanol	Methyl acetate
2-Butanol	3-Methyl-1-butanol
Butyl acetate	Methylethylketone
tert-Butylmethyl ether	Methylisobutylketone
Cumene	2-Methyl-1-propanol
Dimethyl sulfoxide	Pentane
Ethanol	1-Pentanol
Ethyl acetate	1-Propanol
Ethyl ether	2-Propanol
Ethyl formate	Propyl acetate
Formic acid	

### Other Residual Solvents

The residual solvents listed in Table 4 may also be of interest to manufacturers of drug substances, excipients, or drug products. However, no adequate toxicological data on which to base a PDE was found.

Table 4. Other Residual Solvents  
(for which no adequate toxicological data was found)

1,1-Diethoxypropane	Methyl isopropyl ketone
1,1-Dimethoxymethane	Methyltetrahydrofuran
2,2-Dimethoxypropane	Solvent hexane
Isooctane	Trichloroacetic acid
Isopropyl ether	Trifluoroacetic acid

### IDENTIFICATION, CONTROL, AND QUANTIFICATION OF RESIDUAL SOLVENTS

Whenever possible, the substance under test needs to be dissolved to release the residual solvent. Because the USP deals with drug products, as well as active ingredients and excipients, it may be acceptable that in cases some of the components of the formulation will not dissolve completely. In those cases, the drug product may first need to be pulverized into a fine powder so that any residual solvent that may be present can be released. This operation should be as fast as possible to prevent the loss of volatile solvents during the procedure.

note—The organic-free water specified in the following procedures produces no significantly interfering peaks when chromatographed.

#### Class 1 and Class 2 Residual Solvents

The following procedures are useful to identify and quantify residual solvents when the information regarding which solvents are likely to be present in the material is not available. When the information about the presence of specific residual solvents is available, only Procedure C is needed to quantify the amount of residual solvents present.

##### water-soluble articles

###### Procedure A—

Class 1 Standard Stock Solution—Transfer 1.0 mL of USP Class 1 Residual Solvents Mixture RS to a 100-mL volumetric flask, add 9 mL of dimethyl sulfoxide, dilute with water to volume, and mix. Transfer 1.0 mL of this solution to a 100-mL volumetric flask, dilute with water to volume, and mix. Transfer 1.0 mL of this solution to a 10-mL volumetric flask, dilute with water to volume, and mix.

Class 1 Standard Solution—Transfer 1.0 mL of Class 1 Standard Stock Solution to an appropriate headspace vial, add 5.0 mL of water, apply the stopper, cap, and mix.

Class 2 Standard Stock Solutions—Transfer 1.0 mL of USP Residual Solvents Class 2—Mixture A RS to a 100-mL volumetric flask, dilute with water to volume, and mix. This is Class 2 Standard Stock Solution A. Transfer 1.0 mL of USP Residual Solvents Class 2—Mixture B RS to a 100-mL volumetric flask, dilute with water to volume, and mix. This is Class 2



## Standard Stock Solution B.

Class 2 Mixture A Standard Solution—Transfer 1.0 mL of Class 2 Standard Stock Solution A to an appropriate headspace vial, add 5.0 mL of water, apply the stopper, cap, and mix.

Class 2 Mixture B Standard Solution—Transfer 5.0 mL of Class 2 Standard Stock Solution B to an appropriate headspace vial, add 1.0 mL of water, apply the stopper, cap, and mix.

Test Stock Solution—Transfer about 250 mg of the article under test, accurately weighed, to a 25-mL volumetric flask, dissolve in and dilute with water to volume, and mix.

Test Solution—Transfer 5.0 mL of Test Stock Solution to an appropriate headspace vial, add 1.0 mL of water, apply the stopper, cap, and mix.

Class 1 System Suitability Solution—Transfer 1.0 mL of Class 1 Standard Stock Solution to an appropriate headspace vial, add 5.0 mL of Test Stock Solution, apply the stopper, cap, and mix.

Chromatographic System (see [Chromatography 621](#))—The gas chromatograph is equipped with a flame-ionization detector, a 0.32-mm × 30-m fused-silica column coated with a 1.8-μm layer of phase G43 or a 0.53-mm × 30-m wide-bore column coated with a 3.0-μm layer of phase G43. The carrier gas is nitrogen or helium with a linear velocity of about 35 cm per second, and a split ratio of 1:5. [note—Split ratio can be modified in order to optimize sensitivity.] The column temperature is maintained at 40° for 20 minutes, then raised at a rate of 10° per minute to 240°, and maintained at 240° for 20 minutes. The injection port and detector temperatures are maintained at 140° and 250°, respectively. Chromatograph the Class 1 Standard Solution, Class 1 System Suitability Solution, and Class 2 Mixture A Standard Solution, and record the peak responses as directed for Procedure: the signal-to-noise ratio of 1,1,1-trichloroethane in the Class 1 Standard Solution is not less than 5; the signal-to-noise ratio of each peak in the Class 1 System Suitability Solution is not less than 3; and the resolution, R, between acetonitrile and methylene chloride in the Class 2 Mixture A Standard Solution is not less than 1.0.

Procedure—Separately inject (following one of the headspace operating parameter sets described in the table below) equal volumes of headspace (about 1.0 mL) of the Class 1 Standard Solution, Class 2 Mixture A Standard Solution, Class 2 Mixture B Standard Solution, and the Test Solution into the chromatograph, record the chromatograms, and measure the responses for the major peaks. If a peak response of any peak, other than a peak for 1,1,1-trichloroethane, in the Test Solution is greater than or equal to a corresponding peak in either the Class 1 Standard Solution or either of the two Class 2 Mixture Standard Solutions, or a peak response of 1,1,1-trichloroethane is greater than or equal to 150 times the peak response corresponding to 1,1,1-trichloroethane in the Class 1 Standard Solution, proceed to Procedure B to verify the identity of the peak; otherwise the article meets the requirements of this test.

Table 5. Headspace Operating Parameters

	Headspace Operating Parameter Sets		
	1	2	3
Equilibration temperature (°)	80	105	80
Equilibration time (min.)	60	45	45
Transfer-line temperature (°)	85	110	105
Carrier gas: nitrogen or helium at an appropriate pressure			
Pressurization time (s)	30	30	30
Injection volume (mL)	1	1	1

## Procedure B—

Class 1 Standard Stock Solution, Class 1 Standard Solution, Class 2 Standard Stock Solutions, Class 2 Mixture A Standard Solution, Class 2 Mixture B Standard Solution, Test Stock Solution, Test Solution, and Class 1 System Suitability Solution—Prepare as directed for Procedure A.

Chromatographic System (see [Chromatography 621](#))—The gas chromatograph is equipped with a flame-ionization detector, a 0.32-mm × 30-m fused-silica column coated with a 0.25-μm layer of phase G16, or a 0.53-mm × 30-m wide-bore column coated with a 0.25-μm layer of phase G16. The carrier gas is nitrogen or helium with a linear velocity of about 35 cm per second and a split ratio of 1:5. [note—Split ratio can be modified in order to optimize sensitivity.] The column temperature is maintained at 50° for 20 minutes, then raised at a rate of 6° per minute to 165°, and maintained at 165° for 20 minutes. The injection port and detector temperatures are maintained at 140° and 250°, respectively. Chromatograph the Class 1 Standard Solution and the Class 1 System Suitability Solution, and record the peak responses as directed for Procedure: the signal-to-noise ratio of benzene in the Class 1 Standard Solution is not less than 5; the signal-to-noise ratio of each peak in the Class 1 System Suitability Solution is not less than 3; and the resolution, R, between acetonitrile and cis-dichloroethene in the Class 2 Mixture A Standard Solution is not less than 1.0.

Procedure—Separately inject (following one of the headspace operating parameter sets described in [Table 5](#)) equal volumes of headspace (about 1.0 mL) of the Class 1 Standard Solution, the Class 2 Mixture A Standard Solution, the Class 2 Mixture B Standard Solution, and the Test Solution into the chromatograph, record the chromatograms, and measure the responses for the major peaks. If the peak response(s) in the Test Solution of the peak(s) identified in Procedure A is/are greater than or equal to a corresponding peak(s) in either the Class 1 Standard Solution or either of the two Class 2 Mixture Standard Solutions, proceed to Procedure C to quantify the peak(s); otherwise the article meets the requirements of this test.

## Procedure C—

Class 1 Standard Stock Solution, Class 1 Standard Solution, Class 2 Standard Stock Solution A, Class 2 Mixture A Standard Solution, Test Stock Solution, Test Solution, and Class 1 System Suitability Solution—Prepare as directed for Procedure A.

Standard Solution—[note—Prepare a separate Standard Solution for each peak identified and verified by Procedures A and B. For the Class 1 solvents other than 1,1,1-trichloroethane, prepare the first dilution as directed for the first dilution under Class 1 Standard Stock Solution in Procedure A.] Transfer an accurately measured volume of each individual USP Reference Standard corresponding to each residual solvent peak identified and verified by Procedures A and B to a suitable container, and dilute quantitatively, and stepwise if necessary, with water to obtain a solution having a final concentration of 1/20 of the value stated in [Table 1](#) or [2](#) (under Concentration Limit). Transfer 1.0 mL of this solution to an appropriate headspace vial, add 5.0 mL of water, apply the stopper, cap, and mix.

Spiked Test Solution—[note—Prepare a separate Spiked Test Solution for each peak identified and verified by Procedures A and B.] Transfer 5.0 mL of Test Stock Solution to an appropriate headspace vial, add 1.0 mL of the Standard Solution, apply the stopper, cap, and mix.

Chromatographic System (see [Chromatography 621](#))—[note—If the results of the chromatography from Procedure A are found to be inferior to those found with Procedure B, the Chromatographic System from Procedure B may be substituted.] The gas chromatograph is equipped with a flame-ionization detector, a 0.32-mm × 30-m fused-silica column coated with a 1.8-μm layer of phase G43 or a 0.53-mm × 30-m wide-bore column coated with a 3.0-μm layer of phase G43. The carrier gas is nitrogen or helium with a linear velocity of about 35 cm per second, and a split ratio of 1:5. [note—The split ratio can be modified in order to optimize sensitivity.] The column temperature is maintained at 40° for 20 minutes, then raised at a rate of 10° per minute to 240°, and maintained at 240° for 20 minutes. The injection port and detector temperatures are maintained at 140° and 250°, respectively. Chromatograph the Class 1 Standard Solution, the Class 1 System Suitability Solution, and the Class 2 Mixture A Standard Solution, and record the peak responses as directed for Procedure: the signal-to-noise ratio of 1,1,1-trichloroethane in the Class 1 Standard Solution is not less than 5; the signal-to-noise ratio of each peak in the Class 1 System Suitability Solution is not less than 3; and the resolution, R, between acetonitrile and methylene chloride in the Class 2 Mixture A Standard Solution is not less than 1.0.

Procedure—Separately inject (following one of the headspace operating parameters described in [Table 5](#)) equal volumes of headspace (about 1.0 mL) of the Standard Solution, the Test Solution, and the Spiked Test Solution into the chromatograph, record the chromatograms, and measure the responses for the major peaks. Calculate the amount, in ppm, of each residual solvent found in the article under test by the formula:

$$5(C/W)[rU / (rST - rU)]$$

in which C is the concentration, in μg per mL, of the appropriate USP Reference Standard in the Standard Solution; W is the weight, in g, of the article under test taken to prepare the



Test Stock Solution; and rU and rST are the peak responses of each residual solvent obtained from the Test Solution and the Spiked Test Solution, respectively.

#### water-insoluble articles

Procedure A— [note—Dimethyl sulfoxide may be substituted as an alternative solvent to dimethylformamide.]

Class 1 Standard Stock Solution— Transfer 1.0 mL of USP Class 1 Residual Solvents Mixture RS to a 100-mL volumetric flask previously filled with about 80 mL of dimethylformamide, dilute with dimethylformamide to volume, and mix. Transfer 1.0 mL of this solution to a 100-mL volumetric flask, previously filled with about 80 mL of dimethylformamide, dilute with dimethylformamide to volume, and mix (reserve a portion of this solution for the Class 1 System Suitability Solution). Transfer 1.0 mL of this solution to a 10-mL volumetric flask, dilute with dimethylformamide to volume, and mix.

Class 1 Standard Solution— Transfer 1.0 mL of Class 1 Standard Stock Solution to an appropriate headspace vial, containing 5.0 mL of water, apply the stopper, cap, and mix.

Class 2 Standard Stock Solutions— Transfer 1.0 mL of USP Residual Solvents Class 2—Mixture A RS to a 100-mL volumetric flask, previously filled with about 80 mL of dimethylformamide, dilute with dimethylformamide to volume, and mix. This is Class 2 Standard Stock Solution A. Transfer 0.5 mL of USP Residual Solvents Class 2—Mixture B RS to a 10-mL volumetric flask, dilute with dimethylformamide to volume, and mix. This is Class 2 Standard Stock Solution B.

Class 2 Mixture A Standard Solution— Transfer 1.0 mL of Class 2 Standard Stock Solution A to an appropriate headspace vial, containing 5.0 mL of water, apply the stopper, cap, and mix.

Class 2 Mixture B Standard Solution— Transfer 1.0 mL of Class 2 Standard Stock Solution B to an appropriate headspace vial, containing 5.0 mL of water, apply the stopper, cap, and mix.

Test Stock Solution— Transfer about 500 mg of the article under test, accurately weighed, to a 10-mL volumetric flask, dissolve in and dilute with dimethylformamide to volume, and mix.

Test Solution— Transfer 1.0 mL of Test Stock Solution to an appropriate headspace vial, containing 5.0 mL of water, apply the stopper, cap, and mix.

Class 1 System Suitability Solution— Mix 5 mL of Test stock solution with 0.5 mL of the intermediate dilution reserved from Class 1 Standard Stock Solution. Transfer 1.0 mL of this solution to an appropriate headspace vial, containing 5.0 mL of water, apply the stopper, cap, and mix.

Chromatographic System (see [Chromatography](#) 621) — The gas chromatograph is equipped with a flame-ionization detector, a 0.53-mm × 30-m wide-bore column coated with a 3.0-μm layer of phase G43. The carrier gas is helium with a linear velocity of about 35 cm per second, and a split ratio of 1:3 [note—Split ratio can be modified in order to optimize sensitivity.] The column temperature is maintained at 40° for 20 minutes, then raised at a rate of 10° per minute to 240°, and maintained at 240° for 20 minutes. The injection port and detector temperatures are maintained at 140° and 250°, respectively. Chromatograph the Class 1 Standard Solution, Class 1 System Suitability Solution, and Class 2 Mixture A Standard Solution, and record the peak responses as directed for Procedure: the signal-to-noise ratio of 1,1,1-trichloroethane in the Class 1 Standard Solution is not less than 5; the signal-to-noise ratio of each peak in the Class 1 System Suitability Solution is not less than 3; and the resolution, R, between acetonitrile and methylene chloride in the Class 2 Mixture A Standard Solution is not less than 1.0.

Procedure— Separately inject (use headspace operating parameters 3 in [Table 5](#) with a vial pressure of 10 psi) equal volumes of headspace (about 1.0 mL) of the Class 1 Standard Solution, Class 2 Mixture A Standard Solution, Class 2 Mixture B Standard Solution, and Test Solution into the chromatograph, record the chromatograms, and measure the responses for the major peaks. If a peak response of any peak, other than a peak for 1,1,1-trichloroethane, in the Test Solution is greater than or equal to a corresponding peak in either the Class 1 Standard Solution or either of the two Class 2 Mixture Standard Solutions, or a peak response of 1,1,1-trichloroethane is greater than or equal to 150 times the peak response corresponding to 1,1,1-trichloroethane in the Class 1 Standard Solution, proceed to Procedure B to verify the identity of the peak; otherwise the article meets the requirements of this test.

#### Procedure B—

Class 1 Standard Stock Solution, Class 1 Standard Solution, Class 1 System Suitability Solution, Class 2 Standard Stock Solutions, Class 2 Mixture A Standard Solution, and Class 2 Mixture B Standard Solution, Test Stock Solution, and Test Solution— Proceed as directed for Procedure A.

Chromatographic System— Proceed as directed for Procedure B under Water-Soluble Articles with a split ratio of 1:3 [note—The split ratio can be modified in order to optimize sensitivity.]

Procedure— Separately inject (use headspace operating parameters 3 in [Table 5](#) with a vial pressure of 10 psi) equal volumes of headspace (about 1.0 mL) of the Class 1 Standard Solution, Class 2 Mixture A Standard Solution, Class 2 Mixture B Standard Solution, and Test Solution, into the chromatograph, record the chromatograms, and measure the responses for the major peaks. If the peak response(s) in Test Solution of the peak(s) identified in Procedure A is/are greater than or equal to a corresponding peak(s) in either the Class 1 Standard Solution or any of the two Class 2 Mixture Standard Solutions, proceed to Procedure C to quantify the peak(s); otherwise the article meets the requirements of this test.

#### Procedure C—

Class 1 Standard Stock Solution, Class 1 Standard Solution, Class 1 System Suitability Solution, Class 2 Standard Stock Solution A, and Class 2 Mixture A Standard Solution— Proceed as directed for Procedure A.

Standard Stock Solution— [note—Prepare a separate Standard Solution for each peak identified and verified by Procedures A and B.] Transfer an accurately measured volume of each individual USP Reference Standard corresponding to each residual solvent peak identified and verified by Procedures A and B to a suitable container, and dilute quantitatively, and stepwise if necessary, with water to obtain a solution having a final concentration of 1/20 of the value stated in [Table 1](#) or [Table 2](#) (under Concentration Limit).

Standard Solution— Transfer 1.0 mL of the Standard Stock Solution to an appropriate headspace vial, containing 5.0 mL of water, apply the stopper, cap, and mix.

Test Stock Solution— Proceed as directed for Procedure A.

Test Solution— Transfer 1.0 mL of the Test Stock Solution to an appropriate headspace vial, containing 5.0 mL of water, apply the stopper, cap, and mix.

Spiked Test Solution— [note—Prepare a separate Spiked Test Solution for each peak identified and verified by Procedures A and B.] Transfer 1.0 mL of Test Stock Solution to an appropriate headspace vial, add 1 mL of Standard Stock Solution and 4.0 mL of water, apply the stopper, cap, and mix.

Chromatographic System— Proceed as directed for Procedure C under Water-Soluble Articles.

Procedure— Separately inject (use headspace operating parameters 3 in [Table 5](#) with a vial pressure of 10 psi) equal volumes of headspace (about 1.0 mL) of the Standard Solution, Test Solution, and Spiked Test Solution into the chromatograph, record the chromatograms, and measure the responses for the major peaks. Calculate the amount, in ppm, of each residual solvent found in the article under test by the formula:

$$10(C/W)[rU / (rST - rU)]$$

in which C is the concentration, in  $\mu\text{g}$  per mL, of the appropriate USP Reference Standard in the Standard Solution; W is the weight, in g, of the article under test taken to prepare the Test Stock Solution; and rU and rST are the peak responses of each residual solvent obtained from Test Solution and Spiked Test Solution, respectively.

#### Class 3 Residual Solvents

If Class 3 solvents are present, the level of residual solvents may be determined as directed under [Loss on Drying](#) 731 when the monograph for the article under test contains a loss on drying procedure, or a specific determination of the solvent may be made. If there is no loss on drying procedure in the monograph for the article under test or if a Class 3 solvent limit in an individual monograph is greater than 50 mg per day (corresponding to 5000 ppm or 0.5% under Option 1), the individual Class 3 residual solvent or solvents present in the article under test should be identified and quantified, and the procedures as described above, with appropriate modifications to the standard solutions, are to be applied wherever possible. Otherwise an appropriate validated procedure is to be employed. USP Reference Standards, where available, should be used in these procedures. A flow diagram for the application of residual solvent limit tests is shown in [Figure 1](#).

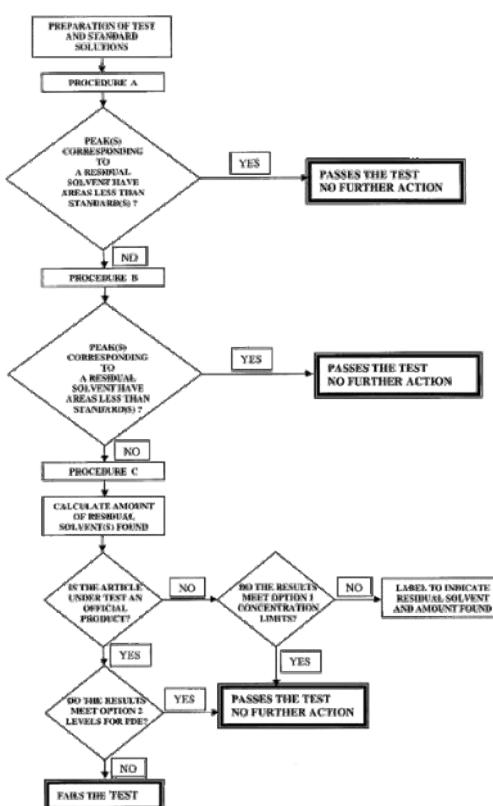


Figure 1. Diagram relating to the identification of residual solvents and the application of limit tests.

#### OTHER ANALYTICAL PROCEDURES

The following procedures, with any necessary variations, are used where specified in the individual monographs.

##### Method I

A gas chromatograph capable of temperature programming and equipped with a wide-bore, wall-coated open tubular column and a flame-ionization detector is used in the following procedure.

**Standard Solution**— Prepare a solution, in organic-free water, or the solvent specified in the monograph, containing in each mL, 12.0 µg of methylene chloride, 7.6 µg of 1,4-dioxane, 1.6 µg of trichloroethylene, and 1.2 µg of chloroform. [note—Prepare fresh daily.]

**Test Solution**— Dissolve in organic-free water, or the solvent specified in the monograph, an accurately weighed portion of the material to be tested to obtain a final solution having a known concentration of about 20 mg of the test material per mL.

**Chromatographic System** (see [Chromatography](#) 621)— The gas chromatograph is equipped with a flame-ionization detector, a 0.53-mm × 30-m fused silica analytical column coated with a 5-µm chemically cross-linked G27 stationary phase and a 0.53-mm × 5-m silica guard column deactivated with phenylmethyl siloxane. The carrier gas is helium with a linear velocity of about 35 cm per second. [note—When a makeup gas is used, nitrogen is recommended.] The injection port temperature and the detector temperature are maintained at 70° and 260°, respectively. The column temperature is programmed as follows. Initially, the column temperature is maintained at 35° for 5 minutes, then increased at a rate of 8° per minute to 175°, followed by an increase at a rate of 35° per minute to 260°, and maintained at 260° for at least 16 minutes.

Inject the Standard Solution, and record the peak responses as directed for Procedure: a suitable system is one that yields chromatograms in which all of the components in the Standard Solution are resolved; the resolution, R, between any two components is not less than 1.0; and the relative standard deviation of the individual peak responses from replicate injections is not more than 15%.

**Procedure**— Separately inject equal volumes (about 1 µL) of the Standard Solution and the Test Solution into the chromatograph, record the chromatograms, and measure the peak responses.

Identify, on the basis of retention time, any peaks present in the chromatogram of the Test Solution. The identity and peak response in the chromatogram may be established as being from any of the organic volatile impurities listed in the table shown below or from some other volatile impurity eluting with a comparable retention time as determined by mass spectrometric relative abundance procedures or by the use of a second validated column containing a different stationary phase.

Unless otherwise specified in the individual monograph, the amount of each organic volatile impurity present in the material does not exceed the limit given in the table shown below.

Organic Volatile Impurity	Limit (µg per g)
Chloroform	60
1,4-Dioxane	380
Methylene Chloride	600
Trichloroethylene	80

##### Method IV

**Standard Solution**— Prepare as directed for Standard Solution in Method I. Pipet 5 mL of the solution into a vial fitted with a septum and crimp cap, containing 1 g of anhydrous sodium sulfate, and seal. Heat the sealed vial at 80° for 60 minutes.

**Test Solution**— Transfer 100 mg, accurately weighed, of the material under test to a vial, add 5.0 mL of water, or the solvent specified in the monograph, and 1 g of anhydrous sodium sulfate, and seal with a septum and crimp cap. Heat the sealed vial at 80° for 60 minutes, or as specified in the individual monograph.

**Chromatographic System and Procedure**— [note—The use of headspace apparatuses that automatically transfer a measured amount of headspace is allowed. Also, the use of a



guard column in this headspace procedure is not necessary.] Proceed as directed for Method V, except to inject, using a heated gas-tight syringe, 1 mL of the headspace.

#### Method V

Standard Solution and Test Solution— Prepare as directed for Method I.

Chromatographic System (see [Chromatography \(621\)](#))—The gas chromatograph is equipped with a flame-ionization detector, a 0.53-mm × 30-m fused silica analytical column coated with a 3.0-μm G43 stationary phase, and a 0.53-mm × 5-m silica guard column deactivated with phenylmethyl siloxane. The carrier gas is helium with a linear velocity of about 35 cm per second. The injection port and detector temperatures are maintained at 140° and 260°, respectively. The column temperature is programmed according to the following steps. It is maintained at 40° for 20 minutes, then increased rapidly to 240°, and maintained at 240° for 20 minutes.

Inject the Standard Solution, and record the peak responses as directed for Procedure: a suitable system is one that yields chromatograms in which all of the components in the Standard Solution are resolved; the resolution, R, between any two components is not less than 3; and the relative standard deviation of the individual peak responses from replicate injections is not more than 15%.

Procedure— Proceed as directed for Method I, the injection volume being about 1 μL.

#### Method VI

Standard Solution and Test Solution— Prepare as directed for Method I.

Chromatographic System (see [Chromatography \(621\)](#))—The gas chromatograph is equipped with a flame-ionization detector. The column and column temperature conditions, as chosen from the list below (see [Table 6](#)), are specified in the individual monograph. The carrier gas, linear velocity or flow rate, and detector and injection port temperatures are appropriate to the column dimensions and column temperatures chosen from the list below.

Inject the Standard Solution, and record the peak responses as directed for Procedure: a suitable system is one that yields the chromatograms in which all of the components in the Standard Solution are resolved; the resolution, R, between any two components is not less than 1.0; and the relative standard deviation of the individual peak responses from replicate injections is not more than 15%.

Procedure— Proceed as directed for Method I, the injection volume being about 1 μL.

Table 6. Chromatographic Conditions for Method VI

Chromatographic Conditions	USP Column Designation	Column Size	Column Temperature
A	S3	3-mm × 2-m	190°
B	S2	3-mm × 2.1-m	160°
C	G16	0.53-mm × 30-m	40°
D	G39	3-mm × 2-m	65°
E	G16	3-mm × 2-m	70°
F	S4	2-mm × 2.5-m	Hold 120° (35 min.) Gradient 120°–200° (2°/min.) Hold 20 min.
H	G14	2-mm × 2.5-m	Hold 45° (3 min.) Gradient 45°–120° (8°/min.) Hold 15 min.
I	G27	0.53-mm × 30-m	Hold 35° (5 min.) 35°–175° (8°/min.) 175°–260° (35°/min.) Hold 16 min.
J	G16	0.33-mm × 30-m	Hold 50° (20 min.) 50°–165° (6°/min.) Hold 20 min.

#### GLOSSARY

Acceptable daily intake (ADI): The maximum acceptable intake of toxic chemicals per day. This term is used by the World Health Organization (WHO).

Genotoxic carcinogens: Carcinogens that produce cancer by affecting genes or chromosomes.

Lowest-observed-effect level (LOEL): The lowest dose of a substance in a study or group of studies that produces biologically significant increases in frequency or severity of any effects in exposed humans or animals.

Modifying factor: A factor determined by professional judgment of a toxicologist and applied to bioassay data so that the data can be safely related to humans.

Neurotoxicity: The ability of a substance to cause adverse effects on the nervous system.

No-observed-effect level (NOEL): The highest dose of a substance at which there are no biologically significant increases in frequency or severity of any effects in exposed humans or animals.

Permitted daily exposure (PDE): The maximum acceptable intake per day of a residual solvent in pharmaceutical products.

Reversible toxicity: The occurrence of harmful effects that are caused by a substance and that disappear after exposure to the substance ends.

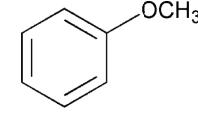
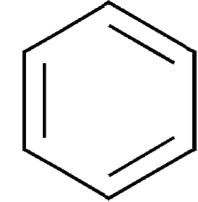
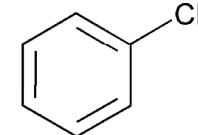
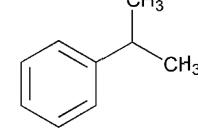
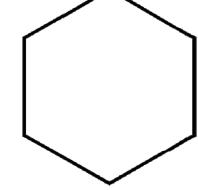
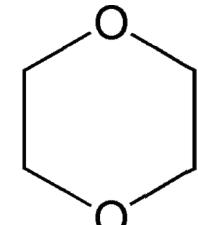
Strongly suspected human carcinogen: A substance for which there is no epidemiological evidence of carcinogenesis but for which there are positive genotoxicity data and clear evidence of carcinogenesis in rodents.

Teratogenicity: The occurrence of structural malformations in a developing fetus when a substance is administered during pregnancy.

Tolerable daily intake (TDI): Tolerable daily exposure to toxic chemicals. Term used by the International Program on Chemical Safety (IPCS).

#### APPENDIX 1. LIST OF RESIDUAL SOLVENTS INCLUDED IN THIS GENERAL CHAPTER

Solvent	Other Names	Structure	Class
Acetic acid	Ethanoic acid	CH <sub>3</sub> COOH	Class 3
Acetone	2-Propanone Propan-2-one	CH <sub>3</sub> COCH <sub>3</sub>	Class 3

Cetonitrile		CH3CN	
Anisole	Methoxybenzene		Class 2 Class 3
Benzene	Benzol		Class 1
1-Butanol	n-Butyl alcohol Butan-1-ol	CH3(CH2)3OH	Class 3
2-Butanol	sec-Butyl alcohol Butan-2-ol	CH3CH2CH(OH)CH3	Class 3
Butyl acetate	Acetic acid butyl ester	CH3COO(CH2)3CH3	Class 3
tert-Butylmethyl ether	2-Methoxy-2-methylpropane	(CH3)3COCH3	Class 3
Carbon tetrachloride	Tetrachloromethane	CCl4	Class 1
Chlorobenzene			Class 2
Chloroform	Trichloromethane	CHCl3	Class 2
Cumene	Isopropylbenzene (1-Methylethyl)benzene		Class 3
Cyclohexane	Hexamethylene		Class 2
1,2-Dichloroethane	sym-Dichloroethane Ethylene dichloride Ethylene chloride	CH2ClCH2Cl	Class 1
1,1-Dichloroethene	1,1-Dichloroethylene Vinylidene chloride	H2C=CCl2	Class 1
1,2-Dichloroethene	1,2-Dichloroethylene Acetylene dichloride	CHCl=CHCl	Class 2
1,2-Dimethoxyethane	Ethyleneglycol dimethyl ether Monoglyme Dimethyl cellosolve	H3COCH2CH2OCH3	Class 2
N,N-Dimethylacetamide	DMA	CH3CON(CH3)2	Class 2
N,N-Dimethylformamide	DMF	HCON(CH3)2	Class 2
Dimethyl sulfoxide	Methylsulfinylmethane Methyl sulfoxide DMSO	(CH3)2SO	Class 3
1,4-Dioxane	p-Dioxane [1,4]Dioxane		Class 2
Ethanol	Ethyl alcohol	CH3CH2OH	Class 3
2-Ethoxyethanol	Cellosolve	CH3CH2OCH2CH2OH	Class 2
Ethyl acetate	Acetic acid ethyl ester	CH3COOCH2CH3	Class 3

Methylene glycol	1,2-Dihydroxyethane 1,2-Ethanediol	HOCH2CH2OH	Class 2
Ethyl ether	Diethyl ether Ethoxyethane 1,1'-Oxybisethane	CH3CH2OCH2CH3	Class 3
Ethyl formate	Formic acid ethyl ester	HCOOCH2CH3	Class 3
Formamide	Methanamide	HCONH2	Class 2
Formic acid		HCOOH	Class 3
Heptane	n-Heptane	CH3(CH2)5CH3	Class 3
Hexane	n-Hexane	CH3(CH2)4CH3	Class 2
Isobutyl acetate	Acetic acid isobutyl ester	CH3COOCH2CH(CH3)2	Class 3
Isopropyl acetate	Acetic acid isopropyl ester	CH3COOCH(CH3)2	Class 3
Methanol	Methyl alcohol	CH3OH	Class 2
2-Methoxyethanol	Methyl cellosolve	CH3OCH2CH2OH	Class 2
Methyl acetate	Acetic acid methyl ester	CH3COOCH3	Class 3
3-Methyl-1-butanol	Isoamyl alcohol Isopentyl alcohol 3-Methylbutan-1-ol	(CH3)2CHCH2CH2OH	Class 3
Methylbutylketone	2-Hexanone Hexan-2-one	CH3(CH2)3COCH3	Class 2
Methylcyclohexane	Cyclohexylmethane		Class 2
Methylene chloride	Dichloromethane	CH2Cl2	Class 2
Methylethylketone	2-Butanone MEK Butan-2-one	CH3CH2COCH3	Class 3
Methyl isobutyl ketone	4-Methylpentan-2-one 4-Methyl-2-pentanone MIBK	CH3COCH2CH(CH3)2	Class 3
2-Methyl-1-propanol	Isobutyl alcohol 2-Methylpropan-1-ol	(CH3)2CHCH2OH	Class 3
N-Methylpyrrolidone	1-Methylpyrrolidin-2-one 1-Methyl-2-pyrrolidinone		Class 2
Nitromethane		CH3NO2	Class 2
Pentane	n-Pentane	CH3(CH2)3CH3	Class 3
1-Pentanol	Amyl alcohol Pantan-1-ol Pentyl alcohol	CH3(CH2)3CH2OH	Class 3
1-Propanol	Propan-1-ol Propyl alcohol	CH3CH2CH2OH	Class 3
2-Propanol	Propan-2-ol Isopropyl alcohol	(CH3)2CHOH	Class 3
Propyl acetate	Acetic acid propyl ester	CH3COOCH2CH2CH3	Class 3
Pyridine			Class 2
Sulfolane	Tetrahydrothiophene 1,1-dioxide		Class 2

Tetrahydrofuran	Tetramethylene oxide Oxacyclopentane		Class 2
Tetralin	1,2,3,4-Tetrahydronaphthalene		Class 2
Toluene	Methylbenzene		Class 2
1,1,1-Trichloroethane	Methylchloroform	CH <sub>3</sub> CCl <sub>3</sub>	Class 1
Trichloroethylene	1,1,2-Trichloroethene	HCIC=CCl <sub>2</sub>	Class 2
Xylene <sup>a</sup>	Dimethylbenzene Xylool		Class 2

\* Usually 60% m-xylene, 14% p-xylene, 9% o-xylene with 17% ethyl benzene.

## APPENDIX 2. ADDITIONAL BACKGROUND

### A2.1. Environmental Regulation of Organic Volatile Solvents

Several of the residual solvents frequently used in the production of pharmaceuticals are listed as toxic chemicals in Environmental Health Criteria (EHC) monographs and in the Integrated Risk Information System (IRIS). The objectives of such groups as the International Programme on Chemical Safety (IPCS), the United States Environmental Protection Agency (EPA), and the United States Food and Drug Administration (FDA) include the determination of acceptable exposure levels. The goal is maintenance of environmental integrity and protection of human health against the possible deleterious effects of chemicals resulting from long-term environmental exposure. The procedures involved in the estimation of maximum safe exposure limits are usually based on long-term studies. When long-term study data are unavailable, shorter term study data can be used with modification of the approach, such as use of larger safety factors. The approach described therein relates primarily to long-term or lifetime exposure of the general population in the ambient environment (i.e., ambient air, food, drinking water, and other media).

### A2.2. Residual Solvents in Pharmaceuticals

Exposure limits in this General Chapter are established by referring to methodologies and toxicity data described in EHC and IRIS monographs. However, the following specific assumptions about residual solvents to be used in the synthesis and formulation of pharmaceutical products should be taken into account in establishing exposure limits.

1. Patients (not the general population) use pharmaceuticals to treat their diseases or for prophylaxis to prevent infection or disease.
2. The assumption of lifetime patient exposure is not necessary for most pharmaceutical products but may be appropriate as a working hypothesis to reduce risk to human health.
3. Residual solvents are unavoidable components in pharmaceutical production and will often be a part of medicinal products.
4. Residual solvents should not exceed recommended levels except in exceptional circumstances.
5. Data from toxicological studies that are used to determine acceptable levels for residual solvents should have been generated using appropriate protocols such as those described, for example, by the Organization for Economic Cooperation and Development (OECD), EPA, and the FDA Red Book.

## APPENDIX 3. PROCEDURES FOR ESTABLISHING EXPOSURE LIMITS

The Gaylor-Kodell method of risk assessment (Gaylor, D. W. and Kodell, R. L. Linear Interpolation Algorithm for Low Dose Assessment of Toxic Substance. *Journal of Environmental Pathology and Toxicology*, 4:305, 1980) is appropriate for Class 1 carcinogenic solvents. Only in cases where reliable carcinogenicity data are available should extrapolation by the use of mathematical models be applied to setting exposure limits. Exposure limits for Class 1 residual solvents could be determined with the use of a large safety factor (i.e., 10,000 to 100,000) with respect to the no-observed-effect level (NOEL). Detection and quantification of these residual solvents should be performed by state-of-the-art analytical techniques.

Acceptable exposure levels in this General Chapter for Class 2 residual solvents were established by calculation of PDE values according to the procedures for setting exposure limits in pharmaceuticals (page 5748 of PF 15(6) [Nov.–Dec. 1989]), and the method adopted by IPCS for Assessing Human Health Risk of Chemicals (Environmental Health Criteria 170, WHO, 1994). These procedures are similar to those used by the U.S. EPA (IRIS) and the U.S. FDA (Red Book) and others. The method is outlined here to give a better understanding of the origin of the PDE values. It is not necessary to perform these calculations in order to use the PDE values presented in [Table 2](#) of this document.



PDE is derived from the no-observed-effect level (NOEL), or the lowest-observed effect level (LOEL), in the most relevant animal study as follows:

$$PDE = \frac{NOEL \times \text{Weight Adjustment}}{F1 \times F2 \times F3 \times F4 \times F5} \quad (1)$$

The PDE is derived preferably from a NOEL. If no NOEL is obtained, the LOEL may be used. Modifying factors proposed here, for relating the data to humans, are the same kind of "uncertainty factors" used in Environmental Health Criteria (Environmental Health Criteria 170, WHO, Geneva, 1994) and "modifying factors" or "safety factors" in Pharmacopeial Forum. The assumption of 100 percent systemic exposure is used in all calculations regardless of route of administration.

The modifying factors are as follows:

F1 = A factor to account for extrapolation between species

F1 = 2 for extrapolation from dogs to humans

F1 = 2.5 for extrapolation from rabbits to humans

F1 = 3 for extrapolation from monkeys to humans

F1 = 5 for extrapolation from rats to humans

F1 = 10 for extrapolation from other animals to humans

F1 = 12 for extrapolation from mice to humans

F1 takes into account the comparative surface area to body weight ratios for the species concerned and for man. Surface area (S) is calculated as:

$$S = kM^{0.67} \quad (2)$$

in which M = body weight, and the constant k has been taken to be 10. The body weights used in the equation are those shown below in [Table A3-1](#).

F2 = A factor of 10 to account for variability between individuals. A factor of 10 is generally given for all organic solvents, and 10 is used consistently in this General Chapter.

F3 = A variable factor to account for toxicity studies of short-term exposure.

F3 = 1 for studies that last at least one half-lifetime (1 year for rodents or rabbits; 7 years for cats, dogs, and monkeys).

F3 = 1 for reproductive studies in which the whole period of organogenesis is covered.

F3 = 2 for a 6-month study in rodents, or a 3.5-year study in nonrodents.

F3 = 5 for a 3-month study in rodents, or a 2-year study in nonrodents.

F3 = 10 for studies of a shorter duration.

In all cases, the higher factor has been used for study durations between the time points (e.g., a factor of 2 for a 9-month rodent study).

F4 = A factor that may be applied in cases of severe toxicity, e.g., nongenotoxic carcinogenicity, neurotoxicity, or teratogenicity. In studies of reproductive toxicity, the following factors are used:

F4 = 1 for fetal toxicity associated with maternal toxicity

F4 = 5 for fetal toxicity without maternal toxicity

F4 = 5 for a teratogenic effect with maternal toxicity

F4 = 10 for a teratogenic effect without maternal toxicity

F5 = A variable factor that may be applied if the no-effect

level was not established.

When only a LOEL is available, a factor of up to 10 can be used depending on the severity of the toxicity. The weight adjustment assumes an arbitrary adult human body weight for either sex of 50 kilograms (kg). This relatively low weight provides an additional safety factor against the standard weights of 60 kg or 70 kg that are often used in this type of calculation. It is recognized that some adult patients weigh less than 50 kg; these patients are considered to be accommodated by the built-in safety factors used to determine a PDE. If the solvent was present in a formulation specifically intended for pediatric use, an adjustment for a lower body weight would be appropriate.

As an example of the application of this equation, consider a toxicity study of acetonitrile in mice that is summarized in *Pharneuropa*, Vol. 9, No. 1, Supplement, April 1997, page S24.

The NOEL is calculated to be 50.7 mg kg<sup>-1</sup> day<sup>-1</sup>. The PDE for acetonitrile in this study is calculated as follows:

$$PDE = \frac{50.7 \text{ mg kg}^{-1} \text{ day}^{-1} \times 50 \text{ kg}}{12 \times 10 \times 5 \times 1 \times 1} = 4.22 \text{ mg day}^{-1}$$

In this example,

F1 = 12 to account for the extrapolation from mice to humans

F2 = 10 to account for differences between individual humans

F3 = 5 because the duration of the study was only 13 weeks

F4 = 1 because no severe toxicity was encountered

F5 = 1 because the no-effect level was determined

#### A3-1. Values Used in the Calculations in This Document

Rat body weight	425 g
Pregnant rat body weight	330 g
Mouse body weight	28 g
Pregnant mouse body weight	30 g
Guinea-pig body weight	500 g
Rhesus monkey body weight	2.5 kg
Rabbit body weight (pregnant or not)	4 kg
Beagle dog body weight	11.5 kg
Rat respiratory volume	290 L/day
Mouse respiratory volume	43 L/day
Rabbit respiratory volume	1440 L/day

Guinea-pig respiratory volume	430 L/day
Human respiratory volume	28,800 L/day
Dog respiratory volume	9000 L/day
Monkey respiratory volume	1150 L/day
Mouse water consumption	5 mL/day
Rat water consumption	30 mL/day
Rat food consumption	30 g/day

The equation for an ideal gas,  $PV = nRT$ , is used to convert concentrations of gases used in inhalation studies from units of ppm to units of mg/L or mg/m<sup>3</sup>. Consider as an example the rat reproductive toxicity study by inhalation of carbon tetrachloride (molecular weight 153.84) summarized in Pharmeuropa, Vol. 9, No. 1, Supplement, April 1997, page S9.

$$\frac{n}{V} = \frac{P}{RT} = \frac{300 \times 10^{-6} \text{ atm} \times 153.84 \text{ g/mol}^{-1}}{0.082 \text{ L atm K}^{-1} \text{ mol}^{-1} \times 298 \text{ K}} = \frac{46.15 \text{ mg}}{24.45 \text{ L}} = 1.89 \text{ mg/L}$$

The relationship 1000 L = 1 m<sup>3</sup> is used to convert to mg/m<sup>3</sup>.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Horacio N. Pappa, Ph.D.</a> Senior Scientist and Latin American Liaison 1-301-816-8319	(GC05) General Chapters 05

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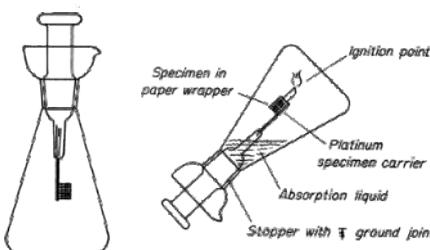
Pharmacopeial Forum: Volume No. 34(5) Page 1232

#### 471 OXYGEN FLASK COMBUSTION

The oxygen flask combustion procedure is provided as the preparatory step in the determination of bromine, chlorine, iodine, selenium, and sulfur in some Pharmacopeial articles. Combustion of the material under test (usually organic) yields water-soluble inorganic products, which are analyzed for specific elements as directed in the individual monograph or general chapter.

The caution statement given for Procedure covers minimum safety precautions only, and serves to emphasize the need for exceptional care throughout.

**Apparatus**— The apparatus<sup>1</sup> consists of a heavy-walled conical, deeply lipped or cupped 500-mL flask (unless a larger flask is specified), fitted with a ground-glass stopper to which is fused a test specimen carrier consisting of heavy-gauge platinum wire and a piece of welded platinum gauze measuring about 1.5 × 2 cm.



Apparatus for Oxygen Flask Combustion

**Procedure**— [Caution—Wear safety glasses and use a suitable safety shield between yourself and the apparatus. Exercise care to ensure that the flask is scrupulously clean and free from even traces of organic solvents.] Weigh the substance, if a solid, on a piece of halide-free filter paper measuring about 4 cm square, and fold the paper to enclose it. Liquid substances are weighed in tared capsules, polycarbonate capsules<sup>1</sup> being used for liquids in volumes not exceeding 200 µL, and gelatin capsules being satisfactory for use for larger volumes. [note—Gelatin capsules may contain significant amounts of combined halide or sulfur. If such capsules are used, perform a blank determination, and make any necessary correction.] Place the specimen, together with a filter paper fuse-strip, in the platinum gauze specimen holder. Place the absorbing liquid specified in the individual monograph or general chapter in the flask, moisten the joint of the stopper with water, and flush the air from the flask with a stream of rapidly flowing oxygen, swirling the liquid to favor its taking up oxygen. [note—Saturation of the liquid with oxygen is essential for the successful performance of the combustion procedure.] Ignite the fuse-strip by suitable means. If the strip is ignited outside the flask, immediately plunge the specimen holder into the flask, invert the flask so that the absorption solution makes a seal around the stopper, and hold the stopper firmly in place. If the ignition is carried out in a closed system, the inversion of the flask may be omitted. After combustion is complete, shake the flask vigorously, and allow to stand for not less than 10 minutes with intermittent shaking. Then proceed as directed in the individual monograph or general chapter.

<sup>1</sup> A suitable apparatus [Catalog Nos. 6513-C20 (500-ml capacity) and 6513-C30 (1000-ml capacity)] and suitable capsules [Catalog No. 6513-84 (1000 capsules)] are obtainable from Thomas Scientific, 99 High Hill Road, Swedesboro, NJ 08085.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Horacio N. Pappa, Ph.D.</a> Senior Scientist and Latin American Liaison 1-301-816-8319	(GC05) General Chapters 05

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#### 481 RIBOFLAVIN ASSAY

The following procedure is suitable for preparations in which riboflavin is a constituent of a mixture of several ingredients. In employing it, keep the pH of solutions below 7.0, and protect the solutions from direct sunlight at all stages.

[USP Reference Standards](#) [11](#)—[USP Riboflavin RS](#).



Standard Riboflavin Stock Solution— To 50.0 mg of [USP Riboflavin RS](#), previously dried and stored protected from light in a desiccator over phosphorus pentoxide, add about 300 mL of 0.02 N acetic acid, and heat the mixture on a steam bath, with frequent agitation, until the riboflavin has dissolved. Then cool, add 0.02 N acetic acid to make 500 mL, and mix. Store under toluene in a refrigerator.

Dilute an accurately measured portion of this solution, using 0.02 N acetic acid, to a concentration of 10.0  $\mu$ g of the dried [USP Riboflavin RS](#) per mL, to obtain the Standard Riboflavin Stock Solution. Store under toluene in a refrigerator.

Standard Preparation— Dilute 10.0 mL of Standard Riboflavin Stock Solution with water in a 100-mL volumetric flask to volume, and mix. Each mL represents 1.0  $\mu$ g of [USP Riboflavin RS](#). Prepare fresh Standard Preparation for each assay.

Assay Preparation— Place an amount of the material to be assayed in a flask of suitable size, and add a volume of 0.1 N hydrochloric acid equal in mL to not less than 10 times the dry weight of the material in g, but the resulting solution shall contain not more than 100  $\mu$ g of riboflavin per mL. If the material is not readily soluble, comminute it so that it may be evenly dispersed in the liquid. Then agitate vigorously, and wash down the sides of the flask with 0.1 N hydrochloric acid.

Heat the mixture in an autoclave at 121° to 123° for 30 minutes, and cool. If clumping occurs, agitate the mixture until the particles are evenly dispersed. Adjust the mixture, with vigorous agitation, to a pH of 6.0 to 6.5 with sodium hydroxide solution,<sup>1</sup> then add hydrochloric acid solution<sup>2</sup> immediately until no further precipitation occurs (usually at a pH of approximately 4.5, the isoelectric point of many of the proteins present). Dilute the mixture with water to make a measured volume that contains about 0.11  $\mu$ g of riboflavin in each mL, and filter through paper known not to adsorb riboflavin. To an aliquot of the filtrate add, with vigorous agitation, sodium hydroxide solution<sup>1</sup> to produce a pH of 6.6 to 6.8, dilute the solution with water to make a final measured volume that contains approximately 0.1  $\mu$ g of riboflavin in each mL, and if cloudiness occurs, filter again.

Procedure— To each of four or more tubes (or reaction vessels) add 10.0 mL of the Assay Preparation. To each of two or more of these tubes add 1.0 mL of the Standard Preparation, and mix, and to each of two or more of the remaining tubes add 1.0 mL of water, and mix. To each tube add 1.0 mL of glacial acetic acid, mix, then add, with mixing, 0.50 mL of potassium permanganate solution (1 in 25), and allow to stand for 2 minutes. To each tube add, with mixing, 0.50 mL of hydrogen peroxide solution, whereupon the permanganate color is destroyed within 10 seconds. Shake the tubes vigorously until excess oxygen is expelled. Remove any gas bubbles remaining on the sides of the tubes after foaming has ceased, by tipping the tubes so that the solution flows slowly from end to end.

In a suitable fluorophotometer, having an input filter of narrow transmittance range with a maximum at about 440 nm and an output filter of narrow transmittance range with a maximum at about 530 nm, measure the fluorescence of all tubes, designating the average reading from the tubes containing only the Assay Preparation as IU and the average from the tubes containing both the Assay Preparation and the Standard Preparation as IS. Then to each of one or more tubes of each kind add, with mixing, 20 mg of sodium hydrosulfite, and within 5 seconds again measure the fluorescence, designating the average reading as IB.

Calculation— Calculate the quantity, in mg, of C17H20N4O6 in each mL of the Assay Preparation taken by the formula:

$$0.0001(IU - IB) / (IS - IU)$$

Calculate the quantity, in mg, of C17H20N4O6 in each capsule or tablet.

\* The concentrations of the hydrochloric acid and sodium hydroxide solutions used are not stated in each instance because these concentrations may be varied depending upon the amount of material taken for assay, volume of test solution, and buffering effect of material.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Curtis Phinney</a>  1-301-816-8540	(DSN05) Dietary Supplements - Non-Botanicals
Reference Standards	Lili Wang, Technical Services Scientist 1-301-816-8129 <a href="mailto:RSTech@usp.org">RSTech@usp.org</a>	

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## 501 SALTS OF ORGANIC NITROGENOUS BASES

Standard Preparation— Unless otherwise directed, prepare a solution in dilute sulfuric acid (1 in 70) containing, in each mL, about 500  $\mu$ g of the specified USP Reference Standard, calculated on the anhydrous basis, and accurately weighed.

Assay Preparation— If the dosage form is a tablet, weigh and finely powder not less than 20 tablets, weigh accurately a portion of the powder, equivalent to about 25 mg of the active ingredient, and transfer to a 125-mL separator; or, if the dosage form is a liquid, transfer a volume of it, equivalent to about 25 mg of the active ingredient and accurately measured, to a 125-mL separator. Then to the separator add 20 mL of dilute sulfuric acid (1 in 350), and shake vigorously for 5 minutes. Add 20 mL of ether, shake carefully, and filter the acid phase into a second 125-mL separator. Shake the ether phase with two 10-mL portions of dilute sulfuric acid (1 in 350), filter each portion of acid into the second separator, and discard the ether. To the acid extract add 10 mL of [sodium hydroxide TS](#) and 50 mL of ether, shake carefully, and transfer the aqueous phase to a third 125-mL separator containing 50 mL of ether. Shake the third separator carefully, and discard the aqueous phase. Wash the two ether solutions, in succession, with a single 20-mL portion of water, and discard the water. Extract each of the two ether solutions with 20-, 20-, and 5-mL portions of dilute sulfuric acid (1 in 70), in the order listed, but each time extract first the ether solution in the third separator and then that in the second separator. Combine the acid extracts in a 50-mL volumetric flask, dilute with the acid to volume, and mix.

note—Hexane or heptane may be substituted for ether if the distribution ratio of the nitrogenous base between water and hexane, or between water and heptane, favors complete extraction by the organic phase.

Procedure— Unless otherwise directed, dilute 5.0 mL each of the Standard Preparation and the Assay Preparation with dilute sulfuric acid (1 in 70) to 100.0 mL, and determine the absorbance of each solution at the specified wavelength, using dilute sulfuric acid (1 in 70) as the blank. Designate the absorbance of the solution from the Standard Preparation as AS and that from the Assay Preparation as AU, and calculate the result of the assay as directed in the individual monograph.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Antonio Hernandez-Cardoso, B.S.</a>  Scientist, Latin American Specialist 1-301-816-8308	(GC05) General Chapters 05

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## 503 ACETIC ACID IN PEPTIDES



The following procedure is to be used to determine the amount of acetate or acetic acid in peptides. Acetate is a common counterion in many peptide preparations.

USP Reference Standards (11) — [USP Glacial Acetic Acid RS](#).

Strong Sodium Hydroxide Solution— Dissolve 42 g of sodium hydroxide in water, and dilute with water to 100 mL.

Solution A— Add 0.7 mL of phosphoric acid to 1000 mL of water, and adjust with Strong Sodium Hydroxide Solution to a pH of 3.0.

Solution B— Use methanol.

Diluent— Prepare a mixture of Solution A and Solution B (95:5).

Standard Solution— [note—The concentration can be adjusted depending on the amount of acetate or acetic acid expected to be present in the test material.] Dissolve an accurately weighed quantity of USP Acetic Acid RS in Diluent to obtain a solution having a known concentration of about 0.1 mg per mL.

Test Solution— Prepare as directed in the individual monograph. The amount of material used can be adapted depending on the amount of acetic acid expected.

Chromatographic System (see [Chromatography \(621\)](#))— The liquid chromatograph is equipped with a 210-nm detector and a 4.6-mm × 25-cm column that contains not greater than 5-μm packing L1. The flow rate is about 1.2 mL per minute. The chromatograph is programmed as follows.

Time (minutes)	Solution A (%)	Solution B (%)	Elution
0	95	5	equilibration
0-5	95	5	isocratic
5-10	95→50	5→50	linear gradient
10-20	50	50	isocratic
20-22	50→95	50→5	linear gradient

Chromatograph the Standard Solution, and record the peak responses as directed for Procedure: the retention time of acetic acid is between 3 and 4 minutes; and the relative standard deviation for replicate injections is not more than 5%.

Procedure— Separately inject equal volumes (about 10 μL) of the Standard Solution and the Test Solution into the chromatograph, record the chromatograms, and measure the responses for the acetic acid peaks. Calculate the percentage of acetic acid in the portion of test material taken by the formula:

$$100(CS / M)(rU / rS)$$

in which CS is the concentration of acetic acid in the Standard Solution; M is the concentration, in mg per mL, of the Test Solution, based on the weight of test material taken and the extent of dilution; and rU and rS are the acetic acid peak responses obtained from the Test Solution and the Standard Solution, respectively.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Larry N. Callahan, Ph.D.</a> Senior Scientist 1-301-816-8385	(BBPP05) Biologics and Biotechnology - Proteins and Polysaccharides
Reference Standards	Lili Wang, Technical Services Scientist 1-301-816-8129 <a href="mailto:RSTech@usp.org">RSTech@usp.org</a>	

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### 511 SINGLE-STEROID ASSAY

In the following procedure, the steroid to be assayed is separated from related foreign steroids and excipients by thin-layer chromatography and determined following recovery from the chromatogram.

Preparation of the Plate— Prepare a slurry from 30 g of chromatographic silica gel with a suitable fluorescing substance by the gradual addition, with mixing, of about 65 mL of a mixture of water and alcohol (5:2). Transfer the slurry to a clean, 20- × 20-cm plate, spread to make a uniform layer 250 μm thick, and allow to dry at room temperature for 15 minutes.

Heat the plate at 105° for 1 hour, and store in a desiccator.

Solvent A— Mix methylene chloride with methanol (180:16).

Solvent B— Mix chloroform with acetone (4:1).

Standard Preparation— Dissolve in a mixture of equal volumes of chloroform and alcohol a suitable quantity of the USP Reference Standard specified in the individual monograph, previously dried as directed (see [USP Reference Standards \(11\)](#)) and accurately weighed, to obtain a solution having a known concentration of about 2 mg per mL.

Assay Preparation— Prepare as directed in the individual monograph.

Procedure— Divide the area of the chromatographic plate into three equal sections, the left and right sections to be used for the Assay Preparation and the Standard Preparation, respectively, and the center section for the blank. Apply 200 μL each of the Assay Preparation and the Standard Preparation as streaks 2.5 cm from the bottom of the appropriate section of the plate. Dry the solution as it is being applied, with the aid of a stream of air. Using the Solvent specified in the individual monograph, develop the chromatogram in a suitable chamber, previously equilibrated and lined with absorbent paper, until the solvent front has moved 15 cm above the initial streaks.



Remove the plate, evaporate the solvent, and locate the principal band occupied by the Standard Preparation by viewing under UV light. Mark this band, as well as corresponding bands in the Assay Preparation and blank sections of the plate. Remove the silica gel from each band separately, either by scraping onto glazed weighing papers or by using a suitable vacuum collecting device, and transfer it to a glass-stoppered, 50-mL centrifuge tube. To each tube add 25.0 mL of alcohol, and shake for not less than 2 minutes. Centrifuge the tubes for 5 minutes, pipet 20 mL of the supernatant from each tube into a glass-stoppered, 50-mL conical flask, add 2.0 mL of a solution prepared by dissolving 50 mg of blue tetrazolium in

10 mL of methanol, and mix. Proceed as directed for Procedure under [Assay for Steroids](#) (351), beginning with "Then to each flask."

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Daniel K. Bempong, Ph.D.</a> Senior Scientist 1-301-816-8143	(MDPS05) Monograph Development-Pulmonary and Steroids

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## 521 SULFONAMIDES

### Identification of Individual Sulfonamides in Mixed Sulfonamides

note—The following instructions for preparations and procedure are applicable to all sulfonamides except sulfadiazine. When testing for sulfadiazine proceed in the same manner, except to use sulfadiazine preparations having one-half the designated concentration, and apply twice the designated volumes of sulfadiazine preparations to the chromatographic plates.

Standard Preparation— Transfer a quantity of the pertinent USP Reference Standard to a suitable glass-stoppered, conical flask, dissolve in methanol to obtain a solution having a concentration of about 2 mg per mL, and mix. A separate Standard Preparation is required for each sulfonamide present in mixed sulfonamides.

Test Preparation— Transfer a portion of the thoroughly mixed suspension or finely powdered tablets, equivalent to about 100 mg of each sulfonamide, to a 50-mL volumetric flask containing 10 mL of [ammonia TS](#), and swirl. Add methanol to volume, mix, filter, and use the filtrate in the Procedure.

Preparation of Chromatographic Plates— Prepare three identical chromatographic plates according to the following directions. Apply separately, and 2 cm apart along a spotting line 1.5 cm from the bottom of the plate and parallel to it, 2  $\mu$ L of each Standard Preparation and 2  $\mu$ L of the Test Preparation to a suitable thin-layer chromatographic plate (see [Chromatography](#) (621)) coated with a 0.25-mm layer of chromatographic silica gel mixture. On another spot, 2 cm along the spotting line from the application of the Test Preparation, apply, successively, 2  $\mu$ L of each Standard Preparation to obtain a mixed standard. Dry the spots immediately with the aid of a stream of nitrogen.

Procedure— Prepare a chromatographic chamber lined with filter paper and containing a solvent system consisting of ethyl acetate, methanol, and a 1 in 4 aqueous solution of ammonium hydroxide (17:6:5), and allow to equilibrate for 1 hour. Similarly prepare a second chamber to contain a solvent system consisting of solvent hexane, chloroform, and butyl alcohol (1:1:1), and a third chamber to contain a solvent system consisting of chloroform and methanol (95:5). Place one prepared chromatographic plate in each equilibrated chamber, and develop the chromatograms until the solvent front has moved about three-fourths of the length of each plate. Remove each plate from its developing chamber, mark the solvent front, and allow the solvent to evaporate. Locate the spots on the plates by viewing under short-wavelength UV light. Spray the plates with a 1 in 100 solution of p-dimethylaminobenzaldehyde in dilute hydrochloric acid (1 in 20), and heat at 110° for 5 minutes or until bright yellow spots become visible. The RF values of the yellow spots obtained from each Test Preparation correspond to those obtained from the mixed Standard Preparations on the respective plates. The individual sulfonamides may be identified by comparison of the RF values of the yellow spots obtained from the Test Preparations and individual Standard Preparations on the respective plates.

### Determination of Individual Sulfonamides in Mixed Sulfonamides

Standard Preparation— A separate Standard Preparation is required for each sulfonamide being determined. Transfer about 50 mg, accurately weighed, of the pertinent USP Reference Standard to a 50-mL volumetric flask containing 1.5 mL of ammonium hydroxide, add methanol, dissolve in methanol, dilute with methanol to volume, and mix. Transfer 1.0 mL of this solution to a 100-mL volumetric flask, add dilute hydrochloric acid (1 in 100) to volume, and mix. [note—Retain the methanol solutions for the Mixed Standard Preparation. The methanol solutions are stable for at least 1 week, and the acid solutions for at least 1 month.]

Mixed Standard Preparation— Transfer 1.0 mL of each methanol solution, prepared as required for each Standard Preparation, to a small glass-stoppered flask, and mix. [note—This Standard is used to identify the components of the Assay Preparation on the chromatogram.]

Assay Preparation— Prepare as directed in the individual monograph.

Procedure— Prepare the necessary number of chromatographic sheets (Whatman No. 1 filter paper, or equivalent), about 20  $\times$  20 cm in size, by drawing a pencil line parallel to and 2.5 cm from one edge of the paper. Mark the line at points 2.5 and 5 cm from each edge of the paper. Impregnate the paper by dipping it in the immobile solvent (prepared fresh by dissolving 30 mL of redistilled formamide in 70 mL of acetone) for 30 seconds. Remove the paper, drain for 10 seconds, and blot between filter paper. Place the impregnated paper on dry filter paper, and air-dry for 3 to 5 minutes. With a micropipet, and with repeated applications, streak 100  $\mu$ L of the Assay Preparation along the starting line, applying the volume in five streaks of about 20  $\mu$ L each and evaporating the solvent with a gentle stream of nitrogen between applications. [note—Make the streak as narrow as possible along the starting line, and keep within the 5-cm border.] Rinse the tip of the pipet with a drop of methanol-ammonia TS mixture (9:1), and then streak the rinse along the starting line between the 5- and 2.5-cm points at the right edge. Repeat the rinsing with two additional drops, and then blow out the pipet.

Apply 10  $\mu$ L of the Mixed Standard Preparation at the mark 2.5 cm from the left edge.

Place 50 mL of methylene chloride (mobile solvent) in a tray in a 23-  $\times$  23-  $\times$  7.5-cm chromatographic chamber arranged for ascending chromatography (see [Chromatography](#) (621)), and allow the chamber to equilibrate for about 15 minutes. Remove the cover, place from 7 to 10 mL of water in a second tray, and without delay, suspend the prepared chromatographic paper sheet so that it dips into the mobile solvent. Cover and seal the chamber, and allow the chromatogram to develop for 1 hour. Remove the paper from the chamber, and allow to air-dry for 5 minutes. Place the chromatogram on a dry sheet of filter paper, and view it under short-wavelength UV light. [note—Conduct the following identification and marking without delay to avoid excessive exposure of the sulfonamide spots to UV irradiation.] Identify and mark the respective spots by matching RF values with those of the spots produced by the Mixed Standard Preparation. [note—Sulfadiazine and sulfamerazine are chromatographed with increasing RF, respectively.]

Cut the marked zones from the paper, cut each zone into five or six pieces, and place the pieces from each spot in separate, glass-stoppered, 50-mL flasks. Add 20.0 mL of dilute hydrochloric acid (1 in 100) to each flask, and allow to stand for about 30 minutes, swirling each flask at least five times during this period. Filter the solutions through dry glass wool into separate test tubes, discarding the first 5 mL of the filtrate. Transfer 5.0 mL of the subsequent filtrate from each solution into separate 10-mL volumetric flasks. Transfer 3.0 mL of each required Standard Preparation into separate, 10-mL volumetric flasks. To each flask, and to a blank flask containing 5 mL of dilute hydrochloric acid (1 in 100), add 1.0 mL of sodium nitrite solution (1 in 1000) and 0.10 mL of hydrochloric acid, and allow to stand for 5 minutes with frequent swirling. To each flask add 1.0 mL of ammonium sulfamate solution (1 in 200), and allow to stand for 5 minutes, swirling frequently. Finally, to each flask add 1.0 mL of freshly prepared N-(1-naphthyl)ethylenediamine dihydrochloride solution (1 in 1000), mix, dilute with water to volume, and mix. Allow each solution to stand between 15 and 60 minutes, and then concomitantly determine the absorbances of the solutions, in 1-cm cells, recording the spectra from 440 to 700 nm, with a suitable spectrophotometer, using the blank to set the instrument. Draw a baseline, and determine the corrected absorbance for each solution at the wavelength of maximum absorbance at about 545 nm.

Calculate the concentration, in mg per mL, of each sulfonamide in the Assay Preparation by the formula:

$$0.12C(AU / AS)$$

in which C is the concentration, in  $\mu$ g per mL, of the pertinent USP Reference Standard in the Standard Preparation; AU is the corrected absorbance of the Assay Preparation; and AS is the corrected absorbance of the pertinent Standard Preparation. From the concentration of the Assay Preparation thus determined, and applying appropriate dilution factors,



calculate the percentage of sulfonamide in the specimen taken.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Behnam Davani, Ph.D., M.B.A.</a> Senior Scientist 1-301-816-8394	(MDAA05) Monograph Development-Antivirals and Antimicrobials

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### 525 SULFUR DIOXIDE

The following methods are provided for the determination of sulfur dioxide in pharmaceutical excipients.

#### METHOD I

##### Procedure

Mix 20 g of the test specimen, accurately weighed, with 200 mL of an appropriate solvent as indicated in each individual monograph, and stir until a smooth suspension is obtained. Allow the test specimen mixture to remain undisturbed until most of the test specimen has settled, and filter the aqueous portion through paper (Whatman No. 1 or equivalent). To 100 mL of the clear filtrate add an additional solvent as indicated in each individual monograph, add 3 mL of starch TS, and titrate with 0.01 N iodine solution VS to the first permanent blue or purple color. Each 1.0 mL of 0.01 N iodine solution VS consumed corresponds to 0.003% of sulfur dioxide found.

#### METHOD II

##### Procedure

Transfer about 50 to 100 g of the substance to be tested, accurately weighed, to a 250-mL conical flask, add 100 to 150 mL of water, and mix. Cool to between 5° and 10°. While stirring with a magnetic stirrer, add 10 mL of cold 1.5 N sodium hydroxide (at a temperature between 5° and 10°). Stir for an additional 20 seconds, and add 10 mL of starch indicator solution, prepared as follows: mix 10 g of soluble starch with 50 mL of cold water, transfer to 1000 mL of boiling water, stir until completely dissolved, cool, and add 1 g of salicylic acid preservative. [note—Discard the solution after 1 month.] Add 10 mL of 2.0 N sulfuric acid (at a temperature between 5° and 10°), and titrate immediately with 0.005 N iodine VS until a light blue color persists for 1 minute (see [Titrimetry](#) 541). Perform a blank determination, using 200 mL of water treated similarly to the solution under test, and make any necessary correction. Each mL of 0.005 N iodine is equivalent to 0.16 mg of SO<sub>2</sub>.

#### METHOD III

##### Procedure

Dissolve 20.0 g of the test specimen in 150 mL of hot water in a flask having a round bottom and a long neck, add 5 mL of phosphoric acid and 1 g of sodium bicarbonate, and at once connect the flask to a condenser. [note—Excessive foaming can be alleviated by the addition of a few drops of a suitable antifoaming agent.] Distill 50 mL, receiving the distillate under the surface of 50 mL of 0.1 N iodine. Acidify the distillate with a few drops of hydrochloric acid, add 2 mL of [barium chloride TS](#), and heat on a steam bath until the liquid is nearly colorless. The precipitate of barium sulfate, if any, when filtered, washed, and ignited, weighs not more than 3 mg, corresponding to not more than 0.004% of sulfur dioxide, correction being made for any sulfate that may be present in 50 mL of the 0.1 N iodine.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Hong Wang, Ph.D.</a> Scientist 1-301-816-8351	(EGC05) Excipient General Chapters

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### 531 THIAMINE ASSAY

[USP Reference Standards](#) 11 — [USP Thiamine Hydrochloride RS](#).

The following procedure is provided for the determination of thiamine as an ingredient of Pharmacopeial preparations containing other active constituents.

#### Special Solutions and Solvents—

POTASSIUM FERRICYANIDE SOLUTION— Dissolve 1.0 g of potassium ferricyanide in water to make 100 mL. Prepare fresh on the day of use.

OXIDIZING REAGENT— Mix 4.0 mL of Potassium Ferricyanide Solution with sufficient 3.5 N sodium hydroxide to make 100 mL. Use this solution within 4 hours.

QUININE SULFATE STOCK SOLUTION— Dissolve 10 mg of quinine sulfate in 0.1 N sulfuric acid to make 1000 mL. Preserve this solution, protected from light, in a refrigerator.

QUININE SULFATE STANDARD SOLUTION— Dilute 0.1 N sulfuric acid with Quinine Sulfate Stock Solution (39:1). This solution fluoresces to approximately the same degree as the thiochrome obtained from 1 µg of thiamine hydrochloride and is used to correct the fluorometer at frequent intervals for variation in sensitivity from reading to reading within an assay. Prepare this solution fresh on the day of use.

Standard Thiamine Hydrochloride Stock Solution— Transfer about 25 mg of [USP Thiamine Hydrochloride RS](#), accurately weighed, to a 1000-mL volumetric flask. Dissolve the weighed Standard in about 300 mL of dilute alcohol solution (1 in 5) adjusted with 3 N hydrochloric acid to a pH of 4.0, and add the acidified, dilute alcohol to volume. Store in a light-resistant container, in a refrigerator. Prepare this stock solution fresh each month.

Standard Preparation— Dilute a portion of Standard Thiamine Hydrochloride Stock Solution quantitatively and stepwise with 0.2 N hydrochloric acid to obtain the Standard Preparation, each mL of which represents 0.2 µg of [USP Thiamine Hydrochloride RS](#).

Assay Preparation— Place in a suitable volumetric flask sufficient amount of the material to be assayed, accurately weighed or measured by volume as directed, such that when diluted to volume with 0.2 N hydrochloric acid, the resulting solution will contain about 100 µg of thiamine hydrochloride (or mononitrate) per mL. If the sample is difficultly soluble, the solution may be heated on a steam bath, and then cooled and diluted with the acid to volume. Dilute 5 mL of this solution, quantitatively and stepwise, using 0.2 N hydrochloric acid, to an estimated concentration of 0.2 µg of thiamine hydrochloride (or mononitrate) per mL.



Procedure— Into each of three or more tubes (or other suitable vessels), of about 40-mL capacity, pipet 5 mL of Standard Preparation. To each of two of these tubes add rapidly (within 1 to 2 seconds), with mixing, 3.0 mL of Oxidizing Reagent, and within 30 seconds add 20.0 mL of isobutyl alcohol, then mix vigorously for 90 seconds by shaking the capped tubes manually, or by bubbling a stream of air through the mixture. Prepare a blank in the remaining tube of the standard by substituting for the Oxidizing Reagent an equal volume of 3.5 N sodium hydroxide and proceeding in the same manner.

Into each of three or more similar tubes pipet 5 mL of the Assay Preparation. Treat these tubes in the same manner as directed for the tubes containing the Standard Preparation.

Into each of the six tubes pipet 2 mL of dehydrated alcohol, swirl for a few seconds, allow the phases to separate, and decant or draw off about 10 mL of the clear, supernatant isobutyl alcohol solution into standardized cells, then measure the fluorescence in a suitable fluorometer, having an input filter of narrow transmittance range with a maximum at about 365 nm and an output filter of narrow transmittance range with a maximum at about 435 nm.

Calculation— The number of  $\mu$ g of C<sub>12</sub>H<sub>17</sub>CIN<sub>4</sub>OS-HCl in each 5 mL of the Assay Preparation is given by the formula:

$$(A - b) / (S - d)$$

in which A and S are the average fluorometer readings of the portions of the Assay Preparation and the Standard Preparation treated with Oxidizing Reagent, respectively, and b and d are the readings for the blanks of the Assay Preparation and the Standard Preparation, respectively.

Calculate the quantity, in mg, of thiamine hydrochloride (C<sub>12</sub>H<sub>17</sub>CIN<sub>4</sub>OS-HCl) in the assay material on the basis of the aliquots taken. Where indicated, the quantity, in mg, of thiamine mononitrate (C<sub>12</sub>H<sub>17</sub>N<sub>5</sub>O<sub>4</sub>S) may be calculated by multiplying the quantity of C<sub>12</sub>H<sub>17</sub>CIN<sub>4</sub>OS-HCl found by 0.9706.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Curtis Phinney</a>  1-301-816-8540	(DSN05) Dietary Supplements - Non-Botanicals
Reference Standards	Lili Wang, Technical Services Scientist 1-301-816-8129 <a href="mailto:RSTech@usp.org">RSTech@usp.org</a>	

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541 TITRIMETRY

Direct Titrations— Direct titration is the treatment of a soluble substance, contained in solution in a suitable vessel (the titrate), with an appropriate standardized solution (the titrant), the endpoint being determined instrumentally or visually with the aid of a suitable indicator.

The titrant is added from a suitable buret and is so chosen, with respect to its strength (normality), that the volume added is between 30% and 100% of the rated capacity of the buret. [note—Where less than 10 mL of titrant is required, a suitable microburet is to be used.] The endpoint is approached directly but cautiously, and finally the titrant is added dropwise from the buret in order that the final drop added will not overrun the endpoint. The quantity of the substance being titrated may be calculated from the volume and the normality or molarity factor of the titrant and the equivalence factor for the substance given in the individual monograph.

Residual Titrations— Some Pharmacopeial assays require the addition of a measured volume of a volumetric solution, in excess of the amount actually needed to react with the substance being assayed, the excess of this solution then being titrated with a second volumetric solution. This constitutes a residual titration and is known also as a "back titration."

The quantity of the substance being titrated may be calculated from the difference between the volume of the volumetric solution originally added, corrected by means of a blank titration, and that consumed by the titrant in the back titration, due allowance being made for the respective normality or molarity factors of the two solutions, and the equivalence factor for the substance given in the individual monograph.

Complexometric Titrations— Successful complexometric titrations depend on several factors. The equilibrium constant for formation of the titrant-analyte complex must be sufficiently large that, at the endpoint, very close to 100% of the analyte has been complexed. The final complex must be formed rapidly enough that the analysis time is practical. When the analytical reaction is not rapid, a residual titration may sometimes be successful.

In general, complexometric indicators are themselves complexing agents. The reaction between metal ion and indicator must be rapid and reversible. The equilibrium constant for formation of the metal-indicator complex should be large enough to produce a sharp color change but must be less than that for the metal-titrant complex. Indicator choice is also restricted by the pH range within which the complexation reaction must be carried out and by interference of other ions arising from the sample or the buffer. Interfering ions may often be masked or "screened" via addition of another complexing agent. (The masking technique is also applicable to redox titrations.)

Oxidation-Reduction (Redox) Titrations— Determinations may often be carried out conveniently by the use of a reagent that brings about oxidation or reduction of the analyte. Many redox titration curves are not symmetric about the equivalence point, and thus graphical determination of the endpoint is not possible; but indicators are available for many determinations, and a redox reagent can often serve as its own indicator. As in any type of titration, the ideal indicator changes color at an endpoint that is as close as possible to the equivalence point. Accordingly, when the titrant serves as its own indicator, the difference between the endpoint and the equivalence point is determined only by the analyst's ability to detect the color change. A common example is the use of permanganate ion as an oxidizing titrant since a slight excess can easily be detected by its pink color. Other titrants that may serve as their own indicators are iodine, cerium (IV) salts, and potassium dichromate. In most cases, however, the use of an appropriate redox indicator will yield a much sharper endpoint.

It may be necessary to adjust the oxidation state of the analyte prior to titration through use of an appropriate oxidizing or reducing agent; the excess reagent must then be removed, e.g., through precipitation. This is nearly always the practice in the determination of oxidizing agents since most volumetric solutions of reducing agents are slowly oxidized by atmospheric oxygen.

Titrations in Nonaqueous Solvents— Acids and bases have long been defined as substances that furnish, when dissolved in water, hydrogen and hydroxyl ions, respectively. This definition, introduced by Arrhenius, fails to recognize the fact that properties characteristic of acids or bases may be developed also in other solvents. A more generalized definition is that of Brönsted, who defined an acid as a substance that furnishes protons, and a base as a substance that combines with protons. Even broader is the definition of Lewis, who defined an acid as any material that will accept an electron pair, a base as any material that will donate an electron pair, and neutralization as the formation of a coordination bond between an acid and a base.

The apparent strength of an acid or a base is determined by the extent of its reaction with a solvent. In water solution all strong acids appear equally strong because they react with the solvent to undergo almost complete conversion to oxonium ion and the acid anion (leveling effect). In a weakly protophilic solvent such as acetic acid the extent of formation of the acetate acidum ion shows that the order of decreasing strength for acids is perchloric, hydrobromic, sulfuric, hydrochloric, and nitric (differentiating effect).

Acetic acid reacts incompletely with water to form oxonium ion and is, therefore, a weak acid. In contrast, it dissolves in a base such as ethylenediamine, and reacts so completely with the solvent that it behaves as a strong acid. The same holds for perchloric acid.

This leveling effect is observed also for bases. In sulfuric acid almost all bases appear to be of the same strength. As the acid properties of the solvent decrease in the series sulfuric acid, acetic acid, phenol, water, pyridine, and butylamine, the bases become progressively weaker until all but the strongest have lost their basic properties. In order of decreasing



strength, the strong bases are sodium 2-aminoethoxide, potassium methoxide, sodium methoxide, and lithium methoxide.

Many water-insoluble compounds acquire enhanced acidic or basic properties when dissolved in organic solvents. Thus the choice of the appropriate solvent permits the determination of a variety of such materials by nonaqueous titration. Furthermore, depending upon which part of a compound is the physiologically active moiety, it is often possible to titrate that part by proper selection of solvent and titrant. Pure compounds can be titrated directly, but it is often necessary to isolate the active ingredient in pharmaceutical preparations from interfering excipients and carriers.

The types of compounds that may be titrated as acids include acid halides, acid anhydrides, carboxylic acids, amino acids, enols such as barbiturates and xanthines, imides, phenols, pyrroles, and sulfonamides. The types of compounds that may be titrated as bases include amines, nitrogen-containing heterocyclic compounds, oxazolines, quaternary ammonium compounds, alkali salts of organic acids, alkali salts of weak inorganic acids, and some salts of amines. Many salts of halogen acids may be titrated in acetic acid or acetic anhydride after the addition of mercuric acetate, which removes halide ion as the unionized mercuric halide complex and introduces the acetate ion.

For the titration of a basic compound, a volumetric solution of perchloric acid in glacial acetic acid is preferred, although perchloric acid in dioxane is used in special cases. The calomel-glass electrode system is useful in this case. In acetic acid solvent, this electrode system functions as predicted by theory.

For the titration of an acidic compound, two classes of titrant are available: the alkali metal alkoxides and the tetraalkylammonium hydroxides. A volumetric solution of sodium methoxide in a mixture of methanol and toluene is used frequently, although lithium methoxide in methanol-benzene solvent is used for those compounds yielding a gelatinous precipitate on titration with sodium methoxide.

The alkali error limits the use of the glass electrode as an indicating electrode in conjunction with alkali metal alkoxide titrants, particularly in basic solvents. Thus, the antimony-indicating electrode, though somewhat erratic, is used in such titrations. The use of quaternary ammonium hydroxide compounds, e.g., tetra-n-butylammonium hydroxide and trimethylhexadecylammonium hydroxide (in benzene-methanol or isopropyl alcohol), has two advantages over the other titrants in that (a) the tetraalkylammonium salt of the titrated acid is soluble in the titration medium, and (b) the convenient and well-behaved calomel-glass electrode pair may be used to conduct potentiometric titrations.

Because of interference by carbon dioxide, solvents for acidic compounds need to be protected from excessive exposure to the atmosphere by a suitable cover or by an inert atmosphere during the titration. Absorption of carbon dioxide may be determined by performing a blank titration. The blank should not exceed 0.01 mL of 0.1 N sodium methoxide VS per mL of solvent.

The endpoint may be determined visually by color change, or potentiometrically, as indicated in the individual monograph. If the calomel reference electrode is used, it is advantageous to replace the aqueous potassium chloride salt bridge with 0.1 N lithium perchlorate in glacial acetic acid for titrations in acidic solvents or potassium chloride in methanol for titrations in basic solvents.

Where these or other mixtures are specified in individual monographs, the calomel reference electrode is modified by first removing the aqueous potassium chloride solution and residual potassium chloride, if any, by rinsing with water, then eliminating residual water by rinsing with the required nonaqueous solvent, and finally filling the electrode with the designated nonaqueous mixture.

In nearly all cases, except those where silver ion might interfere, a silver-silver chloride reference electrode may be substituted for the calomel electrode. The silver-silver chloride electrode is more rugged, and its use helps to eliminate toxic mercury salts from the laboratory. Generally, a salt bridge may be used to circumvent interference by silver ion.

The more useful systems for titration in nonaqueous solvents are listed in [Table 1](#).

Table 1. Systems for Nonaqueous Titrations

Type of Solvent	Acidic (for titration of bases and their salts)	Relatively Neutral (for differential titration of bases)	Basic (for titration of acids)	Relatively Neutral (for differential titration of acids)
Solvent <sup>1</sup>	Glacial Acetic Acid	Acetonitrile	Dimethylformamide	Acetone
	Acetic Anhydride	Alcohols	n-Butylamine	Acetonitrile
	Formic Acid	Chloroform	Pyridine	Methyl Ethyl Ketone
	Propionic Acid	Benzene	Ethylenediamine	Methyl Isobutyl Ketone
	Sulfuryl Chloride	Toluene	Morpholine	tert-Butyl Alcohol
		Chlorobenzene		
		Ethyl Acetate		
		Dioxane		
Indicator	Crystal Violet	Methyl Red	Thymol Blue	Azo Violet
	Quinaldine Red	Methyl Orange	Thymolphthalein	Bromothymol Blue
	p-Naphtholbenzein	p-Naphtholbenzein	Azo Violet	p-Hydroxyazobenzene
	Alphezurine 2-G		o-Nitroaniline	Thymol Blue
	Malachite Green		p-Hydroxyazobenzene	
Electrodes	Glass-calomel	Glass-calomel	Antimony-calomel	Antimony-calomel
	Glass-silver-silver chloride	Calomel-silver-silver chloride	Antimony-glass	Glass-calomel
	Mercury-mercuric acetate		Antimony-antimony <sup>2</sup>	Glass-platinum <sup>2</sup>
			Platinum-calomel	
			Glass-calomel	

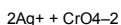
1 Relatively neutral solvents of low dielectric constant such as benzene, toluene, chloroform, or dioxane may be used in conjunction with any acidic or basic solvent in order to increase the sensitivity of the titration end-points.

2 In titrant.

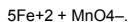
**Indicator and Potentiometric Endpoint Detection**— The simplest and most convenient method by which the equivalence point, i.e., the point at which the stoichiometric analytical reaction is complete, may be determined is with the use of indicators. These chemical substances, usually colored, respond to changes in solution conditions before and after the equivalence point by exhibiting color changes that may be taken visually as the endpoint, a reliable estimate of the equivalence point.

A useful method of endpoint determination results from the use of electrochemical measurements. If an indicator electrode, sensitive to the concentration of the species undergoing titrimetric reaction, and a reference electrode, whose potential is insensitive to any dissolved species, are immersed in the titrate to form a galvanic cell, the potential difference between the electrodes may be sensed by a pH meter and used to follow the course of the reaction. Where such a series of measurements is plotted correctly (i.e., for an acid-base titration, pH versus mL of titrant added; for a precipimetric, complexometric, or oxidation-reduction titration, mV versus mL of titrant added), a sigmoid curve results with a rapidly changing portion (the "break") in the vicinity of the equivalence point. The midpoint of this linear vertical portion or the inflection point may be taken as the endpoint. The equivalence point may also be determined mathematically without plotting a curve. However, it should be noted that in asymmetric reactions, which are reactions in which the number of anions reacting is not the same as the number of cations reacting, the endpoint as defined by the inflection of the titration curve does not occur exactly at the stoichiometric equivalence point.

Thus, potentiometric endpoint detection by this method is not suitable in the case of asymmetric reactions, examples of which are the precipitation reaction,



and the oxidation-reduction reaction,



All acid-base reactions, however, are symmetrical. Thus, potentiometric endpoint detection may be employed in acid-base titrations and in other titrations involving symmetrical reversible reactions where an indicator is specified, unless otherwise directed in the individual monograph.

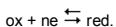
Two types of automatic electrometric titrators are available. The first is one that carries out titrant addition automatically and records the electrode potential differences during the course of titration as the expected sigmoid curve. In the second type, titrant addition is performed automatically until a preset potential or pH, representing the endpoint, is reached, at which point the titrant addition ceases.

Several acceptable electrode systems for potentiometric titrations are summarized in [Table 2](#).

Table 2. Potentiometric Titration Electrode Systems

Titration	Indicating Electrode	Equation <sup>1</sup>	Reference Electrode	Applicability <sup>2</sup>
Acid-base	Glass	$E = k + 0.0591 \text{ pH}$	Calomel or silver-silver chloride	Titration of acids and bases
Precipitometric (silver)	Silver	$E = E^\ominus + 0.0591 \log [\text{Ag}^+]$	Calomel (with potassium nitrate salt bridge)	Titration with or of silver involving halides or thiocyanate
Complexometric	Mercury-mercury(II)	$E = E^\ominus + 0.0296(\log k' - \text{pM})$	Calomel	Titration of various metals (M), e.g., $\text{Mg}^{2+}$ , $\text{Ca}^{2+}$ , $\text{Al}^{3+}$ , $\text{Bi}^{3+}$ , with EDTA
Oxidation-reduction	Platinum	$E = E^\ominus + (0.0591/n) \times \log \frac{[\text{ox}]}{[\text{red}]}$	Calomel or silver-silver chloride	Titrations with arsenite, bromine, cerate, dichromate, exacyanoferrate(III), iodate, nitrite, permanganate, thiosulfate

1 Appropriate form of Nernst equation describing the indicating electrode system: k = glass electrode constant;  $k'$  = constant derived from  $\text{Hg}-\text{Hg(II)}-\text{EDTA}$  equilibrium; M = any metal undergoing EDTA titration; [ox] and [red] from the equation,



2 Listing is representative but not exhaustive.

**Blank Corrections**— As previously noted, the endpoint determined in a titrimetric assay is an estimate of the reaction equivalence point. The validity of this estimate depends upon, among other factors, the nature of the titrate constituents and the concentration of the titrant. An appropriate blank correction is employed in titrimetric assays to enhance the reliability of the endpoint determination. Such a blank correction is usually obtained by means of a residual blank titration, wherein the required procedure is repeated in every detail except that the substance being assayed is omitted. In such instances, the actual volume of titrant equivalent to the substance being assayed is the difference between the volume consumed in the residual blank titration and that consumed in the titration with the substance present. The corrected volume so obtained is used in calculating the quantity of the substance being titrated, in the same manner as prescribed under **Residual Titrations**. Where potentiometric endpoint detection is employed, the blank correction is usually negligible.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Horacio N. Pappa, Ph.D.</a> Senior Scientist and Latin American Liaison 1-301-816-8319	(GC05) General Chapters 05

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### 551 ALPHA TOCOPHEROL ASSAY

The following procedure is provided for the determination of tocopherol as an ingredient.

**Hydrogenator**— A suitable device for low-pressure hydrogenation may be assembled as follows. Arrange in a rack or in clamps two conical centrifuge tubes, connected in series by means of glass and inert plastic tubing and suitable stoppers of glass, polymer, or cork (avoiding all use of rubber). Use one tube for the blank and the other for the assay specimen. Arrange a gas-dispersion tube so that the hydrogen issues as bubbles at the bottom of each tube. Pass the hydrogen first through the blank tube and then through the specimen tube.

**Procedure**— Pipet into a suitable vessel 25 mL of the final washed ether solution of the unsaponifiable fraction obtained as directed for **When Tocopherol Is Present** under [Procedure](#) in the [Vitamin A Assay](#) (571), and evaporate to about 5 mL. Without applying heat, remove the remaining ether in a stream of inert gas or by vacuum. Dissolve the residue in sufficient alcohol to give an expected concentration of about 0.15 mg of alpha tocopherol per mL. Pipet 15 mL into a 50-mL centrifuge tube, add about 200 mg of palladium catalyst, stir with a glass rod, and hydrogenate for 10 minutes in the Hydrogenator, using hydrogen that has been passed through alcohol in a blank tube. Add about 300 mg of chromatographic siliceous earth, stir with a glass rod, and immediately centrifuge until the solution is clear.

Test a 1-mL aliquot of the solution by removing the solvent by evaporation, dissolving the residue in 1 mL of chloroform, and adding 10 mL of [antimony trichloride TS](#): no detectable blue color appears. [note—If a blue color appears, repeat the hydrogenation for a longer time period, or with a new lot of catalyst.]

Pipet 2 mL of the supernatant into a glass-stoppered, opaque flask, add 1.0 mL of a 1 in 500 solution of ferric chloride in dehydrated alcohol,\* and begin timing the reaction, preferably with a stop watch. Add immediately 1.0 mL of a 1 in 200 solution of 2,2'-bipyridine in dehydrated alcohol, mix with swirling, add 21.0 mL of dehydrated alcohol, close the tube, and shake vigorously to ensure complete mixing. When about 9½ minutes have elapsed from the beginning of the reaction, transfer part of the mixture to one of a pair of matched 1-cm spectrophotometer cells. After 10 minutes, accurately timed, following the addition of the ferric chloride-dehydrated alcohol solution, determine the absorbance at 520 nm, with a suitable spectrophotometer, using dehydrated alcohol as the blank. Perform a blank determination with the same quantities of the same reagents and in the same manner, but using 2 mL of dehydrated alcohol in place of the 2 mL of the hydrogenated solution. Subtract the absorbance determined for the blank from that determined for the assay specimen, and designate the difference as AD.

Calculate the alpha tocopherol content, in mg, in the assay specimen taken by the formula:

$$30.2 \text{ AD} / (\text{LCD})$$

in which AD is the corrected absorbance; L is the length, in cm, of the absorption cell; and CD is the content of the assay specimen in the alcohol solution employed for the measurement of absorbance, expressed as g, capsules, or tablets per 100 mL.

\* note—The absorbance of the blank may be reduced, and the precision of the determination thereby improved, by purification of the dehydrated alcohol that is used throughout the assay. Purification may be accomplished by the addition of a few crystals (about 0.02%) of potassium permanganate and of a few pellets of potassium hydroxide to the dehydrated alcohol, and subsequent redistillation.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.



Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Curtis Phinney</a> 1-301-816-8540	(DSN05) Dietary Supplements - Non-Botanicals

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## 561 ARTICLES OF BOTANICAL ORIGIN

## SAMPLING

In order to reduce the effect of sampling bias in qualitative and quantitative results, it is necessary to ensure that the composition of the sample used be representative of the batch of drugs being examined. The following sampling procedures are the minimum considered applicable to vegetable drugs. Some articles, or some tests, may require more rigorous procedures involving more containers being sampled or more samples per container.

## Gross Sample

Where external examination of containers, markings, and labels indicates that the batch can be considered to be homogeneous, take individual samples from the number of randomly selected containers indicated below. Where the batch cannot be considered to be homogeneous, divide it into sub-batches that are as homogeneous as possible, then sample each one as a homogeneous batch.

No. of Containers in Batch (N)	No. of Containers to be Sampled (n)
1 to 10	all
11 to 19	11
>19	$n = 10 + (N/10)$

(Round calculated "n" to next highest whole number.)

Samples are taken from the upper, middle, and lower sections of each container. If the crude material consists of component parts which are 1 cm or less in any dimension, and in the case of all powdered or ground materials, withdraw the sample by means of a sampling device that removes a core from the top to the bottom of the container, not less than two cores being taken in opposite directions. For materials with component parts over 1 cm in any dimension, withdraw samples by hand. In the case of large bales or packs, samples should be taken from a depth of 10 cm because the moisture content of the surface layer may be different from that of the inner layers.

Prepare the gross sample by combining and mixing the individual samples taken from each opened container, taking care not to increase the degree of fragmentation or significantly affect the moisture content.

## Laboratory Sample

Prepare the laboratory sample by repeated quartering of the gross sample.

note—Quartering consists of placing the sample, adequately mixed, as an even and square-shaped heap and dividing it diagonally into four equal parts. The two opposite parts are then taken and carefully mixed. The process is repeated as necessary until the required quantity is obtained.

The laboratory sample should be of a size sufficient for performing all the necessary tests.

## Test Sample

Unless otherwise directed in the individual monograph or test procedure below, prepare the test sample as follows:

Decrease the size of the laboratory sample by quartering, taking care that each withdrawn portion remains representative. In the case of unground or unpowdered drugs, grind the withdrawn sample so that it will pass through a No. 20 standard-mesh sieve, and mix the resulting powder well. If the material cannot be ground, reduce it to as fine a state as possible, mix by rolling it on paper or sampling cloth, spread it out in a thin layer and withdraw the portion for analysis.

## METHODS OF ANALYSIS

## Foreign Organic Matter

Test Sample— Unless otherwise specified in the individual monograph, weigh the following quantities of the laboratory sample, taking care that the withdrawn portion is representative (quartering if necessary):

Roots, rhizomes, bark, and herbs	500 g
Leaves, flowers, seeds, and fruit	250 g
Cut vegetable drugs (average weight of the pieces is less than 0.5 g)	50 g

Spread the sample out in a thin layer, and separate the foreign organic matter by hand as completely as possible. Weigh it, and determine the percentage of foreign organic matter in the weight of drug taken.

## Total Ash

Accurately weigh a quantity of the Test Sample, representing 2 to 4 g of the air-dried material, in a tared crucible, and incinerate, gently at first, and gradually increase the temperature to  $675 \pm 25^\circ$ , until free from carbon, and determine the weight of the ash. If a carbon-free ash cannot be obtained in this way, extract the charred mass with hot water, collect the insoluble residue on an ashless filter paper, incinerate the residue and filter paper until the ash is white or nearly so, then add the filtrate, evaporate it to dryness, and heat the whole to a temperature of  $675 \pm 25^\circ$ . If a carbon-free ash cannot be obtained in this way, cool the crucible, add 15 mL of alcohol, break up the ash with a glass rod, burn off the alcohol, and again heat the whole to a temperature of  $675 \pm 25^\circ$ . Cool in a desiccator, weigh the ash, and calculate the percentage of total ash from the weight of the drug taken.

## Acid-Insoluble Ash

Boil the ash obtained as directed under Total Ash, above, with 25 mL of 3 N hydrochloric acid for 5 minutes, collect the insoluble matter on a tared filtering crucible or ashless filter, wash with hot water, ignite, and weigh. Determine the percentage of acid-insoluble ash calculated from the weight of drug taken.

## Water-Soluble Ash

Boil the ash obtained as directed for Total Ash with 25 mL of water for 5 minutes. Collect the insoluble matter in a sintered-glass crucible or on an ashless filter paper. Wash with hot water, and ignite for 15 minutes at a temperature not exceeding  $450^\circ$ . Subtract the weight of this residue, in mg, obtained under Total Ash, and calculate the percentage of water-soluble ash with reference to the weight of sample as determined under Total Ash.

## Alcohol-Soluble Extractives

Method 1 (hot extraction method)— Transfer about 4 g of air-dried, coarsely powdered material, accurately weighed, to a glass-stoppered conical flask. Add 100 mL of alcohol, and weigh the flask. Shake, and allow to stand for 1 hour. Attach a reflux condenser to the flask, and boil gently for 1 hour, cool, and weigh. Readjust to the original weight with alcohol.



Shake, and filter rapidly through a dry filter. Transfer 25 mL of the filtrate to a tared flat-bottomed dish, and evaporate on a water bath to dryness. Dry at 105° for 6 hours, cool in a desiccator for 30 minutes, and weigh without delay. Calculate the content, in mg per g, of alcohol-extractable matter in the test specimen.

Method 2 (cold extraction method)— Transfer about 4 g of air-dried, coarsely powdered material, accurately weighed, to a glass-stoppered conical flask. Add 100 mL of alcohol, insert a stopper into the flask, and macerate for 24 hours, shaking frequently during the first 8 hours and then allowing to stand for 18 hours. Filter rapidly, taking precautions against loss of alcohol. Evaporate 25 mL of the filtrate to dryness in a tared, flat-bottomed, shallow dish, and dry at 105° to constant weight. Calculate the content, in mg per g, of alcohol-extractable matter in the test specimen.

#### Water-Soluble Extractives

Method 1 (hot extraction method)— Proceed as directed for Method 1 (hot extraction method) under Alcohol-soluble Extractives, except to use water in place of alcohol.

Method 2 (cold extraction method)— Proceed as directed for Method 2 (cold extraction method) under Alcohol-soluble Extractives, except to use water in place of alcohol.

#### Crude Fiber

Exhaust a weighed quantity of the Test Sample, representing about 2 g of the drug, with ether. Add 200 mL of boiling dilute sulfuric acid (1 in 78) to the ether-exhausted marc, in a 500-mL flask, and connect the flask to a reflux condenser. Reflux the mixture for 30 minutes, accurately timed, then filter through a linen or hardened-paper filter, and wash the residue on the filter with boiling water until the effluent washing is no longer acid. Rinse the residue back into the flask with 200 mL of boiling sodium hydroxide solution, adjusted to 1.25 percent by titration and free from sodium carbonate. Again reflux the mixture for 30 minutes, accurately timed, then rapidly filter through a tared filter, wash the residue with boiling water until the last washing is neutral, and dry it at 110° to constant weight. Incinerate the dried residue, ignite to constant weight, cool in a desiccator, and weigh the ash: the difference between the weight obtained by drying at 110° and that of the ash represents the weight of the crude fiber.

note—The boiling with acid and alkali should continue for 30 minutes, accurately timed, from the time that the liquid (which is cooled below the boiling point by being added to the cold flask) again boils. After the solution has been brought to boiling, the heat should be turned low enough just to maintain boiling. During the boiling, the flask should be gently rotated from time to time to wash down any particles that may adhere to the walls of the flask. A slow current of air introduced into the flask during the boiling operation aids in preventing excessive frothing.

#### Starch Content

Method 1— The following is a general procedure for all reducing sugars and may be used to determine the starch content in botanical articles.

Malt Extract— Use clean new barley malt of known efficacy, and grind just before use. Prepare malt extract just prior to use. For every 80 mL of malt extract needed, digest 5 g of ground malt with 100 mL of water at room temperature for 2 hours. [note—If an electric mixer is used, stir the mixture for 20 minutes.] Filter to obtain a clear extract, filtering again, if necessary, and mix the infusion well.

Test Solution— Extract about 5 g of the finely ground test specimen with five 10-mL portions of ether, using a filter that will completely retain the smallest starch granule. Allow the ether to evaporate from the residue, and wash with 250 mL of aqueous alcohol solution (10 in 100). Carefully wash the residue from the paper into a 500-mL beaker with about 100 mL of water. Heat to about 60° (avoiding, if possible, gelatinizing starch), and allow to stand for about 1 hour, stirring frequently to effect complete solution of sugars. Transfer to a wide-mouth bottle, rinse the beaker with a little warm water, and cool. Add an equal volume of alcohol, mix, and allow to stand for not less than 1 hour.

Centrifuge until the precipitate is closely packed on the bottom of the bottle, and decant the supernatant. Wash the precipitate with successive 50-mL portions of alcohol solution (50 in 100) by centrifuging and decanting through a suitable filter until the washings are sugar-free. [note—To test for the presence of sugar, transfer a few drops of the washings to a test tube, add 3 or 4 drops of a 20% solution of 1-naphthol in alcohol, prepared by dissolving 200 mg of 1-naphthol in 1 mL of alcohol and 2 mL of water. Shake the test tube well to allow uniform mixing, allow 2 to 4 mL of sulfuric acid to flow down the sides of the test tube, and hold the test tube upright. If sugar is present, the interface of the two liquids is colored faint to deep violet, and on shaking, the whole solution becomes blue-violet.]

Transfer the residue from the bottle and hardened filter to a beaker with about 50 mL of water. Immerse the beaker in boiling water, and stir constantly for 15 minutes or until all of the starch is gelatinized. Cool the beaker to 55°, add 20 mL of Malt Extract, and hold at this temperature for 1 hour. Heat again to boiling for a few minutes, cool to 55°, add 20 mL of Malt Extract, and hold at this temperature for 1 hour or until the residue when treated with [iodine TS](#) shows no blue tinge upon microscopic examination. Cool, dilute with water to 250 mL, and filter.

General Procedure— Transfer 200 mL of the Test Solution to a flask fitted with a reflux condenser, add 20 mL of hydrochloric acid, and heat in a boiling water bath for 2½ hours. Cool, nearly neutralize with [sodium hydroxide TS](#), complete neutralization with [sodium carbonate TS](#), dilute with water to 500 mL, mix, and filter. The volume of aliquot taken depends on the starch content of the specimen under test (see [Table 1](#)). The aliquot should contain between 100 and 200 mg of dextrose. Transfer 50 mL of the filtrate to a 400-mL alkali-resistant glass beaker, add 50 mL of alkaline cupric tartrate TS, cover the beaker with a water glass, and heat. Adjust the flame in the burner so that the contents of the flask begin to boil in 4 minutes and continue boiling for exactly 2 minutes. Filter the hot solution at once through a sintered-glass filter. Wash the precipitate of cuprous oxide thoroughly with water at about 60°, then with 10 mL of alcohol, and finally with 10 mL of ether.

Table 1. Determination of the Optimum Aliquot

% of Expected Starch Content	Aliquot in mL
60	25
50	35
40	50
30	50
20	50

For solutions of reducing sugars of comparatively high purity, proceed as directed under Method 1A to determine the amount of reduced copper obtained by weighing the dried cuprous oxide. For solutions of reducing sugars containing large amounts of organic impurities, including sucrose, proceed as directed under Method 1B to determine the amount of reduced copper obtained by titration with sodium thiosulfate.

method 1a— Dry the precipitate obtained under General Procedure for 30 minutes in an oven at 110 ± 2°, cool to room temperature in a desiccator, and weigh. Refer to [Table 2](#) to find the quantity of dextrose, in mg, corresponding to the weight of cuprous oxide found. Determine the percentage of dextrose and then the content of starch by the following formula:

$$\text{Percentage of dextrose} =$$

$$\frac{\text{wt. of dextrose in mg} \times 0.1 \times 500}{\text{wt. of sample in g} \times \text{aliquot in mL}}$$

$$\text{Content of starch} = \% \text{ dextrose} \times 0.9.$$

Table 2. Calculating Dextrose (Applicable when Cu<sub>2</sub>O is weighed directly) (Expressed in mg)

Cuprous Oxide (Cu <sub>2</sub> O)	Dextrose (d-Glucose)	Cuprous Oxide (Cu <sub>2</sub> O)	Dextrose (d-Glucose)	Cuprous Oxide (Cu <sub>2</sub> O)	Dextrose (d-Glucose)	Cuprous Oxide (Cu <sub>2</sub> O)	Dextrose (d-Glucose)	Cuprous Oxide (Cu <sub>2</sub> O)	Dextrose (d-Glucose)
10	4.0	90	38.9	170	75.1	250	112.8	330	152.2
12	4.9	92	39.8	172	76.0	252	113.7	332	153.2
14	5.7	94	40.6	174	76.9	254	114.7	334	154.2
16	6.6	96	41.5	176	77.8	256	115.7	336	155.2
									416
									196.8



18	7.5	98	42.4	178	78.8	258	116.6	338	156.3	418	197.9
20	8.3	100	43.3	180	79.7	260	117.6	340	157.3	420	199.0
22	9.2	102	44.2	182	80.6	262	118.6	342	158.3	422	200.1
24	10.0	104	45.1	184	81.5	264	119.5	344	159.3	424	201.1
26	10.9	106	46.0	186	82.5	266	120.5	346	160.3	426	202.2
28	11.8	108	46.9	188	83.4	268	121.5	348	161.4	428	203.3
30	12.6	110	47.8	190	84.3	270	122.5	350	162.4	430	204.4
32	13.5	112	48.7	192	85.3	272	123.4	352	163.4	432	205.5
34	14.3	114	49.6	194	86.2	274	124.4	354	164.4	434	206.5
36	15.2	116	50.5	196	87.1	276	125.4	356	165.4	436	207.6
38	16.1	118	51.4	198	88.1	278	126.4	358	166.5	438	208.7
40	16.9	120	52.3	200	89.0	280	127.3	360	167.5	440	209.8
42	17.8	122	53.2	202	89.9	282	128.3	362	168.5	442	210.9
44	18.7	124	54.1	204	90.9	284	129.3	364	169.6	444	212.0
46	19.6	126	55.0	206	91.8	286	130.3	366	170.6	446	213.1
48	20.4	128	55.9	208	92.8	288	131.3	368	171.6	448	214.1
50	21.3	130	56.8	210	93.7	290	132.3	370	172.7	450	215.2
52	22.2	132	57.7	212	94.6	292	133.2	372	173.7	452	216.3
54	23.0	134	58.6	214	95.6	294	134.2	374	174.7	454	217.4
56	23.9	136	59.5	216	96.5	296	135.2	376	175.8	456	218.5
58	24.8	138	60.4	218	97.5	298	136.2	378	176.8	458	219.6
60	25.6	140	61.3	220	98.4	300	137.2	380	177.9	460	220.7
62	26.5	142	62.2	222	99.4	302	138.2	382	178.9	462	221.8
64	27.4	144	63.1	224	100.3	304	139.2	384	180.0	464	222.9
66	28.3	146	64.0	226	101.3	306	140.2	386	181.0	466	224.0
68	29.2	148	65.0	228	102.2	308	141.2	388	182.0	468	225.1
70	30.0	150	65.9	230	103.2	310	142.2	390	183.1	470	226.2
72	30.9	152	66.8	232	104.1	312	143.2	392	184.1	472	227.4
74	31.8	154	67.7	234	105.1	314	144.2	394	185.2	474	228.3
76	32.7	156	68.6	236	106.0	316	145.2	396	186.2	476	229.6
78	33.6	158	69.5	238	107.0	318	146.2	398	187.3	478	230.7
80	34.4	160	70.4	240	108.0	320	147.2	400	188.4	480	231.8
82	35.3	162	71.4	242	108.9	322	148.2	402	189.4	482	232.9
84	36.2	164	72.3	244	109.9	324	149.2	404	190.5	484	234.1
86	37.1	166	73.2	246	110.8	326	150.2	406	191.5	486	235.2
88	38.0	168	74.1	248	111.8	328	151.2	408	192.6	488	236.3

## METHOD 1B—

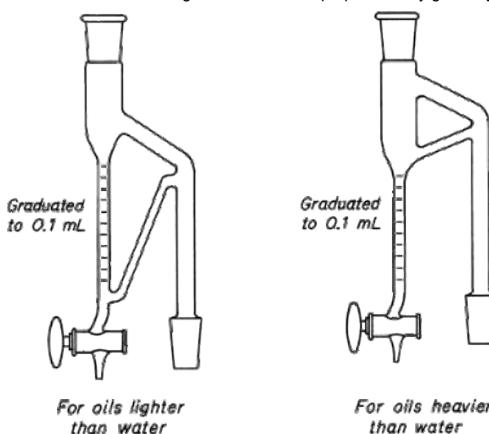
Sodium Thiosulfate Solution— Transfer 3.9 g of sodium thiosulfate, accurately weighed, to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix.

Potassium Iodide Solution— Dissolve 42 g of potassium iodide in 100 mL of water.

Sodium Acetate Solution— Dissolve 5.74 g of sodium acetate in 10 mL of water.

Copper Solution— Transfer about 0.3 g of pure electrolytic copper, accurately weighed, to a 250-mL flask, add 5 mL of nitric acid to dissolve the copper, add about 25 mL of water, and boil to expel red fumes. Add about 5 mL of bromine TS, and boil until the bromine is completely removed. Cool, add 10 mL of Sodium Acetate Solution followed by 10 mL of Potassium Iodide Solution, and titrate with Sodium Thiosulfate Solution to a light yellow color. Add enough starch TS to produce a marked blue color, and continue the titration. As the endpoint

nears, add 2 g of potassium thiocyanate, and stir until completely dissolved. Continue titration until the precipitate is completely white. One mL of sodium thiosulfate solution is equivalent to about 10 mg of copper. [note—It is essential that the concentration of Potassium Iodide Solution be carefully regulated. If the solution contains less than 320 mg of copper at the completion of titration, add 4.2 to 5 g of potassium iodide to make a total solution of 100 mL. If greater amounts of Cu are present, add Potassium Iodide Solution slowly from buret with constant agitation in amounts proportionately greater.]



Traps for Volatile Oil Apparatus



Procedure— Wash the precipitated cuprous oxide obtained under General Procedure with water, cover this filter with a watch glass and dissolve the cuprous oxide with 5 mL of nitric acid directed under the watch glass with a pipet. Collect the filtrate in a 250-mL flask, wash the watch glass and the filter with water. Collect all the washings in the flask. Boil the contents of the flask to expel red fumes. Add about 5 mL of bromine TS, and boil until the bromine is completely removed. Cool, and proceed as directed under Copper Solution beginning with “add 10 mL of Sodium Acetate Solution.” From the volume of Sodium Thiosulfate Solution consumed, obtain the weight of copper, in mg, by multiplying by 1.1259 to obtain the weight, in mg, of cuprous oxide. From [Table 2](#), find the quantity of dextrose, in mg, corresponding to the weight of cuprous oxide. The content of starch is equivalent to the weight, in mg, of dextrose obtained times 0.9. Conduct a blank determination, using 50 mL of [alkaline cupric tartrate TS](#) and 50 mL of Malt Extract. If the weight of the cuprous oxide so obtained exceeds 0.5 mg, correct the result of the determination accordingly. [note—The alkaline cupric tartrate TS deteriorates on standing and the quantity of cuprous oxide obtained in the blank determination increases.]

Method 2— The following method is specific for dextrose (glucose), and because of its extreme sensitivity it may account for differences noted between values obtained from the same specimen. Duplicate determinations do not vary more than 2%.

Glucoamylase Solution— Prepare a solution of glucoamylase in water containing 30 International Units (IU) per mL. Use glucoamylase obtained preferably from *Rhizopus delemar*. The total glucoamylase activity of the test specimen being used should be not less than 150 IU.

Acetate Buffer Solution— Dissolve 16.4 g of sodium acetate in 100 mL of water, add 12.0 mL of glacial acetic acid, and mix. The pH of this solution is 4.8.

Phosphate Buffer— Dissolve 3.63 g of tris (hydroxymethyl) aminomethane and 5.0 g of monobasic sodium phosphate in 50.0 mL of water. At 37°, adjust with phosphoric acid to a pH of 7.0, dilute with water to 100.0 mL, and mix. [note—The pH of the buffer medium is sensitive to temperature and should be adjusted to the desired pH at the temperature to be used during incubation.]

Enzyme Solution— Dissolve 30 mg of glucose oxidase (Type II from *Aspergillus niger*), 3 mg of peroxidase (Type I from horseradish), and 10 mg of potassium ferrocyanide in 100 mL of Phosphate Buffer. [note—This mixture can be stored in a refrigerator for up to 10 days.]

18 N Sulfuric Acid— Add slowly, while stirring, 54 mL of sulfuric acid to 102 mL of water, allow to cool to 25°, and mix.

Standard Solutions— Dissolve an accurately weighed quantity of [USP Dextrose RS](#) in water to obtain a solution containing 1.0 mg of [USP Dextrose RS](#) per mL. Quantitatively dilute a known volume of this solution with water to obtain Standard Solutions A, B, C, D, and E, having known concentrations of 10, 20, 25, 40, and 50 µg per mL of [USP Dextrose RS](#), respectively. [note—Allow 4 hours for complete mutarotation before use.]

Test Solutions— Extract about 5 g of finely ground test specimen with five 25-mL portions of 80% alcohol, and filter. Remove all the alcohol from the residue by drying in an air oven at 105° for about 8 hours. [Note 1—Any traces of alcohol remaining in the residue will inhibit glucoamylase.] Cool, and transfer the flask containing the dried test specimen to a desiccator. Transfer about 1 g, accurately weighed, of the test specimen to a previously tared flask, add 25 mL of water, and adjust with phosphoric acid to a pH between 5.0 and 7.0, if necessary. Boil the suspension for about 3 minutes, transfer the flask to an autoclave, and heat to 135° for 2 hours. Remove the flask from the autoclave, maintain the temperature near 55°, and add 2.5 mL of Acetate Buffer Solution and sufficient water to adjust the total weight of the solution to 45 ± 1 g. Immerse the flask in a water bath maintained at 55 ± 1°, and add 5 mL of Glucoamylase Solution. Continuously swirl the flask for 2 hours to effect hydrolysis, filter through filter paper into a 250-mL volumetric flask, wash quantitatively with water, and collect all the washings in the flask. Dilute the contents of the flask with water to volume, and mix. Transfer 1 mL of an aliquot containing 20 to 60 µg of d-glucose to each of five test tubes. [Note 2—In order to obtain the range of concentration of glucose in the hydrolysate, quantitatively dilute, if necessary, with water to volume.] Add 2 mL of Enzyme Solution to each of the five test tubes, and place the test tubes in the dark at 37 ± 1° for exactly 30 minutes to develop the color. At the end of 30 minutes, add 2 mL of 18 N Sulfuric Acid to each of the test tubes to stop the reaction, and mix.

Control Solution— Transfer an accurately weighed quantity of about 0.4 g of starch to a previously tared flask and proceed as directed under Test Solutions beginning with “add 25 mL of water and, adjust the pH with phosphoric acid.”

Procedure— Concomitantly determine the absorbances of the Standard Solutions and the Test Solutions at the wavelength of maximum absorbance at about 540 nm, with a suitable spectrophotometer, using the Control Solution as the blank to set the instrument. Plot the absorbance values of the Standard Solutions versus concentration, in µg per mL, of dextrose, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in µg per mL, of dextrose in each of the Test Solutions, calculate the average concentration, in µg per mL, of the solution under test. The percentage of starch content in the weight of the test specimen taken by the equation is calculated by the formula:

$$(0.9C / 106)(V1)(250 / V0)(100 / E)(100 / W) = 2.25CV1 / V0EW$$

in which E is the weight, in g, of the test specimen taken; V0 is the volume, in mL, of the aliquot taken from the 250-mL volumetric flask; W is the percentage of dry weight of the test specimen; and V1 is the volume, in mL, if extra dilution is done (see Note 2 under Test Solutions). [note—V0 is 1.0 when no extra dilution is done.]

#### Volatile Oil Determination

Set up a round-bottom, shortneck, 1-liter flask in a heating mantle set over a magnetic stirrer. Insert an egg-shaped stirring bar magnet in the flask, and attach a cold-finger condenser and an appropriate volatile oil trap of the type illustrated.

Coarsely comminute a sufficient quantity of the drug to yield from 1 to 3 mL of volatile oil. Small seeds, fruits, or broken leaves of herbs ordinarily do not need comminution. Very fine powders are to be avoided. If this is not possible, it may be necessary to mix them with purified sawdust or purified sand. Place a suitable quantity of the drug, accurately weighed, in the flask, and fill it one-half with water. Attach the condenser and the proper separator. Boil the contents of the flask, using a suitable amount of heat to maintain gentle boiling for 2 hours, or until the volatile oil has been completely separated from the drug and no longer collects in the graduated tube of the separator.

If a proper quantity of the volatile oil has been obtained in the graduated tube of the separator, it can be read to tenths of 1 mL, and the volume of volatile oil from each 100 g of drug can be calculated from the weight of the drug taken. The graduations on the separator “for oils heavier than water” are so placed that oil remains below the aqueous condensate that automatically flows back into the flask.

#### Water Content

For unground or unpowdered drugs, prepare about 10 g of the Laboratory Sample by cutting, granulating, or shredding, so that the parts are about 3 mm in thickness. Seeds or fruits smaller than 3 mm should be cracked. Avoid the use of high-speed mills in preparing the sample, and exercise care that no appreciable amount of moisture is lost during the preparation and that the portion taken is representative of the Laboratory Sample. Determine the water content as directed for Procedure for Articles of Botanical Origin in the [Method III \(Gravimetric\) under Water Determination](#) (921).

#### TEST FOR AFLATOXINS

Caution—Aflatoxins are highly dangerous, and extreme care should be exercised in handling aflatoxin materials.

This test is provided to detect the possible presence of aflatoxins B1, B2, G1, and G2 in any material of plant origin. Unless otherwise specified in the individual monograph, use the following method.

Zinc Acetate-Aluminum Chloride Reagent— Dissolve 20 g of zinc acetate and 5 g of aluminum chloride in sufficient water to make 100 mL.

Sodium Chloride Solution— Dissolve 5 g of sodium chloride in 50 mL of water.

Test Solution 1— Grind about 200 g of plant material to a fine powder. Transfer about 50 g of the powdered material, accurately weighed, to a glass-stoppered flask. Add 200 mL of a mixture of methanol and water (17:3). Shake vigorously by mechanical means for not less than 30 minutes, and filter. [note—If the solution has interfering plant pigments, proceed as directed for Test Solution 2.] Discard the first 50 mL of the filtrate, and collect the next 40-mL portion. Transfer the filtrate to a separatory funnel. Add 40 mL of Sodium Chloride Solution and 25 mL of solvent hexane, and shake for 1 minute. Allow the layers to separate, and transfer the lower aqueous layer to a second separatory funnel. Extract the aqueous layer in the separatory funnel twice, each time with 25 mL of methylene chloride, by shaking for 1 minute. Allow the layers to separate each time, separate the lower organic layer, and



Collect the combined organic layers in a 125-mL conical flask. Evaporate the organic solvent to dryness on a water bath. Cool the residue. If interferences exist in the residue, proceed as directed for Cleanup Procedure; otherwise, dissolve the residue obtained above in 0.2 mL of a mixture of chloroform and acetonitrile (9.8:0.2), and shake by mechanical means if necessary.

Test Solution 2— Collect 100 mL of the filtrate from the start of the flow, and transfer to a 250-mL beaker. Add 20 mL of Zinc Acetate-Aluminum Chloride Reagent and 80 mL of water. Stir, and allow to stand for 5 minutes. Add 5 g of a suitable filtering aid, such as diatomaceous earth, mix, and filter. Discard the first 50 mL of the filtrate, and collect the next 80-mL portion. Proceed as directed for Test Solution 1, beginning with “Transfer the filtrate to a separatory funnel.”

Cleanup Procedure— Place a medium-porosity sintered-glass disk or a glass wool plug at the bottom of a 10-mm × 300-mm chromatographic tube. Prepare a slurry of 2 g of silica gel with a mixture of ethyl ether and solvent hexane (3:1), pour the slurry into the column, and wash with 5 mL of the same solvent mixture. Allow the absorbent to settle, and add to the top of the column a layer of 1.5 g of anhydrous sodium sulfate. Dissolve the residue obtained above in 3 mL of methylene chloride, and transfer it to the column. Rinse the flask twice with 1-mL portions of methylene chloride, transfer the rinses to the column, and elute at a rate not greater than 1 mL per minute. Add successively to the column 3 mL of solvent hexane, 3 mL of ethyl ether, and 3 mL of methylene chloride; elute at a rate not greater than 3 mL per minute; and discard the eluates. Add to the column 6 mL of a mixture of methylene chloride and acetone (9:1), and elute at a rate not greater than 1 mL per minute, preferably without the aid of vacuum. Collect this eluate in a small vial, add a boiling chip if necessary, and evaporate to dryness on a water bath. Dissolve the residue in 0.2 mL of a mixture of chloroform and acetonitrile (9.8:0.2), and shake by mechanical means if necessary.

Aflatoxin Solution— [Caution—Aflatoxins are highly toxic. Handle with care.] Dissolve accurately weighed quantities of aflatoxin B1, aflatoxin B2, aflatoxin G1, and aflatoxin G2 in a mixture of chloroform and acetonitrile (9.8:0.2) to obtain a solution having concentrations of 0.5 µg per mL each of aflatoxin B1 and aflatoxin G1, and 0.1 µg per mL each of aflatoxin B2 and aflatoxin G2.

Procedure— Separately apply 2.5 µL, 5 µL, 7.5 µL, and 10 µL of the Aflatoxin Solution and three 10-µL applications of either Test Solution 1 or Test Solution 2 to a suitable thin-layer chromatographic plate (see [Chromatography \(621\)](#)) coated with a 0.25-mm layer of chromatographic silica gel mixture. Superimpose 5 µL of the Aflatoxin Solution on one of the three 10-µL applications of the Test Solution. Allow the spots to dry, and develop the chromatogram in an unsaturated chamber containing a solvent system consisting of a mixture of chloroform, acetone, and isopropyl alcohol (85:10:5) until the solvent front has moved not less than 15 cm from the origin. Remove the plate from the developing chamber, mark the solvent front, and allow the plate to air-dry. Locate the spots on the plate by examination under UV light at 365 nm: the four applications of the Aflatoxin Solution appear as four clearly separated blue fluorescent spots; the spot obtained from the Test Solution that was superimposed on the Aflatoxin Solution is no more intense than that of the corresponding Aflatoxin Solution; and no spot from any of the other Test Solutions corresponds to any of the spots obtained from the applications of the Aflatoxin Solution. If any spot of aflatoxins is obtained in the Test Solution, match the position of each fluorescent spot of the Test Solution with those of the Aflatoxin Solution to identify the type of aflatoxin present. The intensity of the aflatoxin spot, if present in the Test Solution, when compared with that of the corresponding aflatoxin in the Aflatoxin Solution will give an approximate concentration of aflatoxin in the Test Solution.

#### GENERAL METHOD FOR PESTICIDE RESIDUES ANALYSIS

Definition— Where used in this Pharmacopeia, the designation pesticide applies to any substance or mixture of substances intended to prevent, destroy, or control any unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport, or marketing of pure articles. The designation includes substances intended for use as growth regulators, defoliants, or desiccants, and any substance applied to crops before or after harvest to protect the product from deterioration during storage and transport.

Limits— Within the United States, many botanicals are treated as dietary supplements and are subject to the statutory provisions of the Federal Food, Drug, and Cosmetic Act that governs foods but not drugs. Limits for pesticides for foods are determined by the Environmental Protection Agency (EPA), and where no limit is set, the limit is zero. The limits contained herein, therefore, are not applicable in the United States when articles of botanical origins are labeled for food purposes. The limits, however, may be applicable in other countries where the presence of pesticide residues is permitted. Unless otherwise specified in the individual monograph, the article under test contains not more than the amount of any pesticide indicated in [Table 3](#). The limits applying to pesticides not listed in [Table 3](#) and whose presence is suspected for any reason comply with the regulations of the EPA.

Where a pesticide is not listed in [Table 3](#) or in EPA regulations, calculate the limit by the formula:

$$AM / 100B$$

in which A is the acceptable daily intake (ADI), in mg per kg of body weight; M is the body weight, in kg; and B is the daily dose of the article, in kg.

Table 3

Substance	Limit (mg/kg)
Alachlor	0.02
Aldrin and Dieldrin (sum of)	0.05
Azinphos-methyl	1.0
Bromopropylate	3.0
Chlordane (sum of cis- and trans- isomers and oxychlordane)	0.05
Chlorfenvinphos	0.5
Chlorpyrifos	0.2
Chlorpyrifos-methyl	0.1
Cypermethrin (and isomers)	1.0
DDT (sum of p,p'-DDT, o,p'-DDT, p,p'-DDE, and p,p'-TDE)	1.0
Deltamethrin	0.5
Diazinon	0.5
Dichlorvos	1.0
Dithiocarbamates (as CS <sub>2</sub> )	2.0
Endosulfan (sum of endosulfan isomers and endosulfan sulfate)	3.0
Endrin	0.05
Ethion	2.0
Fenitrothion	0.5
Fenvalerate	1.5
Fonofos	0.05
Heptachlor (sum of heptachlor and heptachlor epoxide)	0.05
Hexachlorobenzene	0.1
Hexachlorocyclohexane isomers (other than γ)	0.3



Lindane ( $\gamma$ -hexachlorocyclohexane)	0.6
Malathion	1.0
Methidathion	0.2
Parathion	0.5
Parathion-methyl	0.2
Permethrin	1.0
Phosalone	0.1
Piperonyl butoxide	3.0
Pirimiphos-methyl	4.0
Pyrethrins (sum of)	3.0
Quintozene (sum of quintozene, pentachloroaniline and methyl pentachlorophenyl sulfide)	1.0

If the article is intended for the preparation of extracts, tinctures, or other pharmaceutical forms whose preparation method modifies the content of pesticides in the finished product, calculate the limits by the formula:

AME / 100B

in which E is the extraction factor of the preparation method, determined experimentally; and A, M, and B are as defined above.

**Sampling—**

Method— For articles in containers holding less than 1 kg, mix the contents, and withdraw a quantity sufficient for the tests. For articles in containers holding between 1 and 5 kg, withdraw equal portions from the upper, middle, and lower parts of the container, each of the samples being sufficient to carry out the tests. Thoroughly mix the samples, and withdraw an amount sufficient to carry out the tests. For containers holding more than 5 kg, withdraw three samples, each weighing not less than 250 g, from the upper, middle, and lower parts of the container. Thoroughly mix the samples, and withdraw a portion sufficient to carry out the tests.

Size of Sampling— If the number of containers, n, is three or fewer, withdraw samples from each container as indicated above. If the number of containers is more than three, take samples from

$$\sqrt{n} + 1$$

containers, rounding up to the nearest whole number if necessary.

note—Conduct tests without delay to avoid possible degradation of the residues. If this is not possible, store the samples in hermetic containers suitable for food contact, at a temperature below 0°, and protected from light.

Reagents— Use reagents and solvents that are free from any contaminants, especially pesticides, that might interfere with the analysis. It is often necessary to use special grade solvents suitable for pesticide residue analysis or solvents that have recently been redistilled in an apparatus made entirely of glass. In any case, suitable blank tests must be performed.

Preparation of Apparatus— Clean all equipment, especially glassware, to ensure that it is free from pesticides. Soak all glassware for a minimum of 16 hours in a solution of phosphate-free detergent, rinse with copious quantities of distilled water, and then wash with acetone, followed by hexane or heptane.

Qualitative and Quantitative Analysis of Pesticide Residues— Use validated analytical procedures that satisfy the following criteria. The method, especially with respect to its purification steps, is suitable for the combination of pesticide residue and substance under test, and is not susceptible to interference from co-extractives. Measure the limits of detection and quantification for each pesticide matrix combination to be analyzed: the method is shown to recover between 70% and 110% of each pesticide; the repeatability and reproducibility of the method are not less than the appropriate values indicated in [Table 4](#); and the concentrations of test and reference solutions and the setting of the apparatus are such that a linear response is obtained from the analytical detector.

Table 4

Concentration of the Pesticide (mg/kg)	Repeatability (difference, $\pm$ mg/kg)	Reproducibility (difference, $\pm$ mg/kg)
0.010	0.005	0.01
0.100	0.025	0.05
1.000	0.125	0.25

**TEST FOR PESTICIDES**

Unless otherwise specified in the individual monograph, the following methods may be used for the analysis of pesticides. Depending on the substance being examined, it may be necessary to modify, sometimes extensively, the procedure described hereafter. Additionally, it may be necessary to perform another method with another column having a different polarity, another detection method (e.g., mass spectrometry), or a different method (e.g., immunochemical method) to confirm the results.

Extraction— [note—Use the following procedure for the analysis of samples of articles having a water content of less than 15%. Samples having a higher water content may be dried, provided that the drying procedure does not significantly affect the pesticide content.] To 10 g of the coarsely powdered substance under test, add 100 mL of acetone, and allow to stand for 20 minutes. Add 1 mL of a solution in toluene containing 1.8 g of carbophenothion per mL. Mix in a high-speed blender for 3 minutes. Filter this solution, and wash the residue with two 25-mL portions of acetone. Combine the filtrate and the washings, and heat, in a rotary evaporator, maintaining the temperature of the bath below 40° until the solvent has almost completely evaporated. To the residue add a few mL of toluene, and heat again until the acetone is completely removed. Dissolve the residue in 8 mL of toluene. Pass through a membrane filter having a 45- $\mu$ m porosity, rinse the flask and the filter with toluene, dilute with toluene to 10.0 mL (Solution A), and mix.

**Purification—**

Organochlorine, Organophosphorus, and Pyrethroid Insecticides— The size-exclusion chromatograph is equipped with a 7.8-mm  $\times$  30-cm stainless steel column containing 5- $\mu$ m packing L21. Toluene is used as the mobile phase at a flow rate of about 1 mL per minute.

Performance of the Column— Inject 100  $\mu$ L of a solution in toluene containing, in each mL, 0.5 mg of methyl red and 0.5 mg of oracet blue. The column is not suitable unless the color

the eluate changes from orange to blue at an elution volume of about 10.3 mL. If necessary, calibrate the column, using a solution in toluene containing suitable concentrations of the pesticide of interest having the lowest molecular weight (for example, dichlorvos) and that having the highest molecular weight (for example, deltamethrin). Determine which fraction of the eluate contains both pesticides.

Purification of the Test Solution— Inject a suitable volume (100 to 500  $\mu$ L) of Solution A into the chromatograph. Collect the fraction (Solution B) as determined above under Performance of the Column. Organophosphorus pesticides elute between 8.8 and 10.9 mL. Organochlorine and pyrethroid pesticides elute between 8.5 and 10.3 mL.

Organochlorine and Pyrethroid Insecticides— Into a 5-mm  $\times$  10-cm chromatographic column, introduce a piece of fat-free cotton and 0.5 g of silica gel treated as follows. Heat chromatographic silica gel in an oven at 150° for at least 4 hours. Allow to cool, and add dropwise a quantity of water corresponding to 1.5% of the weight of silica gel used. Shake vigorously until agglomerates have disappeared, and continue shaking by mechanical means for 2 hours. Condition the column with 1.5 mL of hexane. [note—Prepacked columns containing about 0.50 g of a suitable silica gel may also be used, provided they have been previously validated.] Concentrate Solution B almost to dryness, with the aid of a stream of helium or oxygen-free nitrogen, and dilute with toluene to a suitable volume (200  $\mu$ L to 1 mL, according to the volume injected in the preparation of Solution B). Quantitatively transfer this solution to the column, and proceed with the chromatography, using 1.8 mL of toluene as the mobile phase. Collect the eluate (Solution C).

#### Quantitative Analysis of Organophosphorus Insecticides—

Test Solution— Concentrate Solution B almost to dryness, with the aid of a stream of helium, dilute with toluene to 100  $\mu$ L, and mix.

Standard Solution— Prepare at least three solutions in toluene containing each of the pesticides of interest and carbophenothion at concentrations suitable for plotting a calibration curve.

Chromatographic System— The gas chromatograph is equipped with an alkali flame-ionization detector or a flame-photometric detector and a 0.32-mm  $\times$  30-m fused silica column coated with a 0.25- $\mu$ m layer of phase G1. Hydrogen is used as the carrier gas. Other gases, such as helium or nitrogen, may also be used. The injection port temperature is maintained at 250°, and the detector is maintained at 275°. The column temperature is maintained at 80° for 1 minute, then increased to 150° at a rate of 30° per minute, maintained at 150° for 3 minutes, then increased to 280° at a rate of 4° per minute, and maintained at this temperature for 1 minute. Use carbophenothion as the internal standard. [Note—If necessary, use a second internal standard to identify any possible interference with the peak corresponding to carbophenothion.] Inject the chosen volume of each solution, record the chromatograms, and measure the peak responses: the relative retention times are approximately those listed in [Table 5](#). Calculate the content of each pesticide from the peak areas and the concentrations of the solution.

Table 5

Substance	Relative Retention Time
Dichlorvos	0.20
Fonofos	0.50
Diazinon	0.52
Parathion-methyl	0.59
Chlorpyrifos-methyl	0.60
Pirimiphos-methyl	0.66
Malathion	0.67
Parathion	0.69
Chlorpyrifos	0.70
Methidathion	0.78
Ethion	0.96
Carbophenothion	1.00
Azinphos-methyl	1.17
Phosalone	1.18

#### Quantitative Analysis of Organochlorine and Pyrethroid Insecticides—

Test Solution— Concentrate Solution C almost to dryness, with the aid of a stream of helium or oxygen-free nitrogen, dilute with toluene to 500  $\mu$ L, and mix.

Standard Solution— Prepare at least three solutions in toluene containing each of the pesticides of interest and carbophenothion at concentrations suitable for plotting a calibration curve.

Chromatographic System— The gas chromatograph is equipped with an electron-capture detector, a device allowing direct on-column cold injection, and a 0.32-mm  $\times$  30-m fused silica column coated with a 0.25- $\mu$ m layer of phase G1. Hydrogen is used as the carrier gas. Other gases, such as helium or nitrogen, may also be used. The injection port temperature is maintained at 275°, and the detector is maintained at 300°. The column temperature is maintained at 80° for 1 minute, then increased to 150° at a rate of 30° per minute, maintained at 150° for 3 minutes, then increased to 280° at a rate of 4° per minute, and maintained at this temperature for 1 minute. Use carbophenothion as the internal standard. [note—If necessary, use a second internal standard to identify any possible interference with the peak corresponding to carbophenothion.] Inject the chosen volume of each solution, record the chromatograms, and measure the peak responses: the relative retention times are approximately those listed in [Table 6](#). Calculate the content of each pesticide from the peak areas and the concentrations of the solutions.

Table 6

Substance	Relative Retention Time
$\alpha$ -Hexachlorocyclohexane	0.44
Hexachlorobenzene	0.45
$\beta$ -Hexachlorocyclohexane	0.49
Lindane	0.49
$\delta$ -Hexachlorocyclohexane	0.54
$\epsilon$ -Hexachlorocyclohexane	0.56
Heptachlor	0.61
Aldrin	0.68
cis-Heptachlor epoxide	0.76
o, p'-DDE	0.81
$\alpha$ -Endosulfan	0.82
Dieldrin	0.87
p, p'-DDE	0.87
o, p'-DDD	0.89
Endrin	0.91



B-Endosulfan	0.92
o, p'-DDT	0.95
Carbophenothion	1.00
p, p'-DDT	1.02
cis-Permethrin	1.29
trans-Permethrin	1.31
Cypermethrin*	1.40
Fenvalerate*	1.47
	1.49
Deltamethrin	1.54

\* The substance shows several peaks.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Yoshiyuki Tokiwa, Ph.D.</a> Senior Scientist 1-301-816-8321	(GC05) General Chapters 05

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#### 563 IDENTIFICATION OF ARTICLES OF BOTANICAL ORIGIN

Identification of raw plant material intended for use in the manufacture of pharmaceuticals, excipients, or dietary supplements is carried out by examining the morphological and histological features of the article under test and by performing diagnostic chemical tests on the article. The botanical and chemical characteristics of the test article are then compared to the known botanical and chemical characteristics of the plant species. Reference articles may be specified to assist in the proper botanical and chemical identification of the plant and plant part. A reference article may be either a USP Authenticated Reference Material, which may be used for both botanical and chemical identification, or a USP Reference Standard, which is used for chemical identification only.

#### USP AUTHENTICATED REFERENCE MATERIALS

USP Authenticated Reference Materials are plant organs or tissues certified to have come from a plant that has been properly identified as belonging to the species listed on the label. The authentication is performed by botanical taxonomists, plant anatomists, phytochemists, or other plant scientists contracted by the USP. A USP Authenticated Reference Material is typically a dried, pulverized plant organ or tissue, and it may be obtained from USP. Herbarium samples that may include roots, stems, leaves, flowers, fruits, and seeds of the authenticated plants are archived and made available for examination upon request. Standard herbarium samples usually consist of the entire mature plant. USP Authenticated Reference Materials undergo the same botanical and chemical diagnostic tests as those applied to the test raw materials. A test article must have all botanical and chemical characteristics specified and found in the USP Authenticated Reference Material. To serve its intended purpose, each USP Authenticated Reference Material is properly stored, handled, and used. Generally, USP Authenticated Reference Materials are stored in their original containers under cool and dry conditions and protected from light and insect infestation. Where special storage conditions are necessary, directions are given on the label. Active principles and marker compounds typically degrade with time; therefore, expiration dates are assigned to USP Authenticated Reference Materials for their use in chemical identification. USP Authenticated Reference Materials are not intended for use in the manufacture of pharmaceuticals, excipients, or dietary supplements.

#### BOTANICAL IDENTIFICATION

The botanical identification of raw plant materials used in the manufacture of pharmaceuticals, excipients, or dietary supplements consists of ascertaining the macroscopic characteristics of the plant part, such as root, stem, leaf, flower, fruit, or seed, used in the manufacture of the article, as well as ascertaining its histological (microscopic) features. It may also include the inspection of organoleptic features of the botanical tissue, such as the presence or absence of a characteristic odor. Individual compendial monographs may include botanical information on possible adulterant species to help ensure their absence in the raw material. For a proper identification of the plant, plant organ, or plant tissue, it is necessary to have a basic knowledge of plant anatomy.

##### Diagnostic Plant Morphology and Anatomy

This section exclusively addresses the diagnostic morphological and anatomical features of vascular plants and the various plant parts, such as roots, stems, leaves, flowers, fruits, and seeds, from which pharmaceuticals, excipients, or dietary supplements are derived. Vascular plants include pteridophytes (ferns and fern allies; for example, genera Aspidium, Equisetum, and Lycopodium), gymnosperms (seed plants, in which the seed is not enclosed within a fruit; for example, genera Ephedra, Gingko, and Pinus), and angiosperms (seed plants, in which the seed is enclosed within a fruit; for example, genera Allium, Digitalis, Panax, Matricaria, and Rauwolfia). Anatomical diagnostic features that are specified in an individual monograph (see Botanic characteristics in individual monographs) may include, but are not limited to, the presence of a particular tissue within an organ; the arrangement and type of cells within a tissue; the presence and type of secretory canal, oil, or resin duct or latexiferous vessels within an organ; the number of epithelial cells surrounding a secretory canal; and the presence and type of ergastic substances such as starch, inulin, fat globules, essential oils, calcium oxalate crystals, cystoliths, polyphenols, fluids, or other materials occurring in the cytoplasm, organelles, vacuoles, cavities, or cell wall.

##### roots

The tissues present in young roots, starting with the most external tissue, include an epidermis with root hairs, cortex, endodermis, pericycle, phloem, xylem, and, in some species, pith. In some species, the outermost layer or layers of cortex are distinct from the inner layers, in which case they are referred to as a hypodermis. In species that undergo secondary growth in the roots, it is typical for all tissues external to the pericycle to be sloughed off. Roots that exhibit secondary growth have a periderm or bark, composed of a phellem (cork), phellogen (cork cambium), and phellem as the outermost tissue. Underneath the periderm, remnants of primary phloem, secondary phloem, vascular cambium, primary xylem, and secondary xylem can be found. Secondary vascular tissues have medullary rays separating clusters of the principal conducting cells of phloem (sieve elements or sieve cells) and the principal conducting cells of xylem (vessels and tracheids). Most species of plants that undergo secondary growth lack pith in the root. The type and arrangement of the principal conducting cells of the vascular tissues may be diagnostic of the species. Roots of many species develop into food storage organs. Abundant parenchyma and large amounts of starch or other polysaccharides characterize these types of roots. The presence, type, and arrangement of fibers, sclereids, and other tissues, and the presence and location of ergastic material may also be diagnostic features. Morphologically, roots may be distinguished from rhizomes (the underground stems) primarily by the absence of nodes and internodes, which are present in rhizomes.

##### stems

Several external macroscopic features of stems that may be diagnostic of the species include the attributes of the nodes, internodes, leaf scars, vascular bundle scars, lenticels, and buds; the growth pattern of the buds; position and arrangement of the leaves along the stem; and the presence of tendrils, spines, thorns, or prickles. Starting with the outermost tissue, the internal arrangement of tissues in the young stems of most species is epidermis, cortex, a concentric ring of vascular bundles separated from each other by parenchymatous medullary rays, and pith. Depending on the species, stomata or trichomes or both structures may be present in the epidermis. The cortex of some species may include a hypodermis or an endodermis or both. In most monocotyledons, the vascular bundles are not arranged concentrically; instead they are scattered throughout a mass of parenchyma tissue internal to



the epidermis. Because of this arrangement, neither cortex, medullary rays, nor pith can be discerned. In woody plant stems that undergo secondary growth, it is typical for the epidermis to be sloughed off and replaced by a periderm composed of a phellem, phellogen, and phelloiderm. Some species are characterized by having multiple periderms (rhytidome). Lenticels may be present in the periderm and their attributes may serve as diagnostic features. Underneath the periderm are the remnants of the cortex, primary phloem, secondary phloem, vascular cambium, and secondary xylem, and the remnants of primary xylem and pith. Medullary rays are also present. As in the root, the type and arrangement of the principal conducting cells of the vascular tissues; the presence, type, and arrangement of fibers, sclereids, and other tissues; and the presence and location of ergastic material may also be diagnostic features. Rhizomes may have some morphological characteristics similar to those of roots and therefore they may be mistaken for roots. However, rhizomes can be correctly identified as stems because they have distinct nodes and internodes.

#### leaves

Several macroscopic features of leaves that may be diagnostic of the species include the attributes of the leaf blade, petiole, and stipules and the phyllotaxy. The outermost tissue of a leaf blade is the epidermis, followed by mesophyll and vascular tissues. Microscopic diagnostic features of epidermal cells include the cuticle thickness and markings, the shape and arrangement of stomata and guard cells, the arrangement and size of subsidiary cells, stomatal number (number of stomata per unit area), and stomatal index (number of stomata per unit number of epidermal cells). Additional features useful in the identification of leaf material include types and arrangement of trichomes (plant hairs) present; type and arrangement of mesophyll and vascular tissues; palisade mesophyll ratio; presence and appearance of accessory tissues such as parenchymatous or sclerenchymatous bundle sheaths, parveinal mesophyll, endodermis, and transfusion tissue; type and arrangement of the principal conducting cells of the vascular tissues; presence, type, and arrangement of fibers, sclereids, and other tissues; and presence, location, and physical appearance of ergastic material.

#### flowers

Flowers are the best diagnostic morphological features of any flowering plant and the floral structure is the principal criterion used in plant taxonomy. The diagnostic features of flowers include type of inflorescence; presence, number, and appearance of the primary floral parts (sepals, petals, stamens, and carpels); type of symmetry displayed by the floral parts; relative position of the ovaries in regards to the other parts of the flower; the number of ovules per ovary; type of placentation of the ovary; physical appearance of the pollen grains; presence of nectaries; presence of covering or glandular trichomes; and physical features of accessory structures, such as the receptacle and bracts. The histological features and the presence of ergastic materials in the tissues of floral parts are also diagnostic of the species.

#### fruits

The identification of the species of plant from which a fruit was derived may be determined by observing several macroscopic criteria. These criteria include the number of pistils found in the fruit, the number of carpels within each pistil, the number of seeds within each carpel, the placentation of the fruit, and the determination whether the fruit is dehiscent, indehiscent, or fleshy. Additional diagnostic features include the number of sutures in a dehiscent fruit, the determination whether the seeds are fused to or free from the pericarp wall, physical features of the three layers of the pericarp of fleshy fruits (epicarp, mesocarp, and endocarp), and presence and physical appearance of accessory tissues such as the receptacle and bracts. Histological features of fruit tissues may aid in identification. The characteristics of the seeds within the fruit are also diagnostic features of the species.

#### seeds

The macroscopic features of seeds used in identification include the shape and size of the seed; appearance of the seed-coat surface; placement of the hilum and micropyle; and presence of accessory structures of the seed coat such as arils, caruncle, or oil bodies. Physical features of the embryo such as its size, shape, position, and the number and appearance of the cotyledons, as well as the presence and appearance of accessory nutritive tissues such as the remnants of a megagametophyte (in gymnosperms), perisperm (nucellus), or endosperm are also diagnostic of the species. Histological features of the seed coat and other structures and tissues of the seed may also be used for species identification.

#### Microtechnique

Histological analysis of botanical specimens can be performed on whole plant material or plant powder. The use of cytological stain or other reagents may be necessary to visualize certain histological features. Crossed polarizers can be used to detect structures that rotate plane-polarized light. These structures include starch grains, calcium oxalate crystals, some fibers, and grains of sand (present as a contaminant) that can be observed as bright objects against a dark background. One polarizer is commonly placed in the condenser or the light source, and the second polarizer is placed in the ocular. Light entering the slide from below is plane polarized, permitting only some light waves in a specific plane to pass through.

When the two polarizers are aligned, the field becomes bright; and when the two polarizers are crossed, the field becomes dark.

#### procedure for temporary mounts and powdered material

General Procedure— Plant samples are observed under the microscope by employing different mounting media, stains, or other solutions to assist in the correct identification of the test article. If a USP Authenticated Reference Material is available, prepare it with the same mounting media or reagent solutions used for the test article. Place one or two drops of water, Glycerin-Alcohol Solution, Chloral Hydrate Solution, or another reagent solution (see Preparation and Use of Reagent Solutions, Optical Devices, and Mountants) in the center of a clean slide. Transfer a small plant tissue section or a portion of plant powder into the mountant or reagent solution, and cover with a clean coverslip. (For specific preparation techniques, see Preparation of Temporary Mounts and Hand Sections, Maceration, or Preparation of Powdered Material, as appropriate.) To prevent the formation of air bubbles, the coverslip may be carefully placed at an appropriate angle with its edge making the first contact with the slide and then pressed until it covers the specimen. Using a piece of filter paper, remove excess fluid from the margin of the coverslip. Air bubbles can be removed by placing the slide in a vacuum desiccator. When using chloral hydrate, air bubbles can be removed by gently boiling the sample over a small flame such as that from an alcohol lamp. To replace the mountant or reagent solution, place drops of the new mountant or reagent solution on one edge of the coverslip. Place a strip of filter paper at the opposite edge of the coverslip to remove the old mountant or reagent solution and to cause the new mountant or reagent solution to be drawn over the powdered material or tissue. Plant oils can be also washed away from the tissue in this manner, when solvent hexane or acetone is washed through the slide followed by water and, if necessary, Chloral Hydrate Solution. Do not use Chloral Hydrate Solution immediately after treating the plant tissue with flammable solvents without thoroughly washing the tissue with water. This is to avoid setting fire to residual solvent when the microscope slide is later placed over a small flame to boil the tissue. Care must be taken when using reagent solutions that are volatile or corrosive to the microscope. To prevent drying of aqueous or chloral hydrate solutions during observation, add a small drop of glycerin to the slide. Observe the mounted sample under an optical microscope (see [Optical Microscopy](#) 776), and examine for histological features.

Preparation and Use of Reagent Solutions, Optical Devices, and Mountants— The following reagents, optical devices, and mounting media are used to assist in the identification of cells, tissues, structural features, and ergastic substances in the tissue or powdered material (see [Tables 1](#) and [2](#)).

Table 1. The Use of Reagent Solutions and Optical Devices

Detection	Reagent Solutions and Optical Devices
Calcium carbonate concretion	Diluted Acetic Acid
Calcium oxalate crystals	Crossed Polarizers
Cellulose	Carmine Alum-Methyl Green Solution Hydriodic Acid Zinc Chloride-Iodine Solution
Cytoplasm	Alcoholic Picric Acid Solution
1,8-Dihydroxyanthra-quinones	1 M Potassium Hydroxide Solution
Essential oils	Osmium Tetroxide Solution Sudan III Solution
Inulin	Naphthol-Sulfuric Acid Solution

Lignin	Carmine Alum–Methyl Green Solution Phloroglucinol–Hydrochloric Acid Solution Universal Reagent
Lipids (cutin, waxes, and suberin included)	Carmine Alum–Methyl Green Solution Osmium Tetroxide Solution Sudan III Solution Universal Reagent
Pectin and mucilage	Ruthenium Red Solution Thionine Solution Toluidine Blue Solution
Phytoglycogen	Ruthenium Red Solution
Protein bodies	Alcoholic Picric Acid Solution Osmium Tetroxide Solution
Saponin	Blood–Gelatin Mixture Iodine–Glycerin Solution (confirm by testing with Blood–Gelatin Mixture)
Starch	Crossed Polarizers Iodine Solution Universal Reagent
Tannins and other polyphenols	Ferric Chloride Solution Osmium Tetroxide Solution

Table 2. Bleaching and Clarifying Agents and Mountants

Use	Mountants and Agents
Bleaching Agents	Sodium Hypochlorite Solution
Clarifying Agents	Chloral Hydrate Solution Lactochloral Solution Lactophenol Solution
Mountants	Glycerin Glycerin–Alcohol Solution Glycerin–Gelatin Mixture Water

Alcoholic Picric Acid Solution— Prepare a 1% solution of picric acid in alcohol. Picric acid is useful to stain cells having dense cytoplasm, such as aleurone cells in seeds. Place a small amount of powdered plant material in a test tube, and shake with about 1 mL of solvent hexane to remove plant oils, which would interfere with the reaction. Centrifuge, and discard solvent hexane. Soak the plant powder in Alcoholic Picric Acid Solution for about 30 minutes. Transfer a portion of the powder to a microscope slide, and observe under a microscope: cytoplasm and protein bodies turn bright yellow. [Caution—Picric acid is explosive when dry. Handle appropriately. ]

Blood–Gelatin Mixture— Add 4.5 g of gelatin powder to 100 mL of a 0.9% sodium chloride solution, and allow to swell for 30 minutes. Heat the gel, while stirring, to about 80° in a water bath. Cool to 40°, and add 6 mL of defibrinated bovine blood. Heat to 45° to 50°, and pour onto a microscope slide in a thin layer of about 1 mm while the slide is in a horizontal position. To prevent loss of blood–gelatin mixture from the sides, seal the microscope slide edge with a 1-cm wide adhesive tape to form a tray. After cooling and solidification, it is ready for use. [note—Store in a humid chamber for not more than 1 to 2 days at 3° to 4°.] To test for saponins, place small clusters of the powdered plant material on the blood–gelatin layer, spacing them a few millimeters apart from each other, transfer to a humidifier for a few hours, and observe: saponin-containing particles will cause light-transparent zones to appear in the blood–gelatin.

Carmine Alum–Methyl Green Solution— Boil 1.5 g of carmine for 30 minutes in a 15% solution of aluminum potassium sulfate. Cool, filter, and add 10 mL of a 0.75% methyl green solution while stirring. Add 1 to 2 drops to plant material: lignin and suberin turn green and cellulose turns red-violet.

Chloral Hydrate Solution— Use chloral hydrate TS. When using the solution as a clarifying agent, add a few drops to the plant material, and boil briefly over a small flame. Chloral hydrate dissolves cellular contents and intercellular substances and allows cell walls and shapes to be easily observed. It can be used to assist in the identification of cork, fibers, vessels, calcium oxalate crystals (with the aid of crossed polarizers), trichomes, stomata, and pollen.

Crossed Polarizers— This optical device is used to detect calcium oxalate crystals and starch grains (amyloplasts). In polarized light, calcium oxalate crystals and starch grains appear as bright, birefringent objects on a dark background. Starch grains observed under polarized light will also have a Maltese-cross effect with the arms of the cross intersecting at the hilum. Calcium oxalate crystals are usually best viewed after the sample has been clarified with Chloral Hydrate Solution or another clarifying agent.

Diluted Acetic Acid— Add 1 to 2 drops to the plant material, and immediately observe under a microscope: calcium carbonate deposits dissolve with effervescence.

Ferric Chloride Solution— Dilute 1 mL of [ferric chloride TS](#) with 9 mL of water. For the detection of phenol hydroxyl groups, such as tannins and flavonoids, from the side of the coverslip add the solution to the aqueous sample: tannins and other polyphenols become blue-black to green.

Glycerin— Use as a mountant to prevent the drying of aqueous and chloral hydrate solutions.

Glycerin–Alcohol Solution— Mix equal volumes of glycerin and alcohol. Use as a mounting medium.

Glycerin–Gelatin Mixture— Add 10.0 g of powdered gelatin to 60 mL of water. Allow to stand for 2 hours, and add 70 mL of glycerin containing 1.5 g of dissolved phenol. Heat in a water bath, and filter through a preheated funnel containing glass wool. The filtered mixture is liquefied before use, and it serves as a mounting medium. Add a few drops to the cut or powdered plant material, and cover with a heated coverslip. This preparation is used for long-term storage of specimen mounts. The margins of the coverslip may be sealed with Canada balsam after a few months of drying.

Hydriodic Acid— Add 1 to 2 drops to plant material: cellulosic cell walls become blue to blue-violet.

Iodine Solution— Add 1 to 2 drops of 0.1 N iodine VS to the plant material: starch particles become dark-blue to blue-violet; this reaction is reversible on heating. [note—Proteins, lipids, and cellulose turn yellow to brown; and guaiac powder particles become green to blue, but this reagent is not used for diagnostic identification of these features.]

Iodine–Glycerin Solution— Dissolve 0.3 g of iodine and 1.0 g of potassium iodide in a small quantity of water, and add 10 mL of a mixture of glycerin and water (1:1). Add 1 to 2 drops to the powdered plant material: samples containing saponins form yellow lumps or aggregates. If a sample tests positive for saponin, the result has to be confirmed by testing the sample with Blood–Gelatin Mixture as well.

Lactochloral Solution— Dissolve 50.0 g of chloral hydrate in 50 mL of lactic acid with gentle heating. Add a few drops to the plant material. Place the microscope slide in a small vacuum desiccator if it is necessary to eliminate air bubbles. Chloral Hydrate Solution and Lactochloral Solution are used for the same type of identification, except that Lactochloral Solution is a stronger clarifying agent and it is used for plant material that is more difficult to clarify.

Lactophenol Solution— Mix 20 g of lactic acid, 40 g of glycerin, and 20 mL of water. Add 20 g of phenol, and mix. This is a strong clarifying agent suitable for the examination of pollen



grains.

Naphthol-Sulfuric Acid Solution— Prepare a 20% solution of 1-naphthol in alcohol. To plant material add 1 drop of 1-naphthol solution and 1 drop of sulfuric acid: inulin crystals turn brownish red and then dissolve.

Osmium Tetroxide Solution— Dissolve 0.1 g of osmium tetroxide in 5 mL of distilled water. Add 1 to 2 drops of the solution so obtained to plant material: essential oils, fatty oils and other lipids, tannins, and protein bodies become brown to black.

Phloroglucinol-Hydrochloric Acid Solution— This solution is used for the identification of lignin and other hydroxyphenylpropane derivatives, lignified tissues such as sclereids, vessels, fibers, and stone cells, and lignified parenchyma. Moisten the powder or the cut sample with [phloroglucinol TS](#), and allow to dry for 2 to 3 minutes before placing the coverslip. Add a few drops of a 25% hydrochloric acid solution, and cover with the coverslip. Lignified cell walls turn carmine red. [note—This stain is not stable.] Cells with hydroxyphenylpropane derivatives, such as vanillin and ferulic acid, also turn red. Alternatively, hydroxyphenylpropane derivatives can be extracted from the plant material and the plant material then examined. To extract hydroxyphenylpropane derivatives repeatedly immerse the untreated material in alcohol, mix on a vortex mixer, centrifuge, and discard the alcohol between washings. Then treat the plant material as specified above, beginning with the addition of phloroglucinol TS.

1 M Potassium Hydroxide Solution— Add 1 drop to plant material: cells containing 1,8-dihydroxyanthraquinones will stain red.

Ruthenium Red Solution— Add a few drops of ammonium hydroxide to [ruthenium red TS](#). [note—Store the solution protected from light.] Add 1 to 2 drops to plant material: pectin-containing cell membranes, acidic mucilage, and phytoglycogen turn red.

Sodium Hypochlorite Solution— This solution is used to bleach deeply colored sections. Immerse the plant material in the solution for a few minutes until sufficiently bleached. Wash the tissue with water, and mount with a suitable mounting agent. [note—Sodium hypochlorite will extract lignin; plant tissue so treated will test negative for lignin.]

Sudan III Solution— Dissolve 0.5 g of Sudan III in 50 mL of alcohol or isopropyl alcohol with reflux boiling. Cool, filter, and add 50 mL of glycerin. Add 1 to 2 drops of this solution to plant powder: essential oils, waxes, cutin, suberin, and fatty oils and other lipids combine with this lipophilic colorant and become orange-red to red after a short time.

Thionine Solution— Prepare a 0.2% thionine acetate solution in 25 percent alcohol. Immerse the dry sample in this solution. After about 15 minutes, wash out the excess of stain with 25 percent alcohol: mucilage will have swollen into spherical globules and turned red-violet, while cellulose, pectin, and lignified septa will turn blue or blue-violet.

Toluidine Blue Solution— Using toluidine blue, proceed as directed for Thionine Solution.

Universal Reagent—

solution a— Dilute 20 mL of a lactic acid-saturated solution of Sudan III with 30 mL of lactic acid.

solution b— Dissolve 0.55 g of aniline sulfate in 35 mL of water.

solution c— Dissolve 0.55 g of potassium iodide and 0.05 g of iodine in 5 mL of water, and add 5 mL of alcohol.

procedure— Combine Solution A, Solution B, and Solution C, and add 2.5 mL of hydrochloric acid while stirring. [note—The solution is used without filtering.] For identification, add 2 to 3 drops to the sample, and gently boil over a small flame. If necessary, small amounts of Universal Reagent may be added during boiling. Cover with the coverslip: lignified elements turn yellow; suberin turns red-brown; lipids turn red; and starch turns blue-violet.

Water— Use as a mounting medium. [note—All grades of water are acceptable for this purpose.]

Zinc Chloride-Iodine Solution— Dissolve 20.0 g of zinc chloride and 6.5 g of potassium iodide in 10.5 mL of water. Add 0.5 g of 0.1 N iodine VS, and shake for 15 minutes. Filter if necessary. Store in low-actinic glassware. Add 1 to 2 drops to plant material, and allow to stand for a few minutes: cellulosic cell walls are stained blue to blue-violet.

Preparation of Temporary Mounts and Hand Sections— When using the dry plant tissue, soak or gently boil in water until soft. Do not soften too much. Material can then be treated like fresh plant material. When appropriate, use the mountants or reagent solutions listed for use with plant powder to help visualize features of the tissue (see Preparation and Use of Reagent Solutions, Optical Devices, and Mountants).

To make an epidermal peel of the leaf, petal, sepal, bract, and other leaf-like appendages, roll the tissue into a cylinder, and nick with a sharp, polytef-coated razor blade that has been wetted with water. Grasp nicked piece of tissue with forceps, and strip back removing a clear section of the epidermis. Mount in water on a microscope slide, place a coverslip over the tissue, and examine under a microscope. If it is difficult to obtain an epidermal peel using the above procedure, proceed as follows. Soak the tissue in a 40% to 60% nitric acid solution at 60° for 3 to 4 minutes or until the epidermis can be easily peeled. The peel is then washed three to five times in water to remove the excess of nitric acid. Neutralize the tissue in a 1% potassium hydroxide solution or a 1% sodium hydroxide solution. Wash the tissue again with water, mount in water on a microscope slide, place a coverslip over the tissue, and examine under a microscope.

An alternative method of preparing leaf tissue for the examination of the epidermis is to heat a leaf fragment (about 5 mm × 5 mm) for 15 minutes in Chloral Hydrate Solution on a water bath. Transfer the tissue to a microscope slide, add a drop of water, and cover with a coverslip. These procedures can be used to determine the stomatal type, distribution, number, and index.

Stomatal number is determined by counting the number of stomata per unit area of a microscopic field. Determine the stomatal number on at least 10 different sites of the specimen, and calculate a mean value. Keep track of which leaf surface is being observed, abaxial or adaxial, as the stomatal number for different surfaces is frequently significantly different.

To calculate the stomatal index, the specimen is observed under a microscope at a low magnification. The size of the surface is determined with a calibrated micrometer ocular, and the number of stomata and the number of epidermal cells for that area are determined. The stomatal index is calculated by the formula:

$$100S/(E + S)$$

in which S is the number of stomata for a given area, and E is the number of epidermal cells of the same area. Determine the stomatal index on at least 10 different sites of the specimen, and calculate a mean value. Again, keep track of which leaf surface is being observed, abaxial or adaxial, as the stomatal indices for different surfaces is frequently significantly different.

To make a cross section of a leaf or thin roots, stems, or other thin appendages, lay the appendage to be sectioned on a microscope slide. Place another microscope slide over the appendage with a portion of the tissue exposed. Using a sharp, polytef-coated razor blade that has been wetted, cut straight down along the edge of upper slide. Without moving the upper slide, cut down again with the razor blade at an angle. Some practice may be necessary for one to be able to get sections thin enough so that when they are mounted and covered with a coverslip, these sections can be used to determine tissue arrangements (for instance, the number of palisade layers in leaf, thickness of cuticle, types of trichomes, types of vascular bundles, and the like). Because razor blades dull quickly, they have to be replaced frequently.

Use the cross section of leaf tissue so obtained to determine the palisade mesophyll ratio. Alternatively, boil leaf fragments of about 2 mm<sup>2</sup> in Chloral Hydrate Solution, mount, cover with a coverslip, and observe under a microscope. Identify groups of four adaxial epidermal cells, and count the palisade mesophyll cells that are lying below and are at least 50% covered by the epidermal cells. This value divided by 4 is the palisade mesophyll ratio. Determine the palisade mesophyll ratio of at least 10 groups of epidermal cells, and calculate a mean value. Palisade mesophyll ratio can also be determined on powdered leaf material.

To make a cross section of thick stems, roots, or other plant parts, including woody tissues, hold the tissue in one hand and using a sharp, polytef-coated razor blade that has been wetted with water, shave a cross section from the appendage. Mount in water, another medium, or reagent solution, place a coverslip over the material, and examine under a microscope. Sections thin enough to determine vascular tissue arrangement, ray type, parenchyma distribution, presence of crystals, and the like can usually be made with a little practice.

Maceration— It is sometimes necessary, for the proper identification of a plant material, to macerate the tissue into its individual cells before microscopic examination. This can be an especially useful technique for woody or other hard tissues. The material is cut into small pieces of about 2-mm thickness and 5-mm length or sliced into pieces of about 1-mm thickness. Depending on the nature of the cell wall, one of the following methods is used. For hard or highly lignified tissues, use Method I. For tissues that are not extensively lignified, use Method II.

**Method I—**

solution a—Use 4 N nitric acid solution.

solution b—Prepare a mixture of 1.2 M chromium trioxide solution and sulfuric acid (7:4).

procedure—Place the plant material in a test tube containing about 5 mL of a mixture of Solution A and Solution B (1:1). Heat in a water bath for 20 minutes. Wash the tissue repeatedly with water, and transfer to a microscope slide. Tease tissue apart with dissecting needle, add 1 to 2 drops of mountant, cover with a coverslip, and examine under a microscope. If necessary, cells can be further separated from each other by pressing down on the coverslip with a gentle, sliding motion. The macerated tissue will test negative for lignin.

**Method II—**

procedure—Place the plant material in a test tube containing about 5 mL of 2 M potassium hydroxide solution. Heat in a water bath for 30 minutes. Wash the tissue repeatedly with water, and transfer to a microscope slide. Add 1 to 2 drops of mountant. Place a coverslip over the tissue, press down, squashing the tissue, and examine under a microscope. The macerated tissue will test negative for lignin.

**Preparation of Powdered Materials**—Place one or two drops of water, another mountant, or a reagent solution in the center of a clean slide. Moisten the tip of a dissecting needle with water, and dip into the powder under test. Transfer a small amount of material that adheres to the needle into the fluid on the slide, and stir thoroughly and carefully. Cover with a clean coverslip. Because the arrangement of the tissue structures within the plant tissue has been destroyed, the important features for observation of the powdered plant material are the chemical and physical features of tissues and cell types, as well as the presence and chemical and physical features of ergastic substances. The specific tissues, cells, and ergastic substances to be examined are specified in the individual monograph.

**procedure for thin, permanent mounts**

When it is necessary to reveal detailed histological features of a plant specimen, thin tissue sections have to be obtained. The sections need to be thin enough to transmit light and they have to be cut in such a plane that the desired features are exposed. The plant material is properly killed, fixed, dehydrated, and embedded in paraffin or other embedding media. The embedding medium is used as a solid-support matrix during tissue sectioning. After sectioning and mounting, staining of the specimen is frequently performed to aid in the differentiation of certain structures. [note—The process of fixing, dehydration, embedding, and staining can be significantly expedited by utilizing a microwave oven specifically designed for histological work.]

**Killing and Fixation**—As a first step in preparing plant material for sectioning, living cells are killed, and the tissue is preserved. This is most frequently done by employing a chemical fixative. A good general purpose fixative for plant material is a mixture of formaldehyde, acetic acid, and alcohol (FAA).

**FAA Solution**—Mix 50 mL of alcohol, 5 mL of glacial acetic acid, 10 mL of formaldehyde solution, and 35 mL of water. [note—Periodically prepare fresh solution, as it loses effectiveness with storage.]

**Procedure**—Completely immerse the plant material in FAA Solution. Allow the material to remain immersed for 18 to 24 hours at room temperature. Plant material can be kept indefinitely in FAA Solution, as long as it remains completely immersed and is not allowed to dry out. Certain plant tissues may require vacuum infiltration to facilitate the penetration of the fixative. Vacuum infiltration is required if the tissue has abundant air spaces or epidermal hairs or if it floats on top of the fixative solution. Place the tissue in a small vial containing the fixative. Place the uncapped vial into a bell jar or desiccator that is connected to a vacuum source, preferably an oil-sealed vacuum pump. The vacuum is vented into a fume hood to prevent fixative vapors from filling the room. Slowly turn on the vacuum. Do not use a strong vacuum because the fixative may start to boil and damage the tissue. As residual air is pulled from the tissue, it will rise to the surface. Turn the vacuum on and off through several cycles until the tissue stays at the bottom of the container during an "on" cycle.

**Tissue Dehydration**—Paraffin and other embedding media are hydrophobic; thus, water must be removed from the plant tissue after fixation. This is accomplished by immersing the fixed tissue in dehydration solutions, which are a series of mixtures of alcohol and water with increasing alcohol concentration. The final solution in the series is dehydrated alcohol. Begin by washing the fixed tissue once or twice with fresh 50 percent alcohol to remove traces of FAA. Remove this solution, and subsequently remove any other dehydration solution, by decanting the solution or removing it with the aid of a glass pipet. Add the first dehydration solution (70 percent alcohol) to the vial, completely immersing the tissue. The graded alcohol-water series and the suggested times for tissue immersion are as follows.

Dehydration Solution	Time (hours)
50 percent alcohol	1–2
70 percent alcohol	1–2
90 percent alcohol	1–2
95 percent alcohol	1–2
Dehydrated alcohol containing 0.1% of safranin O	
safranin O	2–4
Dehydrated alcohol	1

Safranin O is added to the penultimate dehydration solution in the series to visualize the tissue when it has become embedded in paraffin. If the tissue to be sectioned is hard or woody, the time for each step in the series may need to be increased to up to 24 hours. If necessary, the tissue can be stored for several days in 70 percent alcohol or in solutions of even higher alcohol concentrations.

**Embedding—****Preparation for Embedding—**

**alcohol removal**—Paraffin is the most common embedding medium, although other embedding media are available. After dehydration, alcohol is removed from the tissue by using a graded series of dehydrated alcohol-xylene solutions, because paraffin is not soluble in alcohol. The graded dehydrated alcohol-xylene series and the suggested times for tissue immersion are as follows.

Alcohol Removal Solution	Time (hours)
A mixture of dehydrated alcohol and xylene (3:1)	1
A mixture of dehydrated alcohol and xylene (1:1)	1
A mixture of dehydrated alcohol and xylene (1:3)	1
Xylene	1
Xylene	1

**xylene removal**—Once xylene has completely replaced alcohol, paraffin is added slowly to infiltrate the tissue and remove xylene. Proceed as follows:

1. For each mL of xylene add about 1 paraffin chip to the tissue vial, cap, and allow to stand at room temperature for 4 hours. Add additional paraffin chips until no more chips dissolve.
2. Place tissue in an oven maintained at 42° to 45°. Add 2 to 3 paraffin chips every hour until no more chips dissolve at that temperature.



3. Pour off one-third of the volume, and replace with an equal volume of melted paraffin. Do not cap, and transfer vial to an oven maintained at 58° to 60°.
4. After the paraffin remelts (about 4 hours later) pour off one-half of the volume, and replace with equal volume of melted paraffin. Transfer vial to the oven maintained at 58° to 60° if paraffin begins to solidify.
5. Repeat step 4 twice more, then pour off the entire volume of paraffin-xylene. Replace with pure melted paraffin. About 4 hours later, pour off paraffin, and replace with fresh pure melted paraffin. Repeat the pouring off and replacement 4 hours later, and allow to stand overnight. [note—Transfer vial to the oven maintained at 58° to 60° if paraffin begins to solidify at any point.]

**Embedding Procedure**— Pour the tissue with the paraffin to an embedding boat. Paraffin has to completely cover the tissue by about 3 to 5 mm. Place the embedding boat on top of a preheated warming platform that is designed for histology work. Adjust the tissue in the boat to its proper orientation for sectioning. Slowly cool the paraffin by sliding the boat down to the cool side of the platform until the paraffin has solidified. Immerse the paraffin block in ice water to rapidly cool the block and to prevent paraffin crystals from forming. Store the paraffin block at 4°.

**Sectioning and Mounting**— Cut the paraffin block into pieces, each containing one tissue sample. Trim the paraffin block, as close to the tissue mass as possible, to form a rectangle or a slight trapezoid. Such trimming will prevent sectioning problems due to excess paraffin around the tissue. To make transverse sections, orient the tissue at a right angle to a wooden tissue block whose face has been soaked in melted paraffin. Affix the paraffin block to the face of the tissue block. Add a small amount of melted paraffin to the base of the paraffin block to help form a tighter seal. Cool the block to 4°.

Properly mount and adjust the tissue and paraffin block in a microtome. Use a sharp stainless-steel microtome knife that has been properly honed. Set the microtome to cut sections 8 to 15 µm thick (10-µm thickness is optimal for most tissues). Cut individual or serial sections. Prepare a microscope slide as follows. An adhesive may be prepared as a solution containing 1% of gelatin and 0.5% of sodium benzoate that is heated to 30° to 35° to dissolve the gelatin. Smear a thin film of the adhesive so obtained onto the slide, allow to dry, rinse with a 4% solution of formaldehyde TS, and add a small amount of water. Plate the cut sections upside down on the slide, so that they float on water, and flood with a 4% solution of formaldehyde TS. The sections will immediately spread out and wrinkles will disappear.

Place the slide on a warming platform, maintained at 42°, to relax the sections. Pipet, and blot the excess water and formaldehyde solution. Dry overnight in an oven at 42° to ensure adherence of the tissue section to the slide.

#### Staining—

**Preparation for Staining**— Immerse the microscope slide with the affixed tissue twice into xylene, each time for 10 to 15 minutes, to remove paraffin. Then immerse the slide into several solutions, leaving it in each solution for 5 minutes and taking care not to dislodge the tissue, the following sequence of solutions being used: a mixture of dehydrated alcohol and xylene (1:1), dehydrated alcohol, alcohol, and a 70 percent alcohol solution. The tissue is bleached prior to staining if it is opaque because of the presence of tannins or other ergastic materials. To bleach, dip the slide into a 1% potassium permanganate solution for 1 minute, rinse with water, dip into a 5% oxalic acid solution for 1 minute, and rinse thoroughly with water. The material is ready for staining. One of the following two staining procedures is recommended for most botanical identification work. The first staining procedure uses safranin O counterstained with fast green. An alternative staining procedure uses safranin O counterstained with orange G.

#### Safranin O-Fast Green Staining—

**safranin o staining solution**— Prepare a mixture of methoxyethanol, dehydrated alcohol, water, and formaldehyde solution (50:25:25:2). Add a sufficient quantity of sodium acetate to obtain a solution containing 1% of sodium acetate, and mix. Add a sufficient quantity of safranin O to obtain a solution containing 1% of safranin O, and mix.

**fast green staining solution**— Prepare a mixture of methoxyethanol, dehydrated alcohol, and methyl salicylate (1:1:1) containing 0.05% of fast green FCF.

**procedure**— Once the tissue has been rehydrated to 70 percent alcohol as described under Preparation for Staining, immerse for 2 to 24 hours, depending on the tissue, in Safranin O Staining Solution. Remove excess stain by immersing the slide in water several times. Transfer slide to an alcohol solution containing 0.5% of picric acid for 2 to 10 seconds to further remove excess stain from the section and to assist in differentiation of the tissue structures. To stop the action of the picric acid, transfer slide for 10 seconds to 1 minute to an alcohol solution containing 4 drops of ammonium hydroxide in each 100 mL of alcohol. Transfer slide to dehydrated alcohol for 10 seconds. Visually inspect the stained tissue under a microscope to see if further destaining with picric acid is necessary. Counterstain for 10 to 15 seconds in Fast Green Staining Solution. Transfer slide through two changes of a mixture of methyl salicylate, dehydrated alcohol, and xylene (2:1:1), each change lasting for 5 to 10 seconds. Then transfer slide to a mixture of xylene and dehydrated alcohol (95:5) for 1 minute. Transfer through two changes of xylene. Store in xylene until ready to mount the coverslip. Chromosomes, nuclei, and lignified, cutinized, or suberized cell walls will be stained red. Cytoplasm and cellulosic cell walls will be stained green to blue, depending on the pH of the tissue.

#### Safranin O-Orange G Staining—

**safranin o staining solution**— Prepare a 0.004% solution of safranin O.

**orange g staining solution**— Dissolve 2 g of orange G, 5 g of tannic acid, and 4 drops of hydrochloric acid in water, and dilute with water to 100 mL.

**procedure**— Once the tissue has been rehydrated to 70 percent alcohol as described under Preparation for Staining, sequentially transfer slide through the following series of solutions.

Solution	Time
35 Percent alcohol	5 minutes
A filtered 2% zinc chloride solution	1 minute
Water	5 seconds
Safranin O Staining Solution	5 minutes
Water	5 seconds
Orange G Staining Solution	1 minute
Water	5 seconds
A filtered 5% tannic acid solution	5 minutes
Water	3 seconds
A 1% ferric ammonium sulfate solution	2 minutes
Water	15 seconds
45 Percent alcohol	10 seconds
90 Percent alcohol	10 seconds
Dehydrated alcohol	10 seconds
A mixture of dehydrated alcohol and xylene (1:1)	1 to 2 minutes

Finally, store in xylene until ready to mount the coverslip. Cellulosic cell walls will stain blue-black, nuclei will stain yellow, starch grains will stain black, and lignified cell walls will stain red.

**Mounting the Coverslip**— The mounting of a coverslip over the tissue completes the preparation of the slide. Canada balsam, diluted with a small portion of xylene, can be used as an adhesive. Other mountants are also commercially available. Upon drying of the mountant, the slide can then be examined under a microscope. The entire process of making permanent microscope slides can be expected to take 5 or more days.



## CHEMICAL IDENTIFICATION

To help ensure the authenticity of the article, chemical identification is performed in conjunction with botanical identification outlined above. Chemical identification typically employs chromatographic procedures to detect the presence of marker compounds specified in the individual monograph. Spectroscopic or chromatographic profiles can be used to achieve chemical identification by fingerprint comparison against that of a reference sample or standard. Examples of spectroscopic method include UV, IR, and Fourier transformed IR (see [Spectrophotometric Identification Tests](#) (197)). Examples of chromatographic methods include high-pressure liquid chromatography (HPLC), thin-layer chromatography (TLC), 2-dimensional-TLC, and gas chromatography (see [Chromatography](#) (621)). Analytical methods used for fingerprinting should be capable of detecting as many chemical constituents as possible. Multiple fingerprints, using a combination of analytical methods with different separation principles and test conditions, may be useful. In addition to the spectroscopic chromatographic methods, qualitative wet-chemistry methods may also be specified in the individual monograph.

### Chemotaxonomy

Chemotaxonomy is the classification of the plants based on their chemical constituents and it may be useful in botanical articles identification. Metabolic compounds found within plant tissues can be divided into two broad categories based on their functions. The first category comprises primary metabolites—metabolites involved in the physiological plant processes that are absolutely necessary for life and ubiquitous throughout the plant kingdom. These processes include photosynthesis, respiration, and nucleic acid, protein, carbohydrate, and lipid metabolism. The second category comprises secondary metabolites—compounds that are thought not to be absolutely necessary for plant processes, although they may have important functions in the plant's interactions with other organisms, such as allelopathic interactions; in chemical defense against herbivores and plant pathogens; and in signaling to attract pollinating and seed-dispersing animals. Many secondary metabolites are known to have pharmacological activity. They are also the basis for the chemotaxonomy of plants.

Secondary metabolites fall into several different chemical classes such as nonprotein amino acids, flavonoids, xanthones, coumarins, polyacetyles, cyclic polyketides, monoterpenes, sesquiterpenes, iridoids, triterpenes, sterols, nitrogen-containing terpenes, and alkaloids. These chemical classes are not ubiquitous throughout the plant kingdom, but tend to be specific to certain botanical classes, orders, and families. Moreover, many chemical subclasses and individual secondary compounds are specific to certain subfamilies, genera, or species. It is these chemical subclasses and individual compounds that can be used as marker compounds to aid in the proper identification of plant material.

### Active Principles and Marker Compounds

For chemical identification of botanical articles, extracts are prepared. Such extracts are usually complex mixtures of several chemical constituents. For a large majority of botanical extracts it is not known with certainty which of the various components is responsible for the reported pharmacological effect. It is generally believed that several constituents act synergistically to provide the reported effect. For articles for which compendial monographs are provided, certain chemical constituents of the article are chosen and quantitative test procedures for determining their content are provided. The choice of such constituents, known generally as marker compounds, is based on certain considerations. Currently, the following types of marker compounds are specified in compendial monographs and may be identified in raw materials:

**Active Principles**— These are constituents that have proven clinical activity. A minimum content or range for the active principles is usually specified in the individual monograph. A quantitative determination of active principles during stability studies of botanical dosage forms provides necessary information for arriving at suitable expiration dates.

**Active Markers**— These are constituents that have known pharmacological activity contributing in some extent to efficacy. However, the clinical efficacy for these constituents may not be proven. A minimum content or range for active markers is usually specified in individual monographs. A quantitative determination of active markers during stability studies of botanical dosage forms provides necessary information for arriving at suitable expiration dates.

**Analytical Markers**— Where neither defined active principles nor active markers are known, other constituents of the botanical extract amenable to quantitative determination are chosen. These markers aid in the positive identification of the article under test. In addition, maintaining a minimum content or a specified range of the analytical markers helps to achieve standardization of the plant extract and to arrive at a suitable expiration date during stability studies.

**Negative Markers**— These are constituents that may have allergenic or toxic properties, rendering their presence in the botanical extract undesirable. For example ginkgolic acids from ginkgo belong to this category. A stringent limit for these negative markers may be specified in individual monographs.

### Use of USP Reference Articles

Reference articles are used to assist in the identification of marker compounds within the test article. Reference articles are either USP Authenticated Reference Materials or USP Reference Standards (see [USP Reference Standards](#) (11)), whichever is specified in the individual monograph. USP Reference Standards used to identify marker compounds in the test articles may be a single purified chemical entity, a mixture of purified chemical entities, or a standardized extract prepared from the authenticated plant article. USP Reference Standards may also be used to quantitate marker compounds, as specified in the individual monograph.

A pulverized test article undergoes a specified extraction procedure (see [Methods of Extraction under Botanical Extracts](#) (565)) and is prepared for chromatographic or wet-chemistry analysis. If a USP Authenticated Reference Material is available, then it undergoes the same extraction procedure as the test article. The test preparation and reference articles then undergo the same chromatographic or wet-chemistry procedure specified in the individual monograph. The response of the test preparation is compared to the response of reference articles to determine the presence of the marker compounds in the test article.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Maged H. Sharaf, Ph.D.</a> Senior Scientist 1-301-816-8318	(DSB05) Dietary Supplements - Botanicals

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### 565 BOTANICAL EXTRACTS

In the extraction practice for articles of botanical origin, the constituents of interest are completely or partially separated from other components with the aid of water, alcohol, alcohol-water mixtures, or other suitable solvents. This extraction process involves the removal of the desired constituents from the plant matter with suitable menstrua, the evaporation of all or nearly all of the solvent, and the adjustment of the residual fluids, masses, or powders to the prescribed standards. Suitable inert substances may be added as carriers or diluents to improve physical characteristics. Suitable antimicrobials and other preservatives may be added to preserve the integrity. Extracts may be subjected to processes that increase the content of characterized constituents, decrease the content of unwanted constituents, or both. Extracts with no added inert substances and no processing beyond the extraction are called native extracts. In some preparations, the plant matter may be pretreated by inactivation of enzymes and microbial contaminants, grinding, defatting, or a similar procedure. Extracts may be defined as preparations with liquid, solid, or semisolid consistency. The products obtained by extraction are fluidextracts, powdered extracts, semisolid extracts, and tinctures.

## METHODS OF EXTRACTION

### Percolation

In the manufacture of extracts, percolation is a commonly used method. The crude material being extracted is reduced to pieces of suitable size, if necessary, then mixed thoroughly with a portion of the specified solvent, and allowed to stand for about 15 minutes. The mixture is transferred to a percolator, sufficient amount of the specified solvent is added to cover the entire solid mass, and the mixture is allowed to percolate slowly (at a rate of not more than 1 mL per minute for 1000 g of material), the matter to be extracted being always covered with a layer of solvent. The residue may be pressed, and the obtained fluid is combined with the percolate. The entire percolates are concentrated, generally by distillation under reduced pressure, so as to subject the constituents of interest in the article under extraction to as little heat as possible.



## Maceration

Unless otherwise specified, the crude material being extracted is reduced to pieces of suitable size, mixed thoroughly with the specified extracting solvent, and allowed to stand at room temperature in a closed container for an appropriate time, with frequent agitation until soluble matter is dissolved. The mixture is filtered, the insoluble material is washed with the same solvent used for maceration, and the filtrates are combined and concentrated, usually under reduced pressure, to the desired consistency.

## PREPARATIONS

### Fluidextracts

Fluid extracts, also known as liquid extracts, are preparations of plant matter, containing alcohol as a solvent or as a preservative, or both, and are so made that each mL contains the extracted constituents of 1 g of the crude material that it represents, unless otherwise specified in the individual monograph. They may be prepared from suitable extracts and may contain suitable antimicrobial or other preservatives.

Pharmacopeial fluidextracts are made by percolation, often following a period of maceration. The required solvent is specified in the individual monograph. The common manufacturing procedure includes concentration of the more diluted portion of percolate by evaporation or distillation under vacuum at temperatures below 60°. The time of maceration and the rate of flow during percolation may be varied to adjust for the quantity and nature of the crude material under extraction, provided that the composition of the extracted constituents of interest is not adversely affected.

The rate of flow of the percolate can be slow, moderate, or rapid. With reference to the extraction of 1000 g of the starting material, at a slow rate, not more than 1 mL of percolate is produced per minute; at a moderate rate, between 1 and 3 mL per minute is produced; and at a rapid rate, between 3 and 5 mL per minute is produced. A fluidextract that tends to deposit sediment may be aged and filtered, or the clear portion may be decanted, provided that the resulting clarified liquid conforms to the Pharmacopeial standards.

### Powdered Extracts

Powdered extracts are solid preparations having a powdery consistency obtained by evaporation of the solvent used for extraction. They may contain suitable added substances such as excipients, stabilizers, and preservatives. Standardized powdered extracts are adjusted to the defined content of constituents, using suitable inert materials or a powdered extract of the plant matter used for preparation. Where applicable, a limit for the solvent used for extraction is specified in the individual monograph.

### Semisolid Extracts

Semisolid extracts, also known as soft extracts or pillular extracts, are preparations having consistencies between those of fluidextracts and those of powdered extracts, and are obtained by partial evaporation of the solvent, water, alcohol, or hydroalcoholic mixtures being used as extracting solvents. They may contain suitable antimicrobial or other preservatives. A semisolid extract and a powdered extract obtained from the same material are interchangeable as drugs or as supplements, but each has its own advantages.

### General Pharmacopeial Requirements

Unless otherwise specified in the individual monographs, Pharmacopeial requirements for the fluidextracts, powdered extracts, and semisolid extracts are as follows.

**Packaging and Storage**— Store in tight, light-resistant containers. [note—See Preservation, Packaging, Storage, and Labeling under General Notices and Requirements.]

**Labeling**— Label it to indicate the name of the plant part used; the names of solvents, other than the hydroalcoholic solvents, used in preparation; the content, in percentage, of active principles or marker compounds identified in the individual monograph; and the name and concentration of any added antimicrobial or other preservative. Where active principles are unknown, the ratio of starting material to final product is stated. For semisolid extracts and powdered extracts, the identity and quantity of any added excipient is also indicated. In such cases the percentage of native extract may also be stated.

**Residue on Evaporation**— Transfer promptly about 2 mL, accurately measured, of Fluidextract, about 0.5 g of Powdered Extract, or about 2 g of Semisolid Extract to a suitable tared, round-bottom flask. Evaporate to dryness on a water bath, and dry the residue at 100° to 105° for 3 hours. Allow to cool in a desiccator over phosphorus pentoxide, and determine the weight of the residue obtained: not less than 95% of Powdered Extract specimen remains as residue; or not less than 70% of Semisolid Extract specimen remains as residue. [note—Limits for Fluidextracts are specified in the individual monographs.]

**Residual Solvents**— If prepared with solvents other than alcohol, water, or alcohol-water mixtures, it meets the requirements for [Residual Solvents](#) (467). [note—See ICH document Impurities: Residual Solvents for related information.]

**Pesticide Residues**— Proceed as directed under [Articles of Botanical Origin](#) (561): meets the requirements.

[Heavy Metals, Method II](#) (231): 20 µg per g.

[Alcohol Content, Method II](#) (611) (if present): between 90% and 110% of the labeled amount of C2H5OH is found in Fluidextract and Semisolid Extract.

### Tinctures

Tinctures are liquid preparations usually prepared by extracting plant materials with alcohol or hydroalcoholic mixtures. Traditionally, tinctures of potent articles of botanical origin represent the activity of 10 g of the drug in each 100 mL of tincture, the strength being adjusted following the test for content of active principles or marker compounds. Most other plant tinctures represent 20 g of the respective plant material in each 100 mL of tincture.

Different tinctures are not always diluted to obtain the same ratio of starting plant material to final tincture. This ratio will depend on the requirements prescribed in the specific tests for content of active principles or marker compounds included in the individual monographs. As tinctures are being prepared, they are assayed in accordance with these content tests.

Using the values obtained from such assays, the final concentration of a tincture is adjusted by adding more solvent or by evaporating part of the solvent.

Unless otherwise specified, tinctures are usually prepared from coarse powder or fine cuttings of plant materials either by a percolation process or a maceration process.

### percolation process

Carefully mix the ground mixture of ingredients with a sufficient quantity of the prescribed extracting solvent to render it evenly and distinctly damp, allow it to stand for 15 minutes, transfer it to a suitable percolator, and pack the mass firmly. Pour on enough of the specified extracting solvent to saturate the drug, and cover the top of the percolator. When the liquid is about to drip from the percolator, close the lower orifice, and allow the drug to macerate for 24 hours or for the time specified in the monograph. If the test for content of active principles or marker compounds is not required in the individual monograph, allow the percolation to proceed slowly or at the specified rate (for definitions of flow rates, see under [Fluidextracts](#)), gradually adding sufficient quantity of extracting solvent to produce 1000 mL of tincture, and mix. If a test for content of active principles or marker compounds is required, collect only 950 mL of percolate, mix, and test a portion of it as directed in the individual monograph. Dilute the remainder of the percolate with as much of the prescribed extracting solvent as calculation from the content test indicates is necessary to produce a tincture that conforms to the requirements, and mix.

### maceration process

Macerate the drug with 750 mL of the prescribed extracting solvent in a closed container, and put in a warm place. Agitate it frequently during 3 days or until the soluble matter is dissolved. Transfer the mixture to a filter. When most of the liquid has drained, wash the residue on the filter with a sufficient quantity of the prescribed extracting solvent, combining the filtrates, to produce 1000 mL of tincture, and mix.

### general pharmacopeial requirements

Unless otherwise specified in the individual monographs, Pharmacopeial requirements for the tinctures are as follows.

**Packaging and Storage**— Store in tight, light-resistant containers, and avoid exposure to direct sunlight and excessive heat. [note—See Preservation, Packaging, Storage, and Labeling under General Notices and Requirements.]



Labeling—Label it to indicate the name of the plant part used for preparation; the name of the solvent or solvent mixture used for extraction; and the content of the constituents of interest and the ratio of starting material to final product.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Maged H. Sharaf, Ph.D.</a> Senior Scientist 1-301-816-8318	(DSB05) Dietary Supplements - Botanicals

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### 571 VITAMIN A ASSAY

#### CHEMICAL METHOD

The following procedure is provided for the determination of vitamin A as an ingredient of Pharmacopeial preparations. It conforms to that which was adopted in 1956 for international use by the International Union of Pure and Applied Chemistry.

Complete the assay promptly, and exercise care throughout the procedure to keep to a minimum the exposure to actinic light and to atmospheric oxygen and other oxidizing agents, preferably by the use of low-actinic glassware and an atmosphere of an inert gas.

##### Special Reagents—

ether—Use ethyl ether, and use it within 24 hours after opening the container.

isopropyl alcohol—Use spectrophotometric-grade isopropyl alcohol (see Isopropyl Alcohol under Reagent Specifications in the section [Reagents, Indicators, and Solutions](#)).

Procedure—Accurately weigh, count, or measure a portion of the test specimen expected to contain the equivalent of not less than 0.15 mg of retinol but containing not more than 1 g of fat. If in the form of capsules, tablets, or other solid, so that it cannot be saponified efficiently by the ensuing instructions, reflux the portion taken in 10 mL of water on a steam bath for about 10 minutes, crush the remaining solid with a blunt glass rod, and warm for about 5 minutes longer.

Transfer to a suitable borosilicate glass flask, and add 30 mL of alcohol, followed by 3 mL of potassium hydroxide solution (9 in 10). Reflux in an all-borosilicate glass apparatus for 30 minutes. Cool the solution, add 30 mL of water, and transfer to a conical separator. Add 4 g of finely powdered sodium sulfate decahydrate. Extract by shaking with one 150-mL portion of ether for 2 minutes, and then, if an emulsion forms, with three 25-mL portions of ether. Combine the ether extracts, if necessary, and wash by swirling gently with 50 mL of water.

Repeat the washing more vigorously with three additional 50-mL portions of water. Transfer the washed ether extract to a 250-mL volumetric flask, add ether to volume, and mix.

Evaporate a 25.0-mL portion of the ether extract to about 5 mL. Without applying heat and with the aid of a stream of inert gas or vacuum, continue the evaporation to about 3 mL. Dissolve the residue in sufficient isopropyl alcohol to give an expected concentration of the equivalent of 3 µg to 5 µg of vitamin A per mL or to give an absorbance in the range 0.5 to 0.8 at 325 nm. Determine the absorbances of the resulting solution at the wavelengths 310 nm, 325 nm, and 334 nm, with a suitable spectrophotometer fitted with matched quartz cells, using isopropyl alcohol as the blank.

when tocopherol is present—Transfer to a suitable borosilicate glass flask a test specimen, accurately measured, or not less than 5 previously crushed capsules or tablets. Reflux in an all-borosilicate glass apparatus with 30 mL of alcohol and 3 mL of potassium hydroxide solution (9 in 10) for 30 minutes. Add through the condenser 2.0 g of citric acid monohydrate, washing the walls of the condenser with 10 mL of water. Cool, and transfer the solution to a conical separator with the aid of 20 mL of water. Add 4 g of finely powdered sodium sulfate decahydrate. Extract with one 150-mL portion of ether and then, if an emulsion forms, with three 25-mL portions of ether. Combine the ether extracts, if necessary, and wash by swirling gently with 50 mL of water. Repeat the washing more vigorously with three additional 50-mL portions of water. Transfer the washed ether extract to a 250-mL volumetric flask, add ether to volume, and mix.

Evaporate a 50.0-mL aliquot of the ether solution of the unsaponifiable extract to about 5 mL. Without applying heat and with the aid of a stream of inert gas or vacuum, remove the residual ether. Dissolve the residue in 50.0 mL of isopropyl alcohol.

Hydrogenated Portion—Pipet 15.0 mL of the isopropyl alcohol solution into a 50-mL centrifuge tube, add approximately 200 mg of palladium catalyst, stir with a glass rod, and hydrogenate for 10 minutes in a Hydrogenator such as is described in the [Alpha Tocopherol Assay](#) (551), using isopropyl alcohol in the blank tube. Add about 300 mg of chromatographic siliceous earth, stir with a glass rod, and immediately centrifuge until the solution is clear.

Test a 1-mL aliquot of the solution by removing the solvent by evaporation, dissolving the residue in 1 mL of chloroform, and adding 10 mL of [phosphomolybdic acid TS](#): no detectable blue-green color appears. [note—If a blue-green color appears, repeat the hydrogenation for a longer time period, or with a new lot of catalyst.]

Into two separate flasks pipet equal volumes of the Hydrogenated Portion and the untreated isopropyl alcohol solution, respectively, and add sufficient isopropyl alcohol to give an expected concentration of vitamin A equivalent to 3 µg to 5 µg per mL. Determine the absorbances of the untreated solution against the solution from the Hydrogenated Portion as a blank, at the wavelengths 310 nm, 325 nm, and 334 nm, with a suitable spectrophotometer fitted with matched quartz cells.

Calculation—Calculate the vitamin A content as follows:

$$\text{Content (in mg)} = 0.549A325 / LC$$

in which A325 is the observed absorbance at 325 nm; L is the length, in cm, of the absorption cell; and C is the amount of test specimen expressed as g, capsule, or tablet in each 100 mL of the final isopropyl alcohol solution, provided that A325 has a value not less than [A325]/1.030 and not more than [A325]/0.970, where [A325] is the corrected absorbance at 325 nm and is given by the equation:

$$[A325] = 6.815A325 - 2.555A310 - 4.260A334$$

in which A designates the absorbance at the wavelength indicated by the subscript.

Where [A325] has a value less than A325/1.030, apply the following equation:

$$\text{Content (in mg)} = 0.549[A325] / LC$$

in which the values are as defined herein. Each mg of vitamin A (alcohol) represents 3333 USP Units of vitamin A.

Confidence Interval—The range of the limits of error, indicating the extent of discrepancy to be expected in the results of different laboratories at P = 0.05, is approximately  $\pm 8\%$ .

#### CHROMATOGRAPHIC METHOD

The following pressurized liquid chromatographic procedure is provided for the determination of Vitamin A. Where the use of vitamin A ester (retinyl acetate or retinyl palmitate) is specified in the following procedure, use the chemical form present in the raw material. Use low-actinic glassware throughout this procedure.

[USP Reference Standards](#) (11)—[note—Use [USP Vitamin A RS](#), all-trans retinyl acetate, for assaying pharmaceutical dosage forms that are labeled to contain retinol or vitamin A ester (retinyl acetate or retinyl palmitate).]

Mobile Phase—Use n-hexane.



System Suitability Preparation— Dissolve an accurately weighed quantity of retinyl palmitate and [USP Vitamin A RS](#) in n-hexane to obtain a solution containing about 7.5 µg per mL each.

Standard Preparation— Dissolve an accurately weighed quantity of [USP Vitamin A RS](#) in n-hexane, and dilute quantitatively, and stepwise if necessary, to obtain a solution having a known concentration of about 15 µg of retinyl acetate per mL.

Assay Preparation— Transfer about 15 mg of vitamin A ester (retinyl acetate or retinyl palmitate), accurately weighed, to a 100-mL volumetric flask, dissolve in and dilute with n-hexane to volume, and mix. Pipet 5.0 mL of this solution into a 50-mL volumetric flask, dilute with n-hexane to volume, and mix.

Chromatographic System (see [Chromatography \(621\)](#))—The liquid chromatograph is equipped with a 325-nm detector and a 4.6-mm × 15-cm column that contains packing L8. The flow rate is about 1 mL per minute. Chromatograph the System Suitability Preparation, and record the peak responses as directed for Procedure: the resolution, R, between retinyl acetate and retinyl palmitate is not less than 10; and the relative standard deviation for replicate injections is not more than 3.0%.

Procedure— Separately inject equal volumes (about 40 µL) of the Standard Preparation and the Assay Preparation into the chromatograph, record the chromatograms, and measure the responses for retinyl acetate obtained from the Standard Preparation and the peak area for retinyl acetate or retinyl palmitate in the chromatogram of the Assay Preparation.

Calculate the quantity, in mg, of vitamin A as the retinol equivalent (C<sub>20</sub>H<sub>30</sub>O) in the portion of vitamin A taken by the formula:

$$0.872CD(rU / rS)$$

in which 0.872 is the factor used to convert retinyl acetate, obtained from [USP Vitamin A RS](#), to its retinol equivalent; C is the concentration, in mg per mL, of [USP Vitamin A RS](#) in the Standard Preparation; D is the dilution factor, in mL, for the Assay Preparation; and rU and rS are the peak responses of the retinyl ester obtained from the Assay Preparation and the Standard Preparation, respectively. [note—The molar responses of retinyl acetate and retinyl palmitate are equivalent.]

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Curtis Phinney</a>  1-301-816-8540	(DSN05) Dietary Supplements - Non-Botanicals
Reference Standards	Lili Wang, Technical Services Scientist 1-301-816-8129 <a href="mailto:RSTech@usp.org">RSTech@usp.org</a>	

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### 581 VITAMIN D ASSAY

#### Chromatographic Method

The following pressurized liquid chromatographic procedure is provided for the determination of vitamin D, as cholecalciferol or as ergocalciferol, as an ingredient of Pharmacopeial multiple-vitamin preparations.

Throughout this assay, protect solutions containing, and derived from, the test specimen and the Reference Standard from the atmosphere and light, preferably by the use of a blanket of inert gas and low-actinic glassware.

USP Reference Standards (11)—[note—Use [USP Ergocalciferol RS](#), or [USP Cholecalciferol RS](#), for assaying pharmaceutical dosage forms that are labeled to contain vitamin D as ergocalciferol, or as cholecalciferol, respectively.] USP Cholecalciferol RS. [USP A<sub>4,6</sub>-Cholestadienol RS](#). [USP Ergocalciferol RS](#). [USP Vitamin D Assay System Suitability RS](#).

#### Special Reagents and Solutions—

Ether— Use ethyl ether. Use within 24 hours after opening container.

Dehydrated Hexane— Prepare a chromatographic column by packing a chromatographic tube, 60 cm × 8 cm in diameter, with 500 g of 50- to 250-µm chromatographic siliceous earth, activated by drying at 150° for 4 hours (see Column Adsorption Chromatography under [Chromatography \(621\)](#)). Pass 500 mL of hexanes through the column, and collect the eluate in a glass-stoppered flask.

Butylated Hydroxytoluene Solution— Dissolve a quantity of butylated hydroxytoluene in chromatographic hexane to obtain a solution containing 10 mg per mL.

Aqueous Potassium Hydroxide Solution— Dissolve 500 g of potassium hydroxide in 500 mL of freshly boiled water, mix, and cool. Prepare this solution fresh daily.

Alcoholic Potassium Hydroxide Solution— Dissolve 3 g of potassium hydroxide in 50 mL of freshly boiled water, add 10 mL of alcohol, dilute with freshly boiled water to 100 mL, and mix. Prepare this solution fresh daily.

Sodium Ascorbate Solution— Dissolve 3.5 g of ascorbic acid in 20 mL of 1 N sodium hydroxide. Prepare this solution fresh daily.

Sodium Sulfide Solution— Dissolve 12 g of sodium sulfide in 20 mL of water, dilute with glycerin to 100 mL, and mix.

Mobile Phase A— Prepare a mixture of acetonitrile, methanol, and water (25:25:1). The amount of water and the flow rate may be varied to meet system suitability requirements.

Mobile Phase B— Prepare a 3 in 1000 mixture of n-amyl alcohol in Dehydrated Hexane. The ratio of components and the flow rate may be varied to meet system suitability requirements.

Internal Standard Solution— Transfer 15 mg of [USP A<sub>4,6</sub>-Cholestadienol RS](#), accurately weighed, to a 200-mL volumetric flask, add a 1 in 10 mixture of toluene and Mobile Phase B to volume, and mix.

Standard Preparation— Transfer about 25 mg of [USP Ergocalciferol RS](#) or [Cholecalciferol RS](#), accurately weighed, to a 50-mL volumetric flask, dissolve without heat in toluene, add toluene to volume, and mix. Pipet 10 mL of this stock solution into a 100-mL volumetric flask, dilute with toluene to volume, and mix. Prepare stock solution fresh daily.

#### Assay Preparation—

For oily solutions— Accurately weigh a portion of the specimen to be assayed, preferably more than 0.5 g and equivalent to about 125 µg of cholecalciferol or ergocalciferol (5000 USP Units). Add 1 mL of Sodium Ascorbate Solution, 25 mL of alcohol, and 2 mL of Aqueous Potassium Hydroxide Solution, and mix.

For capsules or tablets— Reflux not less than 10 capsules or tablets with a mixture of 10 mL of Sodium Ascorbate Solution and 2 drops of Sodium Sulfide Solution on a steam bath for 10 minutes, crush any remaining solids with a blunt glass rod, and continue heating for 5 minutes. Cool, add 25 mL of alcohol and 3 mL of Aqueous Potassium Hydroxide Solution, and mix.

For dry preparations and aqueous dispersions— Accurately weigh a portion of the specimen to be assayed, preferably more than 0.5 g and equivalent to about 125 µg of cholecalciferol or ergocalciferol (5000 USP Units). Add, in small quantities and with gentle swirling, 25 mL of alcohol, 5 mL of Sodium Ascorbate Solution, and 3 mL of Aqueous Potassium Hydroxide Solution.

Saponification and extraction— Reflux the mixture prepared from the specimen to be assayed on a steam bath for 30 minutes. Cool rapidly under running water, and transfer the saponified mixture to a conical separator, rinsing the saponification flask with two 15-mL portions of water, 10 mL of alcohol, and two 50-mL portions of ether. Shake the combined saponified mixture and rinsings vigorously for 30 seconds, and allow to stand until both layers are clear. Transfer the aqueous phase to a second conical separator, add a mixture of 10



of alcohol and 50 mL of solvent hexane, and shake vigorously. Allow to separate, transfer the aqueous phase to a third conical separator, and transfer the hexane phase to the second separator, rinsing the second separator with two 10-mL portions of solvent hexane, adding the rinsings to the first separator. Shake the aqueous phase in the third separator with 50 mL of solvent hexane, and add the hexane phase to the first separator. Wash the combined ether-hexane extracts by shaking vigorously with three 50-mL portions of Alcoholic Potassium Hydroxide Solution, and wash with 50-mL portions of water vigorously until the last washing is neutral to phenolphthalein. Drain any remaining drops of water from the combined ether-hexane extracts, add 2 sheets of 9-cm filter paper, in strips, to the separator, and shake. Transfer the washed ether-hexane extracts to a round-bottom flask, rinsing the separator and paper with solvent hexane. Combine the hexane rinsings with the ether-hexane extracts, add 5.0 mL of Internal Standard Solution and 100  $\mu$ L of Butylated Hydroxytoluene Solution, and mix. Evaporate to dryness in vacuum by swirling in a water bath maintained at a temperature not higher than 40°. Cool under running water, and introduce nitrogen sufficient to restore atmospheric pressure. Without delay, dissolve the residue in 5.0 mL of a mixture of equal volumes of acetonitrile and methanol, or in a measured portion of the acetonitrile-methanol mixture until the concentration of vitamin D is about 25  $\mu$ g per mL, to obtain the Assay Preparation.

Chromatographic System— Use a chromatograph, operated at room temperature, fitted with an UV detector that monitors absorption at 254 nm, a 30-cm  $\times$  4.6-mm stainless steel cleanup column packed with column packing L7 and using Mobile Phase A, and a 25-cm  $\times$  4.6-mm stainless steel analytical column packed with column packing L3 and using Mobile Phase B.

Cleanup Column System Suitability Test— Pipet 5 mL of the Standard Preparation into a round-bottom flask fitted with a reflux condenser, and add 2 or 3 crystals of butylated hydroxytoluene. Displace the air with nitrogen, and heat in a water bath maintained at a temperature of 90° in subdued light under an atmosphere of nitrogen for 45 minutes, to obtain a solution containing vitamin D and pre-vitamin D. Cool, add 10.0 mL of Internal Standard Solution, mix, and evaporate in vacuum to dryness by swirling in a water bath maintained at a temperature not higher than 40°. Cool under running water, and introduce nitrogen sufficient to restore atmospheric pressure. Without delay, dissolve the residue in 10.0 mL of a mixture of equal volumes of acetonitrile and methanol, and mix. Inject 500  $\mu$ L of this solution into the cleanup column, and record the chromatogram as directed under Procedure. The chromatogram exhibits a peak exhibiting a retention time between 5 and 9 minutes, corresponding to the separation under a single peak of the mixture of vitamin D, pre-vitamin D, and  $\Delta$ 4,6-cholestadienol from other substances. Adjust the water content or other operating parameters, if necessary (see Mobile Phase A).

Analytical Column System Suitability Test— Transfer about 100 mg of USP Vitamin D Assay System Suitability RS to a 100-mL volumetric flask, add a 1 in 20 mixture of toluene and Mobile Phase B to volume, and mix. Heat a portion of this solution, under reflux, at 90° for 45 minutes, and cool. Chromatograph five injections of the resulting solution, and measure the peak responses as directed for Procedure: the resolution, R, between trans-cholecalciferol and pre-cholecalciferol is not less than 1.0, and the relative standard deviation for the cholecalciferol peak response does not exceed 2.0%. [note—Chromatograms obtained as directed for this test exhibit relative retention times of approximately 0.4 for pre-cholecalciferol, 0.5 for trans-cholecalciferol, and 1.0 for cholecalciferol.]

#### Calibration—

Vitamin D Response Factor— Transfer 4.0 mL of the Standard Preparation and 10.0 mL of Internal Standard Solution to a 100-mL volumetric flask, dilute with Mobile Phase B to volume, and mix to obtain the Working Standard Preparation. Store this Working Standard Preparation at a temperature not above 0°, retaining the unused portion for the Procedure. Inject 200  $\mu$ L of the Working Standard Preparation into the analytical column, and measure the peak responses for vitamin D and for  $\Delta$ 4,6-cholestadienol. The relative retention time of  $\Delta$ 4,6-cholestadienol is about 1.3. Calculate the response factor, FD, by the formula:

$$FD = \frac{CS}{R'S}$$

in which CS and CR are the concentrations, in  $\mu$ g per mL, of vitamin D and  $\Delta$ 4,6-cholestadienol, respectively, in the Working Standard Preparation, and RS is the ratio of the peak response of vitamin D to that of  $\Delta$ 4,6-cholestadienol.

Pre-Vitamin D Response Factor— Pipet 4 mL of the Standard Preparation into a round-bottom flask fitted with a reflux condenser, and add 2 or 3 crystals of butylated hydroxytoluene. Displace the air with nitrogen, and heat in a water bath maintained at a temperature of 90° in subdued light under a nitrogen atmosphere for 45 minutes, to obtain a solution containing vitamin D and pre-vitamin D. Cool, transfer with the aid of several portions of Mobile Phase B to a 100-mL volumetric flask containing 10.0 mL of Internal Standard Solution, dilute with Mobile Phase B to volume, and mix to obtain the Working Mixture. Inject 200  $\mu$ L of this Working Mixture into the analytical column, and measure the peak responses for vitamin D, pre-vitamin D, and  $\Delta$ 4,6-cholestadienol. Calculate the concentration, C's, in  $\mu$ g per mL, of vitamin D in the (heated) Working Mixture by the formula:

$$FD \times CS = C'D$$

in which CR is the concentration, in  $\mu$ g per mL, of  $\Delta$ 4,6-cholestadienol, and R'S is the ratio of the peak response for vitamin D to that for  $\Delta$ 4,6-cholestadienol. Calculate the concentration, C'PRE, in  $\mu$ g per mL, of pre-vitamin D, in the Working Mixture by the formula:

$$C'PRE = CS - C'D$$

Calculate the response factor, FPRE, for pre-vitamin D by the formula:

$$(FD \times CS) / (C'PRE)$$

in which R'PRE is the ratio of the peak response of pre-vitamin D to that of  $\Delta$ 4,6-cholestadienol. [note—Value of FPRE determined in duplicate, on different days, can be used during the whole procedure.]

Procedure— Inject 500  $\mu$ L of the Assay Preparation into the cleanup column, and collect the fraction representing 0.7 to 1.3 relative to the retention time of the mixed vitamin D peak (see Cleanup Column System Suitability Test) in a round-bottom flask. Add 50  $\mu$ L of Butylated Hydroxytoluene Solution, mix, and evaporate in vacuum to dryness by swirling in a water bath maintained at a temperature not higher than 40°. Cool under running water, and introduce nitrogen sufficient to restore atmospheric pressure. Without delay, dissolve the residue in 5.0 mL of a 1 in 20 mixture of toluene and Mobile Phase B, and mix. Inject 200  $\mu$ L of this solution into the analytical column, and measure the peak responses for vitamin D, pre-vitamin D, and  $\Delta$ 4,6-cholestadienol. Calculate the concentration, in  $\mu$ g per mL, of cholecalciferol (C27H44O) or ergocalciferol (C28H44O) in the Assay Preparation by the formula:

$$(R'DF + R'PRE)C'D$$

in which R'D is the ratio of the peak response of vitamin D to that of  $\Delta$ 4,6-cholestadienol; R'PRE is the ratio of the peak response of pre-vitamin D to that of  $\Delta$ 4,6-cholestadienol; and C'D is the concentration, in  $\mu$ g per mL, of  $\Delta$ 4,6-cholestadienol in the Assay Preparation.

#### Chemical Method

The following procedure is provided for the determination of vitamin D as an ingredient of Pharmacopeial preparations.

Complete the assay promptly, and exercise care throughout the procedure to keep to a minimum the exposure to air and to actinic light, preferably by the use of a blanket of inert gas and low-actinic glassware.

USP Reference Standards [11]—[note—Use USP Ergocalciferol RS, or USP Cholecalciferol RS, for assaying pharmaceutical dosage forms that are labeled to contain vitamin D as ergocalciferol, or as cholecalciferol, respectively.] USP Cholecalciferol RS, USP Ergocalciferol RS.

#### Special Reagents and Solutions—

Chromatographic Fuller's Earth— Use chromatographic Fuller's earth having a water content corresponding to between 8.5% and 9.0% of loss on drying.

Solvent Hexane— Use solvent hexane (see under Reagents, Indicators, and Solutions), redistilling if necessary so that it meets the following additional specification:

spectral purity—Measure in a 1-cm cell at 300 nm, with a suitable spectrophotometer, against air as the blank: the absorbance is not more than 0.070.

Ethylene Dichloride— Purify by passage through a column of granular (20 to 200 mesh) silica gel.

Potassium Hydroxide Solution— Dissolve 500 g of potassium hydroxide in water to make 1000 mL.

Butylated Hydroxytoluene Solution— Dissolve 10 mg of butylated hydroxytoluene in 100 mL of alcohol. Prepare this solution fresh daily.

Ether— Use freshly distilled ether, discarding the first and last 10% portions of the distillate.



Color Reagent— Prepare two stock solutions as follows.

solution a—Empty, without weighing, the entire contents of a previously unopened 113-g bottle of dry, crystalline antimony trichloride into a flask containing about 400 mL of Ethylene Dichloride. Add about 2 g of anhydrous alumina, mix, and pass through filter paper into a clear-glass, glass-stoppered container calibrated at 500 mL. Dilute with Ethylene Dichloride to 500 mL, and mix: the absorbance of the solution, measured in a 20-mm cell at 500 nm, with a suitable spectrophotometer, against Ethylene Dichloride, does not exceed 0.070.

solution b—Mix, under a hood, 100 mL of acetyl chloride and 400 mL of Ethylene Dichloride.

Mix 45 mL of Solution A and 5 mL of Solution B to obtain the Color Reagent. Store in a tight container, and use within 7 days, but discard any reagent in which a color develops.

Chromatographic Tubes—

First Column— Arrange for descending column chromatography a tube of 2.5-cm (inside) diameter, about 25 cm long, and constricted to 8-mm diameter for a distance of 5 cm at the lower end, by inserting at the point of constriction a coarse-porosity, sintered-glass disk or a small plug of glass wool. The constricted portion may be fitted with an inert, plastic stopcock.

Second Column— Select a tube that is made up of three sections: (1) a flared top section, 18 mm in (inside) diameter and approximately 14 cm long, (2) a middle section, 6 mm in (inside) diameter and approximately 25 cm long, and (3) a tapered, constricted lower exit tube approximately 5 cm long. Insert a small plug of glass wool in the upper 1-cm portion of the constricted section.

Chromatographic Columns—

First Column— To about 125 mL of isooctane contained in a screw-capped, wide-mouth bottle add 25 g of chromatographic siliceous earth, and shake until a slurry is formed. Add, dropwise and with vigorous mixing, 10 mL of polyethylene glycol 600. Replace the bottle cover, and shake vigorously for 2 minutes. Pour about half of the resulting slurry into the chromatographic tube, and allow it to settle by gravity. Then apply gentle suction, and add the remainder of the slurry in small portions, packing each portion with a 20-mm disk plunger. When a solid surface has formed, remove the vacuum, and add about 2 mL of isooctane.

Second Column— Pack the midsection of the tube with 3 g of moderately coarse Chromatographic Fuller's Earth with the aid of gentle suction (about 125 mm of mercury).

Standard Preparation— Dissolve about 25 mg of Reference Standard, accurately weighed, in isooctane to give a known concentration of about 250  $\mu$ g per mL. Store in a refrigerator.

On the day of assay, pipet 1 mL of this solution into a 50-mL volumetric flask, remove the solvent with a stream of nitrogen, and dissolve the residue in and dilute with Ethylene Dichloride to volume, and mix.

Sample Preparation— Accurately weigh or measure a portion of the sample to be assayed, equivalent to not less than 125  $\mu$ g but preferably about 250  $\mu$ g of ergocalciferol (10,000 USP Units). If little or no vitamin A is present in the sample, add about 1.5 mg (the equivalent of 3000 USP Units) of vitamin A acetate to provide the needed pilot bands in the subsequent chromatography.

For capsules or tablets, reflux not fewer than 10 of them in 10 mL of water on a steam bath for about 10 minutes, crush the remaining solid with a blunt glass rod, and warm for 5 minutes longer.

Add a volume of Potassium Hydroxide Solution representing 2.5 mL for each g of the total weight of the sample, but not less than a total of 3.0 mL. Add 10 mL of Butylated Hydroxytoluene Solution and 20 mL of alcohol. Reflux vigorously on a steam bath for 30 minutes. Cool, and transfer the saponified mixture to a conical separator, rinsing the saponification flask with three 10-mL portions of water and three 50-mL portions of Ether, adding each rinse to the separator. Add about 4 g of sodium sulfate decahydrate to the separator, and extract by shaking for 2 minutes. If an emulsion forms, extract with three 25-mL portions of Ether. Combine the ether extracts, if necessary, and wash by swirling gently with 50 mL of water. Repeat the washing more vigorously with additional 50-mL portions of water until the last portion shows no pink color on the addition of *phenolphthalein* TS. Transfer the washed ether extract to a 250-mL volumetric flask, dilute with Ether to volume, and mix. Transfer the entire sample or an accurately measured aliquot containing about 250  $\mu$ g to a tall-form, 400-mL beaker containing about 5 g of anhydrous sodium sulfate. Stir for 2 minutes, then decant the solution into a second 400-mL beaker. Rinse the sodium sulfate with three 25-mL portions of Ether, adding each rinse to the main portion. Reduce the total volume to about 30 mL by evaporation on a steam bath, and transfer the concentrate to a small, round-bottom evaporation flask. Rinse the beaker with three 10-mL portions of Ether, adding the rinsings to the flask. With the aid of vacuum in a water bath at a temperature not exceeding 40°, or with a stream of nitrogen at room temperature, remove the remaining solvent completely. Dissolve the residue in a small amount of Solvent Hexane, transfer to a 10-mL volumetric flask, dilute with Solvent Hexane to volume, and mix to obtain the Sample Preparation.

Procedure—

First Column Chromatography— Just as the 2 mL of isooctane disappears into the surface of the prepared First Column, pipet 2 mL of the Sample Preparation onto the column. As the meniscus of the Sample Preparation reaches the column surface, add the first of three 2-mL portions of Solvent Hexane, adding each succeeding portion as the preceding portion disappears into the column. Continue adding Solvent Hexane in portions of 5 to 10 mL until 100 mL has been added. If necessary, adjust the flow rate to between 3 and 6 mL per minute, by application of gentle pressure at the top of the chromatographic tube.

Discard the first 20 mL of effluent, and collect the remainder. Examine the column under UV light at intervals during the chromatography, and stop the flow when the front of the fluorescent band representing vitamin A is about 5 mm from the bottom of the column. (The UV lamp should provide weak radiation in the 300-nm region. It is frequently necessary to use a narrow aperture or screen with commercial lamps to reduce the amount of radiation to the minimum required to visualize the vitamin A on the column.)

Transfer the eluate to a suitable evaporation flask, and remove the Solvent Hexane completely under vacuum at a temperature not higher than 40° or with a stream of nitrogen at room temperature. Dissolve the residue in about 10 mL of Solvent Hexane.

Second Column Chromatography— Add the solvent hexane solution obtained as directed under First Column Chromatography onto the Second Column. Rinse the evaporation flask with a total of 10 mL of Solvent Hexane in small portions, adding each portion to the Second Column and allowing it to flow through the column, and discard the effluent. When about 1 mL of the hexane remains above the surface of the column, add 75 mL of toluene, and elute with the aid of gentle suction (about 125 mm of mercury), collecting the eluate. Evaporate the toluene under vacuum at a temperature not higher than 40°, or with a stream of nitrogen at room temperature.

Assay Preparation— Dissolve the residue obtained as directed under Second Column Chromatography in a small amount of Ethylene Dichloride, transfer to a 10-mL volumetric flask, dilute with Ethylene Dichloride to volume, and mix to obtain the Assay Preparation.

Color Development— Into each of three suitable, matched colorimeter tubes of about 20-mm (inside) diameter, and designated tubes 1, 2, and 3, respectively, pipet 1 mL of the Assay Preparation. Into tube 1, pipet 1 mL of the Standard Preparation; into tube 2, 1 mL of Ethylene Dichloride; and into tube 3, 1 mL of a mixture of equal volumes of acetic anhydride and Ethylene Dichloride. To each tube add quickly, and preferably from an automatic pipet, 5.0 mL of Color Reagent, and mix. After 45 seconds, accurately timed, following the addition of the Color Reagent, determine the absorbances of the three solutions at 500 nm, with a suitable spectrophotometer, using Ethylene Dichloride as the blank. Similarly, 45 seconds after making the first reading on each solution, determine the absorbances of the solutions in tubes 2 and 3 at 550 nm, in a similar manner. Designate the absorbances as A1500, A2500, A3500, A2550, and A3550, respectively, in which the superscript indicates the number of the tube and the subscript, the wavelength.

Calculation— Calculate the quantity, in  $\mu$ g, of vitamin D in the portion of the sample taken by the formula:

$$(CS / C)(AU / AS)$$

in which CS is the concentration of vitamin D, in  $\mu$ g per mL, of the Standard Preparation; C is the concentration of the sample (as g, capsules, tablets, etc.) in each mL of the final solution; AU has the value of  $(A2500 - A3500) - 0.67(A2550 - A3550)$  determined from the absorbances observed on the solution from the Assay Preparation; and AS has the value of  $A1500 - A2500$  determined on the solutions from the Standard Preparation.

Biological Method

The biological assay of vitamin D comprises the recording and interpretation of observations on groups of rats maintained on specified dietary regimens throughout specified periods of their lives whereby the biological response to the preparation under assay is compared with the response to [USP Vitamin D Capsules RS](#).

USP Reference Standards (11) — USP Cholecalciferol RS.

Preliminary Period— Throughout the preliminary period in the life of a rat, which is not longer than 30 days and extends from birth to the first day of the depletion period, maintain litters of rats under the immediate supervision of, or according to the directions of, the individual responsible for the assay. During the preliminary period, use a dietary regimen that provides for normal development but is limited in its content of vitamin D, so that when placed upon the Rachitogenic Diet in the depletion period the rats develop rickets. At the end of the preliminary period, reject any rat that weighs less than 44 g or more than 60 g, or that shows evidence of injury, disease, or anatomical abnormality.

Depletion Period— Through the depletion period, which extends from the end of the preliminary period to the first day of the assay period, provide each rat ad libitum with the Rachitogenic Diet and water, and allow access to no other food or dietary supplement.

Rachitogenic Diet— The Rachitogenic Diet consists of a uniform mixture of the following ingredients in the proportions shown in the accompanying table.

Rachitogenic Diet	
Ingredient	Parts by weight
Whole yellow corn, ground	76
Wheat gluten, ground	20
Calcium carbonate	3
Sodium chloride	1

When a chemical analysis of the entire ration shows a Ca:P ratio of less than 4:1 or more than 5:1, the proportion of calcium carbonate may be varied to bring the adjusted ratio to a uniform level within this range.

Assigning Rats to Groups for Assay Period— Consider a litter suitable for the assay period when individual rats in the litter show evidence of rickets such as enlarged joints and a distinctive wobbly, rachitic gait, provided that the depletion period is not less than 19 or more than 25 days. The presence of rickets may be established also from the width of the rachitic metaphysis upon X-ray examination or by applying the Line Test (described below) to a leg bone of one member of each litter.

Record the weight of each rat, and assign it to a group, in which each rat will be fed a specified dose of the Reference Standard or of an assay sample that is under examination for its vitamin D potency. For each assay sample provide one or more assay groups and not less than two standard groups. The two standard groups may be used for the concurrent assay of more than one assay sample. Within an interval not exceeding 30 days, complete the assignment of rats to groups according to a design that divides litters among the groups, to achieve a complete balance.

For complete balance, whereby each litter is represented equally in every group, use 7 or more litters containing at least as many depleted rats as there are groups. From a given litter, assign one rat, selected at random, to each group on the same day. If a litter contains twice as many rats as there are groups, assign a second series of rats similarly. The last one or two litters to be assigned may be allotted to groups so that at the start of the assay period the average body weight of any completed groups will not differ by more than 8 g from that of any other group.

Assay Doses— Select two dosage levels of the USP Cholecalciferol RS, spaced so that the ratio of the larger to the smaller dose is not less than 1.5 or more than 2.5. Select one or two dosage levels based upon a single assumed potency for each sample. The dosage levels of the sample are equivalent to those of the standard or to a mid-level equal to the square root of the product of the two dosage levels of the standard.

Select dosage levels such that, when fed to rachitic rats, they are expected to produce degrees of calcification within the range specified under the test of data acceptability. Before feeding, the Reference Standard and/or sample may be diluted with cottonseed oil, provided that not more than 0.2 mL is fed on any one day. Store the oil solutions in well-closed bottles, protected from light, at a temperature not exceeding 10°, and use within 5 weeks.

Assign one group of rats to each dosage level of the standard and of the one or more samples.

Assay Period— During the assay period, which extends from the end of the depletion period for a fixed interval of 7 to 10 days, cage each rat individually and provide it ad libitum with the Rachitogenic Diet and water. Supply a Rachitogenic Diet prepared from the same lots of ingredients to all rats. On the first and on the third (or fourth) day of the assay period, feed each rat one-half of its total assigned dose.

Throughout the assay period, maintain as uniform environmental conditions as possible for all rats, and exclude exposure to antirachitic radiations. At the end of a fixed period of 7 to 10 days, weigh and kill each rat. From those rats that do not weigh less at the end than at the start of the assay period and that have consumed each assigned dose within 24 hours of the time it was fed, dissect out one or more leg bones for examination by the Line Test.

Line Test— Remove the proximal end of a tibia or the distal end of a radius, and clean adhering tissue from it, in any one assay using the same bone from all animals. With a clean, sharp blade cut a median, longitudinal section through the juncture of the epiphysis and diaphysis at the same place on each bone. Rinse both sections in purified water, immerse immediately in silver nitrate solution (1 in 50) for 1 minute, and rinse again in purified water. Expose the cut surface of bone, in water, to daylight or another source of actinic light until the calcified areas develop a clearly defined stain without marked discoloration of the uncalcified areas. The staining procedure may be modified to differentiate more clearly between calcified and uncalcified areas.

Score the degree of calcification of the rachitic metaphysis in each rat, according to a scale that allows the average response to be plotted as a straight line against the logarithm of the dose.

Acceptability— Observations are acceptable for use in calculation of the potency only from those groups in which two-thirds or more but not less than 7 rats show calcification at least as great as the lowest level and not greater than the highest level. If the average score of the standard group on the high dosage level is not greater than the average score of the standard group on the low dosage level, discard the results, and repeat the assay. If an assay sample is represented solely by assay groups that are not acceptable for measuring vitamin D potency and in each of which the average score is less than the average score of the standard group on the low dosage level or more than the average score of the standard group on the high dosage level, its assayed content of vitamin D is respectively less than that represented by the low dose or more than that represented by the high dose of the Reference Standard.

Calculation— Tabulate the scores (y), listing each litter in a separate row with treatment groups in columns. Omit any groups that do not meet the test for Acceptability. Equalize the number of observations in the acceptable groups by disregarding the results on all litters not equally represented in the groups or by other suitable means (see Design and Analysis of Biological Assays (111)). Total the f scores for each of the treatment groups, where f is the number of litters, and designate each total as T with subscripts 1 and 2 for the low and high dosage levels, respectively. Compute the slope b from the sums of T<sub>1</sub>, i.e.,  $\Sigma T_1$ , and of T<sub>2</sub>, i.e.,  $\Sigma T_2$ , for the standard and sample, provided the latter is represented at both dosage levels, from the equation:

$$b = (\Sigma T_2 - \Sigma T_1) / ih'$$

in which i is the logarithm of the ratio of the high dose to the low dose and is the same for each preparation, and h' is the number of preparations represented by two dosage levels and included in the calculation of the value of b.

Compute the logarithm of the relative potency of each specimen under assay from the equation:

$$\begin{aligned} \log (\text{relative potency}) &= M' \\ &= (y_u - \bar{y}_s)/b \\ &= ih'T/2\Sigma T_b \end{aligned}$$

in which each mean score, y<sub>u</sub> for the assay sample and y<sub>s</sub> for the Reference Standard, is the average of the individual scores for an intermediate dosage level or of the two means for



the high and the low dosage levels and where  $T_b = \Sigma T_2 - \Sigma T_1$  and  $T_a$  is as defined (see [Design and Analysis of Biological Assays](#) (111)). Convert each observed  $M'$  to its antilogarithm to obtain the relative potency of the sample. Multiply the relative potency by the assumed potency of the assay oil in Units per g, adopted at the start of the assay, to obtain its assayed content of vitamin D in USP Units per g.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Curtis Phinney</a>  1-301-816-8540	(DSN05) Dietary Supplements - Non-Botanicals
Reference Standards	Lili Wang, Technical Services Scientist 1-301-816-8129 <a href="mailto:RSTech@usp.org">RSTech@usp.org</a>	

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### 591 ZINC DETERMINATION

The need for a quantitative determination of zinc in the Pharmacopeial insulin preparations reflects the fact that the element is an essential component of zinc-insulin crystals. In common with lead, zinc may be determined either by the dithizone method or by atomic absorption.

#### Dithizone Method

Select all reagents for this test to have as low a content of heavy metals as practicable. If necessary, distill water and other solvents into hard or borosilicate glass apparatus. Rinse thoroughly all glassware with warm dilute nitric acid (1 in 2) followed by water. Avoid using on the separator any lubricants that dissolve in chloroform.

#### Special Solutions and Solvents—

ALKALINE AMMONIUM CITRATE SOLUTION— Dissolve 50 g of dibasic ammonium citrate in water to make 100 mL. Add 100 mL of ammonium hydroxide. Remove any heavy metals that may be present by extracting the solution with 20-mL portions of Dithizone Extraction Solution (see [Lead](#) (251)) until the dithizone solution retains a clear green color, then extract any dithizone remaining in the citrate solution by shaking with chloroform.

CHLOROFORM— Distill chloroform in hard or borosilicate glass apparatus, receiving the distillate in sufficient dehydrated alcohol to make the final concentration 1 mL of alcohol for each 100 mL of distillate.

DITHIZONE SOLUTION— Use Standard Dithizone Solution (see [Lead](#) (251)), prepared with the distilled Chloroform.

STANDARD ZINC SOLUTION— Dissolve 625 mg of zinc oxide, accurately weighed, and previously gently ignited to constant weight, in 10 mL of nitric acid, and add water to make 500.0 mL. This solution contains 1.0 mg of zinc per mL.

DILUTED STANDARD ZINC SOLUTION— Dilute 1 mL of Standard Zinc Solution, accurately measured, with 2 drops of nitric acid and sufficient water to make 100.0 mL. This solution contains 10  $\mu$ g of zinc per mL. Use this solution within 2 weeks.

TRICHLOROACETIC ACID SOLUTION— Dissolve 100 g of trichloroacetic acid in water to make 1000 mL.

Procedure— Transfer 1 to 5 mL of the preparation to be tested, accurately measured, to a centrifuge tube graduated at 40 mL. If necessary, add 0.25 N hydrochloric acid, dropwise, to obtain a clear solution. Add 5 mL of Trichloroacetic Acid Solution and sufficient water to make 40.0 mL. Mix, and centrifuge.

Transfer to a hard-glass separator an accurately measured volume of the supernatant believed to contain from 5 to 20  $\mu$ g of zinc, and add water to make about 20 mL. Add 1.5 mL of Alkaline Ammonium Citrate Solution and 35 mL of Dithizone Solution. Shake vigorously 100 times. Allow the chloroform phase to separate. Insert a cotton plug in the stem of the separator to remove any water emulsified with the chloroform. Collect the chloroform extract (discarding the first portion that comes through) in a test tube, and determine the absorbance at 530 nm, with a suitable spectrophotometer.

Calculate the amount of zinc present by reference to a standard absorbance-concentration curve obtained by using 0.5 mL, 1.0 mL, 1.5 mL, and, if the zinc content of the sample extracted exceeds 15  $\mu$ g, 2.0 mL of the Diluted Standard Zinc Solution, corrected as indicated by a blank determination run concomitantly, using all of the reagents but no added zinc.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Kahkashan Zaidi, Ph.D.</a> Senior Scientist 1-301-816-8269	(GC05) General Chapters 05

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### 601 AEROSOLS, NASAL SPRAYS, METERED-DOSE INHALERS, AND DRY POWDER INHALERS

This general chapter contains test methods for propellants, pressurized topical aerosols, nasal sprays, metered-dose inhalers, and propellant-free dry powder inhalers used to aerosolize, or to aerosolize and meter, doses of powders for inhalation. Apply these methods, where indicated, in the testing of the appropriate dosage forms.

#### PROPELLANTS

Caution—Hydrocarbon propellants are highly flammable and explosive. Observe precautions and perform sampling and analytical operations in a well-ventilated fume hood. General Sampling Procedure

This procedure is used to obtain test specimens for those propellants that occur as gases at about 25° and that are stored in pressurized cylinders. Use a stainless steel sample cylinder equipped with a stainless steel valve and having a capacity of not less than 200 mL and a pressure rating of 240 psi or more. Dry the cylinder with the valve open at 110° for 2 hours, and evacuate the hot cylinder to less than 1 mm of mercury. Close the valve, cool, and weigh. Connect one end of a charging line tightly to the propellant container and the other end loosely to the sample cylinder. Carefully open the propellant container, and allow the propellant to flush out the charging line through the loose connection. Avoid excessive flushing, which causes moisture to freeze in the charging line and connections. Tighten the fitting on the sample cylinder, and open the sample cylinder valve, allowing the propellant to flow into the evacuated cylinder. Continue sampling until the desired amount of specimen is obtained, then close the propellant container valve, and finally close the sample cylinder valve. [Caution—Do not overload the sample cylinder; hydraulic expansion due to temperature change can cause overloaded cylinders to explode.] Again weigh the charged sample cylinder, and calculate the weight of the specimen.

#### Approximate Boiling Temperature

Transfer a 100-mL specimen to a tared, pear-shaped, 100-mL centrifuge tube containing a few boiling stones, and weigh. Suspend a thermometer in the liquid, and place the tube in a medium maintained at a temperature of 32° above the expected boiling temperature. When the thermometer reading becomes constant, record as the boiling temperature the thermometer reading after at least 5% of the specimen has distilled. Retain the remainder of the specimen for the determination of High-Boiling Residues.

High-Boiling Residues, Method I



Allow 85 mL of the specimen to distill as directed in the test for Approximate Boiling Temperature, and transfer the centrifuge tube containing the remaining 15 mL of specimen to a medium maintained at a temperature  $10^{\circ}$  above the boiling temperature. After 30 minutes, remove the tube from the water bath, blot dry, and weigh. Calculate the weight of the residue.

#### High-Boiling Residues, Method II

Prepare a cooling coil from copper tubing (about 6 mm outside diameter  $\times$  about 6.1 m long) to fit into a vacuum-jacketed flask. Immerse the cooling coil in a mixture of dry ice and acetone in a vacuum-jacketed flask, and connect one end of the tubing to the propellant sample cylinder. Carefully open the sample cylinder valve, flush the cooling coil with about 50 mL of the propellant, and discard this portion of liquefied propellant. Continue delivering liquefied propellant from the cooling coil, and collect it in a previously chilled 1000-mL sedimentation cone until the cone is filled to the 1000-mL mark. Allow the propellant to evaporate, using a warm water bath maintained at about  $40^{\circ}$  to reduce evaporating time. When all of the liquid has evaporated, rinse the sedimentation cone with two 50-mL portions of pentane, and combine the rinsings in a tared 150-mL evaporating dish. Transfer 100 mL of the pentane solvent to a second tared 150-mL evaporating dish, place both evaporating dishes on a water bath, evaporate to dryness, and heat the dishes in an oven at  $100^{\circ}$  for 60 minutes. Cool the dishes in a desiccator, and weigh. Repeat the heating for 15-minute periods until successive weighings are within 0.1 mg, and calculate the weight of the residue obtained from the propellant as the difference between the weights of the residues in the two evaporating dishes.

#### Water Content

Proceed as directed under [Water Determination \(921\)](#), with the following modifications: (a) Provide the closed-system titrating vessel with an opening through which passes a coarse-porosity gas dispersion tube connected to a sampling cylinder. (b) Dilute the Reagent with anhydrous methanol to give a water equivalence factor of between 0.2 and 1.0 mg per mL; age this diluted solution for not less than 16 hours before standardization. (c) Obtain a 100-g specimen as directed under General Sampling Procedure, and introduce the specimen into the titration vessel through the gas dispersion tube at a rate of about 100 mL of gas per minute; if necessary, heat the sample cylinder gently to maintain this flow rate.

#### Other Determinations

For those aerosols that use propellants, perform the tests specified in the individual NF propellant monographs.

### AEROSOLS

Because leaching of extractable substances into pressurized formulations should be minimized, valve materials and other components that are in contact with the product meet the requirements under [Elastomeric Closures for Injections \(381\)](#) (Note that under Physicochemical Test Procedures in (381) the directions for preparing a sample require pre-extraction, which may cause an underestimate of the amount of extractables from a given component.) See also Aerosols under [Pharmaceutical Dosage Forms \(1151\)](#).

#### TOPICAL AEROSOLS

The following tests are applicable to topical aerosols containing drug, in suspension or solution, packaged under pressure, and released upon activation of an appropriate valve system.

##### Delivery Rate and Delivered Amount

Perform these tests only on containers fitted with continuous valves.

**Delivery Rate**— Select not fewer than four aerosol containers, shake, if the label includes this directive, remove the caps and covers, and actuate each valve for 2 to 3 seconds. Weigh each container accurately, and immerse in a constant-temperature bath until the internal pressure is equilibrated at a temperature of  $25^{\circ}$  as determined by constancy of internal pressure, as directed under the Pressure Test below. Remove the containers from the bath, remove excess moisture by blotting with a paper towel, shake, if the label includes this directive, actuate each valve for 5.0 seconds (accurately timed by use of a stopwatch), and weigh each container again. Return the containers to the constant-temperature bath, and repeat the foregoing procedure three times for each container. Calculate the average Delivery Rate, in g per second, for each container.

**Delivered Amount**— Return the containers to the constant-temperature bath, continuing to deliver 5 second actuations to waste, until each container is exhausted. [note—Ensure that sufficient time is allowed between each actuation to avoid significant canister cooling.] Calculate the total weight loss from each container. This is the Delivered Amount.

##### Pressure Test

Perform this test only on topical aerosols fitted with continuous valves.

Select not fewer than four aerosol containers, remove the caps and covers, and immerse in a constant-temperature bath until the internal pressure is constant at a temperature of  $25^{\circ}$ . Remove the containers from the bath, shake, and remove the actuator and water, if any, from the valve stem. Place each container in an upright position, and determine the pressure in each container by placing a calibrated pressure gauge on the valve stem, holding firmly, and actuating the valve so that it is fully open. The gauge is of a calibration approximating the expected pressure and is fitted with an adapter appropriate for the particular valve stem dimensions. Read the pressure directly from the gauge.

##### Minimum Fill

Topical aerosols meet the requirements for aerosols under [Minimum Fill \(755\)](#).

##### Leakage Test

Perform this test only on topical aerosols fitted with continuous valves.

Select 12 aerosol containers, and record the date and time to the nearest half hour. Weigh each container to the nearest mg, and record the weight, in mg, of each as W1. Allow the containers to stand in an upright position at a temperature of  $25.0 \pm 2.0^{\circ}$  for not less than 3 days, and again weigh each container, recording the weight, in mg, of each as W2, and recording the date and time to the nearest half hour. Determine the time, T, in hours, during which the containers were under test. Calculate the leakage rate, in mg per year, of each container taken by the formula:

$$(365)(24)(T)(W1 - W2)$$

Where plastic-coated glass aerosol containers are tested, dry the containers in a desiccator for 12 to 18 hours, and allow them to stand in a constant-humidity environment for 24 hours prior to determining the initial weight as indicated above. Conduct the test under the same constant-humidity conditions. Empty the contents of each container tested by employing any safe technique (e.g., chill to reduce the internal pressure, remove the valve, and pour). Remove any residual contents by rinsing with suitable solvents, then rinse with a few portions of methanol. Retain as a unit the container, the valve, and all associated parts, and heat them at  $100^{\circ}$  for 5 minutes. Cool, weigh, record the weight as W3, and determine the net fill weight (W1 - W3) for each container tested. [note—If the average net fill weight has been determined previously, that value may be used in place of the value (W1 - W3) above.] The requirements are met if the average leakage rate per year for the 12 containers is not more than 3.5% of the net fill weight, and none of the containers leaks more than 5.0% of the net fill weight per year. If 1 container leaks more than 5.0% per year, and if none of the containers leaks more than 7.0% per year, determine the leakage rate of an additional 24 containers as directed herein. Not more than 2 of the 36 containers leak more than 5.0% of the net fill weight per year, and none of the 36 containers leaks more than 7.0% of the net fill weight per year. Where the net fill weight is less than 15 g and the label bears an expiration date, the requirements are met if the average leakage rate of the 12 containers is not more than 525 mg per year and none of the containers leaks more than 750 mg per year. If 1 container leaks more than 750 mg per year, but not more than 1.1 g per year, determine the leakage rate of an additional 24 containers as directed herein. Not more than 2 of the 36 containers leak more than 750 mg per year, and none of the 36 containers leaks more than 1.1 g per year. This test is in addition to the customary in-line leak testing of each container.

##### Number of Discharges per Container

Perform this test only on topical aerosols fitted with dose-metering valves, at the same time as, and on the same containers used for, the test for Delivered-Dose Uniformity. Determine



the number of discharges or deliveries by counting the number of priming discharges plus those used in determining the spray contents, and continue to fire until the label claim number of discharges. The requirements are met if all the containers or inhalers tested contain not less than the number of discharges stated on the label.

#### Delivered-Dose Uniformity

The test for Delivered-Dose Uniformity is required for topical aerosols fitted with dose-metering valves. For collection of the minimum dose, proceed as directed in the test for Delivered-Dose Uniformity under Metered-Dose Inhalers and Dry Powder Inhalers, as described below, except to modify the dose sampling apparatus so that it is capable of quantitatively capturing the delivered dose from the preparation being tested. Unless otherwise stated in the individual monograph, apply the acceptance criteria for Metered-Dose Inhalers and Dry Powder Inhalers as described below.

#### NASAL SPRAYS

The following test is applicable to nasal sprays, formulated as aqueous suspensions or solutions of drug, presented in multi-dose containers and fitted with dose-metering valves. In all cases, and for all tests, prepare and test the nasal spray as directed on the label and the instructions for use.

#### Delivered-Dose Uniformity

Unless otherwise directed in the individual monograph, the drug content of the minimum delivered doses (minimum number of sprays per nostril as described on the label, or instructions for use) collected at the beginning of unit life (after priming as described on the label, or instructions for use) and at the label claim number of metered sprays, from each of 10 separate containers, must meet the following acceptance criteria: not more than 2 of the 20 doses are outside the range of 80% to 120% of label claim, and none are outside the range of 75% to 125% of label claim, while the mean for each of the beginning and end doses falls within the range of 85% to 115% of label claim. If 3–6 doses of the 20 doses collected are outside of 80% to 120% of the label claim, but none are outside of 75% to 125% of label claim, and the means for each of the beginning and end doses fall within 85% to 115% of label claim, select 20 additional containers for second-tier testing. For second-tier testing, the requirements are met if not more than 6 of the 60 doses collected are outside the range of 80% to 120% of label claim, none are outside the range of 75% to 125% of label claim, and the means for each of the beginning and end doses fall within the range of 85% to 115% of label claim.

#### sampling for delivered-dose uniformity of metered-dose nasal sprays

**General Sampling Procedure**— To ensure reproducible in-vitro dose collection, it is recommended that a mechanical means of actuating the pump assembly be employed to deliver doses for collection. The mechanical actuation procedure should have adequate controls for the critical mechanical actuation parameters (e.g., actuation force, actuation speed, stroke length, rest periods, etc.). The test must be performed on units that have been primed according to the patient-use instructions. The test unit should be actuated in a vertical or near vertical, valve-up, position. The two doses collected at the beginning and end of the container life should be the dose immediately following priming and the dose corresponding to the last label claim number of doses from the container.

For suspension products, the delivered dose should be delivered into a suitable container (e.g., scintillation vial) in which quantitative transfer from the container under test can be accomplished. A validated analytical method is employed to determine the amount of drug in each delivered dose, and data are reported as a percent of label claim. For solution products, the delivered dose can be determined gravimetrically from the weight of the delivered dose, and the concentration and density of the fill solution of the product under test.

#### METERED-DOSE INHALERS AND DRY POWDER INHALERS

The following tests are applicable to metered-dose inhalers that are formulated as suspensions or solutions of active drug in propellants and dry powder inhalers presented as single or multidose units. The following test methods are specific to the aforementioned inhalers and may require modification when testing alternative inhalation technologies (for example, breath-actuated metered-dose inhalers, or dose-metering nebulizers). However, Pharmacopeial requirements for all dose-metering inhalation dosage forms require determination of the delivered dose and Aerodynamic Size Distribution. In all cases, and for all tests, prepare and test the inhaler as directed on the label and the instructions for use. When these directions are not provided by the product manufacturer, follow the precise dose discharge directions included in the tests below.

#### Delivered-Dose Uniformity

The test for Delivered-Dose Uniformity is required for inhalers (e.g., metered-dose inhalers or dry powder inhalers) containing drug formulation (e.g., solution, suspension, or powder) either in reservoirs or in premetered dosage units, and for drug formulations packaged in reservoirs or in premetered dosage units where these containers are labeled for use with a named inhalation device. (For inhalations packaged in premetered dosage units, see also [Uniformity of Dosage Units \(905\)](#).) Note that the target-delivered dose is the expected mean drug content for a large number of delivered doses collected from many inhalers of the chosen product. In many cases, its value may depend upon the manner in which the test for delivered dose is performed. For metered-dose inhalers, the target-delivered dose is specified by the label claim, unless otherwise specified in the individual monograph. For dry powder inhalers, where the label claim is usually the packaged or metered-dose of drug, the target-delivered dose is specified in the individual monograph and is usually less than the label claim. Its value reflects the expected mean drug content for a large number of delivered doses collected from the product, using the method specified in the monograph.

Unless otherwise directed in the individual monograph, the drug content of the minimum delivered dose from each of 10 separate containers is determined in accordance with the procedure described below.

Unless otherwise specified in the individual monograph, the requirements for dosage uniformity are met if not less than 9 of the 10 doses are between 75% and 125% of the specified target-delivered dose and none is outside the range of 65% to 135% of the specified target-delivered dose. If the contents of not more than 3 doses are outside the range of 75% to 125% of the specified target-delivered dose, but within the range of 65% to 135% of the specified target-delivered dose, select 20 additional containers, and follow the prescribed procedure for analyzing 1 minimum dose from each. The requirements are met if not more than 3 results, out of the 30 values, lie outside the range of 75% to 125% of the specified target-delivered dose, and none is outside the range of 65% to 135% of the specified target-delivered dose.

#### sampling the delivered dose from metered-dose inhalers

To determine the content of active ingredient in the discharged spray from a metered-dose inhaler, use the sampling apparatus described below, using a flow rate of 28.3 L of air per minute ( $\pm 5\%$ ), unless otherwise stated in the individual monograph.

**Apparatus A— The apparatus (see [Figure 1](#))**

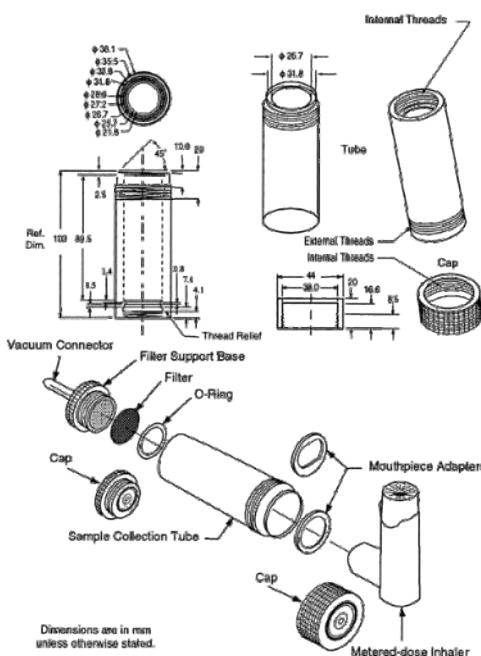


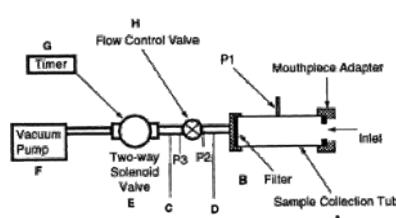
Fig. 1. Sampling apparatus for pressurized metered-dose inhalers.

consists of a filter support base with an open-mesh filter support, such as a stainless steel screen, a collection tube that is clamped or screwed to the filter support base, and a mouthpiece adapter to ensure an airtight seal between the collection tube and the mouthpiece. Use a mouthpiece adapter that ensures that the opening of the inhaler mouthpiece is flush with the front face or 2.5-mm indented shoulder in the sample collection tube, as appropriate. The vacuum connector is connected to a system comprising a vacuum source, flow regulator, and flowmeter. The source should be capable of pulling air through the complete assembly, including the filter and the inhaler to be tested, at the desired flow rate. When testing metered-dose inhalers, air should be drawn continuously through the system to avoid loss of drug into the atmosphere. The filter support base is designed to accommodate 25-mm diameter filter disks. At the airflow being used, the sample collection tube and the filter disk must be capable of quantitatively collecting the Delivered Dose. The filter disk and other materials used in the construction of the apparatus must be compatible with the drug and the solvents that are used to extract the drug from the filter. One end of the collection tube is designed to hold the filter disk tightly against the filter support base. When assembled, the joints between the components of the apparatus are airtight so that when a vacuum is applied to the base of the filter, all of the air drawn through the collection device passes through the inhaler.

**Procedure**— Prepare the inhaler for use according to the label instructions. Unless otherwise specified in the individual monograph, with the vacuum pump running, ensuring an airflow rate through the inhaler of 28.3 L of air per minute ( $\pm 5\%$ ), discharge the minimum recommended dose into the apparatus through the mouthpiece adapter by depressing the valve for a duration sufficient to ensure that the dose has been completely discharged. Detach the inhaler from Apparatus A, and disconnect the vacuum. Assay the contents of the apparatus for drug after rinsing the filter and the interior of the apparatus with a suitable solvent.

sampling the delivered dose from dry powder inhalers

To determine the content of active ingredient emitted from the mouthpiece of a dry powder inhaler, use Apparatus B (see [Figure 2](#)).

Fig. 2. Apparatus B: Sampling apparatus for dry powder inhalers. (See [Table 1](#) for component specifications.)Table 1. Component Specifications for Apparatus B (see [Fig. 2](#))

Code	Item	Description	Dimensions
A	Sample collection tube <sup>a</sup>	See <a href="#">Fig. 2</a>	34.85-mm ID $\times$ 12-cm length
B	Filter <sup>b</sup>	See <a href="#">Fig. 2</a>	47-mm glass fiber filter
C	Connector	(e.g., short metal coupling with low diameter branch to P3)	$\geq 8$ -mm ID
D	Vacuum tubing	(e.g., silicon tubing with an outside diameter of 14 mm and an internal diameter of 8 mm)	a length of suitable tubing $\geq 8$ mm ID with an internal volume of 25 $\pm 5$ mL
E	Two-way solenoid valve <sup>c</sup>	See <a href="#">Fig. 2</a>	2-way, 2-port solenoid valve having an ID $\geq 8$ mm and an opening response time of $\leq 100$ milliseconds.
F	Vacuum pump <sup>d</sup>	See <a href="#">Fig. 2</a>	Pump must be capable of drawing the required flow rate through the assembled apparatus with the dry powder inhaler in the mouthpiece adapter. Connect the pump to the solenoid valve using short and wide ( $\geq 10$ -mm ID) vacuum tubing and connectors to minimize pump capacity requirements.



G	Timer <sup>a</sup>	See <a href="#">Fig. 2</a>	The timer switches current directly to the solenoid valve for the required duration.
P1	pressure tap	See <a href="#">Fig. 2</a>	2.2-mm ID, 3.1-mm OD flush with the internal surface of the sample collection tube, centered and burr free, 59 mm from its inlet. The pressure taps P1, P2, and P3 must not be open to the atmosphere during dose collection.
P1, P2, P3	pressure measurements <sup>f</sup>		
H	Flow-control valve <sup>g</sup>	See <a href="#">Fig. 2</a>	Adjustable regulating valve with maximum Cv $\geq 1^h$ .

a An example being a Millipore product number XX40 047 00 (Millipore Corporation, 80, Ashby Road, Bedford, MA 01732), modified so that the exit tube has an ID  $\geq$  8-mm, fitted with Gelman product number 61631.

b A/E (Gelman Sciences Inc., 600 South Wagner Road, Ann Arbor, MI 48106) or equivalent.

c ASCO product number 8030G13, Automatic Switch Company, 60 Hanover Road, Florham Park, NJ 07932.

d Gast product type 1023, 1423, or 2565 (Gast Manufacturing Inc., PO Box 97, Benton Harbor, MI 49022) or equivalent.

e Eaton Product number 45610-400 (Eaton Corporation, Automotive Products Division, 901, South 12th Street, Watertown, WI 53094) or equivalent.

f An example being a PDM 210 pressure meter (Air-Neotronics Ltd., Neotronics Technology plc, Parsonage Road, Takeley, Bishop's Stortford, CM22 6PU, UK), or equivalent.

g Parker Hannifin type 8FV12LNSS (Parker Hannifin plc., Riverside Road, Barnstaple, Devon EX31 1NP, UK) or equivalent.

h Flow Coefficient, as defined by ISA S75.02 "Control valve capacity test procedure" in Standards and Recommended Practices for Instrumentation and Control, 10th ed., Vol. 2, 1989. Published by Instrument Society of America, 67 Alexander Drive, P.O. Box 1227, Research Triangle Park, NC 27709, U.S.A.

This apparatus is capable of sampling the emitted doses at a variety of airflow rates.

Apparatus B—The apparatus is similar to that described in [Figure 1](#) for testing metered-dose inhalers. In this case, however, the filter and collection tube have a larger internal diameter to accommodate 47-mm diameter filter disks. This feature enables dosage collection at higher airflow rates—up to 100 L of air per minute—when necessary. A mouthpiece adapter ensures an airtight seal between the collection tube and the mouthpiece of the dry powder inhaler being tested. The mouthpiece adapter must ensure that the tip of the inhaler mouthpiece is flush with the open end of the sample collection tube. Tubing connectors, if they are used, should have an internal diameter greater than or equal to 8 mm to preclude their own internal diameters from creating significant airflow resistance. A vacuum pump with excess capacity must be selected in order to draw air, at the designated volumetric flow rate, through both the sampling apparatus and the inhaler simultaneously. A timer-controlled, low resistance, solenoid-operated, two-way valve is interposed between the vacuum pump and the flow-control valve to control the duration of flow. This type of valve enables 4.0 L of air ( $\pm 5\%$ ) to be withdrawn from the mouthpiece of the inhaler at the designated flow rate. Flow control is achieved by ensuring that critical (sonic) flow occurs in the flow-control valve (absolute pressure ratio P3/P2  $\leq 0.5$  under conditions of steady-state flow).

Procedure—Operate the apparatus at an airflow rate that produces a pressure drop of 4 kPa (40.8 cm H<sub>2</sub>O) over the inhaler to be tested and at a duration consistent with the withdrawal of 4 L of air from the mouthpiece of the inhaler. [note—If the flow rate and duration are defined otherwise in the monograph, adjust the system to within 5% of those values.]

Determine the test flow rate using Apparatus B as follows. Insert an inhaler into the mouthpiece adapter to ensure an airtight seal. In cases where the drug packaging modifies the inhaler's resistance to airflow, use a loaded, drug-free inhaler (with previously emptied packaging). In other cases, use an unloaded (drug free) inhaler. Connect one port of a differential pressure transducer to the pressure tap, P1, and leave the other open to the atmosphere. Switch on the pump, and open the two-way solenoid valve. Adjust the flow-control valve until the pressure drop across the inhaler is 4.0 kPa (40.8 cm H<sub>2</sub>O). Ensure that critical (sonic) flow occurs in the flow-control valve by determining the individual values for absolute pressure, P2 and P3, so that their ratio P3/P2 is less than or equal to 0.5. If this criterion cannot be achieved, it is likely that the vacuum pump is worn or of insufficient capacity. Critical (sonic) flow conditions in the flow-control valve are required in order to ensure that the volumetric airflow drawn from the mouthpiece is unaffected by pump fluctuations and changes in airflow resistance of the inhaler. Remove the inhaler from the mouthpiece adapter and without disturbing the flow-control valve, measure the airflow rate drawn from the mouthpiece, Qout, by connecting a flowmeter to the mouthpiece adaptor in an airtight fashion. Use a flowmeter calibrated for the volumetric flow leaving the meter in an airtight fashion to directly determine Qout or, if such a meter is unobtainable, calculate the volumetric flow leaving the meter (Qout) using the ideal gas law. For example, for a meter calibrated for the entering volumetric flow (Qin), use the formula:

$$Qout = QinP0 / (P0 - \Delta P)$$

where P0 is the atmospheric pressure and  $\Delta P$  is the pressure drop over the meter. If the flow rate is greater than 100 L of air per minute, adjust the flow-control valve until Qout equals 100 L per minute; otherwise, record the value of Qout, and leave the flow-control valve undisturbed. Define the test flow duration, T = 240/Qout, in seconds, so that a volume of 4.0 L of air ( $\pm 5\%$ ) is withdrawn from the inhaler at the test flow rate Qout, and adjust the timer controlling the operation of the two-way solenoid valve accordingly. Prime or load the inhaler with powder for inhalation according to the labeled instructions. With the vacuum pump running and the solenoid valve closed, insert the inhaler mouthpiece horizontally into the mouthpiece adapter. Discharge the powder into the sampling apparatus by activating the timer controlling the solenoid valve and withdrawing 4.0 L of air from the inhaler at the previously defined airflow rate. If the labeled instructions so direct, repeat the operation so as to simulate the use of the inhaler by the patient (e.g., inhale two or three times, if necessary, to empty the capsule). Repeat the whole operation  $n - 1$  times beginning with the text, "Prime or load the inhaler with powder," where n is the number of times defined in the labeling as the minimum recommended dose. Detach the dry powder inhaler from the sampling apparatus, and disconnect the vacuum tubing, D. Assay the contents of the apparatus for drug after rinsing the filter and the interior of the apparatus with a suitable solvent. Where specified in individual monographs, perform this test under conditions of controlled temperature and humidity.

#### Delivered-Dose Uniformity over the Entire Contents

The test for Delivered-Dose Uniformity over the Entire Contents is required for inhalers (e.g., metered-dose inhalers or dry powder inhalers) containing multiple doses of drug formulation (e.g., solution, suspension, or dry powder) either in reservoirs or in premetered dosage units (e.g., blisters), and for drug formulations packaged in reservoirs or in multiple-dose assemblies of premetered dosage units that have a predetermined dose sequence, where these multiple-dose assemblies are labeled for use with a named inhalation device.

The test for Delivered-Dose Uniformity over the Entire Contents also ensures that multidose products supply the total number of discharges stated on the label. Unless otherwise directed in the individual monograph, the drug content of at least 9 of the 10 doses collected from one inhaler, in accordance with the procedure below, are between 75% and 125% of the target-delivered dose, and none is outside the range of 65% to 135% of the target-delivered dose. If the contents of not more than 3 doses are outside the range of 75% to 125%, but within the range of 65% to 135% of the target-delivered dose, select 2 additional inhalers, and follow the prescribed procedure for analyzing 10 doses from each. The requirements are met if not more than 3 results, out of the 30 values, lie outside the range of 75% to 125% of the target-delivered dose, and none is outside the range of 65% to 135% of the target-

delivered dose.

#### metered-dose inhalers

Apparatus— Use Apparatus A as directed in Sampling the Delivered-Dose from Metered-Dose Inhalers under Delivered-Dose Uniformity at a flow rate of 28.3 L of air per minute ( $\pm 5\%$ ).

Procedure— A single dose is defined as the number of sprays specified in the product labeling as the minimum recommended dose. Select a single metered-dose inhaler, and follow the labeled instructions for priming, shaking, cleaning, and firing the inhaler throughout. Unless otherwise prescribed in the patient instructions, shake the inhaler for 5 seconds, and fire one minimum recommended dose to waste. Wait for 5 seconds, and collect the next dose. Detach the inhaler from Apparatus A, and disconnect the vacuum. Assay the contents of the apparatus for drug after rinsing the filter and the interior of the apparatus with a suitable solvent. Collect two more doses, allowing at least 5 seconds between doses. Discharge the device to waste, waiting for not less than 5 seconds between actuations (unless otherwise specified in the individual monograph), until  $(n/2) + 1$  minimum recommended doses remain, in which  $n$  is the number of minimum recommended doses on the label. Collect four more doses, allowing at least 5 seconds between doses, unless otherwise specified in the individual monograph. Discharge the device to waste, as before, until three doses remain. Collect the final three doses, allowing at least 5 seconds between doses. Note that the rate of discharges to waste should not be such as to cause excessive canister cooling.

#### dry powder inhalers

Apparatus— Use Apparatus B as directed in Sampling the Delivered Dose from Dry Powder Inhalers under Delivered-Dose Uniformity at the appropriate airflow rate for testing.

Procedure— Proceed as directed for Procedure in Sampling the Delivered Dose from Dry Powder Inhalers under Delivered-Dose Uniformity. A single dose is defined as the number of actuations stated in the product labeling as the minimum recommended dose. Select a single inhaler and follow the labeled instructions for loading with powder, discharging and cleaning throughout. Collect a total of 10 doses—three doses at the beginning, four in the middle [ $(n/2) - 1$  to  $(n/2) + 2$ , where  $n$  is the number of minimum recommended doses on the label], and three at the end—of the labeled contents following the labeled instructions. Prior to collecting each of the doses to be analyzed, clean the inhaler as directed in the labeling.

#### Particle Size

The particle or droplet size distribution in the spray discharged from metered-dose inhalers, and the particle size distribution in the cloud discharged from dry powder inhalers, are important characteristics used in judging inhaler performance. While particle size measurement by microscopy can be used to evaluate the number of large particles, agglomerates, and foreign particulates in the emissions of metered-dose inhalers (e.g., Epinephrine Bitartrate Inhalation Aerosol), whenever possible this test should be replaced with a method to determine the aerodynamic size distribution of the drug aerosol leaving the inhaler. The aerodynamic size distribution defines the manner in which an aerosol deposits during inhalation. When there is a log-normal distribution, the aerodynamic size distribution may be characterized by the mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD). The aerodynamic size distribution of the drug leaving metered-dose and dry powder inhalers is determined using Apparatus 1, 2, 3, 4, 5, or 6 as specified in this chapter. A fine particle dose or fine particle fraction can also be determined as that portion of the inhaler output having an aerodynamic diameter less than the size defined in the individual monograph. This may be expected to correlate with the drug dose or that fraction of the drug dose that penetrates the lung during inhalation. Individual monographs may also define the emitted fractions of the delivered dose in more than one aerodynamic size range.

#### aerodynamic size distribution

Cascade impaction devices classify aerosol particles and droplets on the basis of those particles' aerodynamic diameters. The principle of their operation, whereby they separate aerosol particles and droplets from a moving airstream on the basis of particle or droplet inertia, is shown in [Figure 3](#).

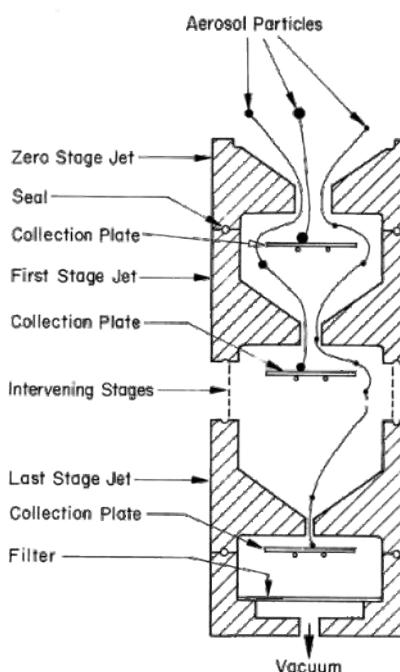


Fig. 3. Schematic representation of the principle of operation of cascade impactors. (A single jet per impactor stage is shown. Impactors with multiple jets in each stage function in the same manner.)

Because the dimensions of the induction port used to connect inhalers to the cascade impactors and impingers (shown in Apparatus 1, 2, 3, 4, 5, and 6) also define the mass of drug that enters the aerodynamic sizing device, these are carefully defined and, where possible, are held constant between each apparatus (see [Figures 4, 6, 7, 8, and 9](#)).

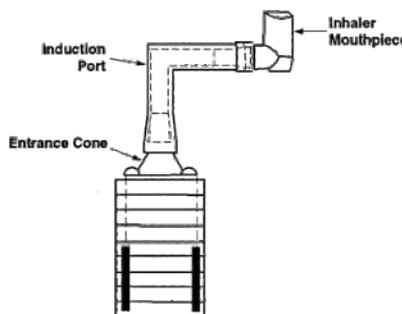


Fig. 4. Apparatus 1: Assembly of induction port and entrance cone mounted on cascade impactor.

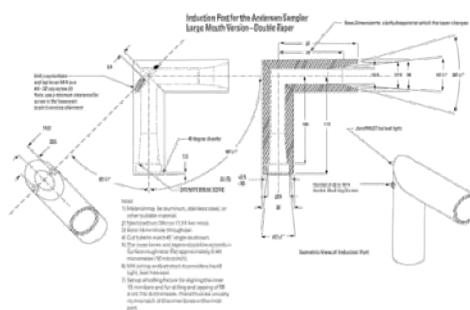


Fig. 4a. Apparatus 1: Expanded view of induction port for use with metered-dose and dry powder inhalers.

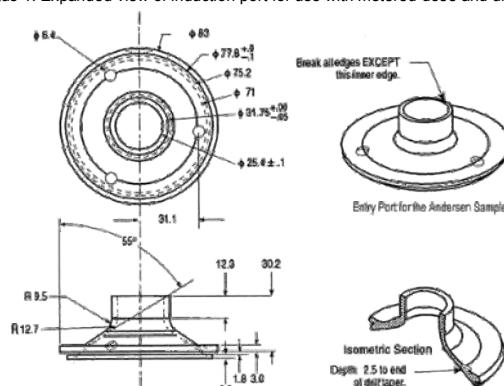


Fig. 4b. Apparatus 1: Expanded view of the entrance cone for mounting induction port on the Andersen cascade impactor without preseparators. Material may be aluminum, stainless steel, or other suitable material. Surface roughness (Ra) should be approximately 0.4  $\mu\text{m}$ .

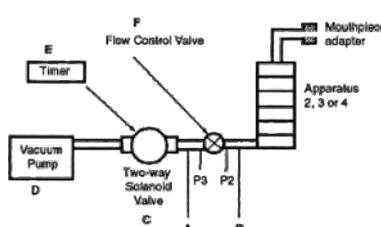


Fig. 5. Apparatus 2, 3, 4, or 5: General control equipment. (See [Table 3](#) for component specifications.)

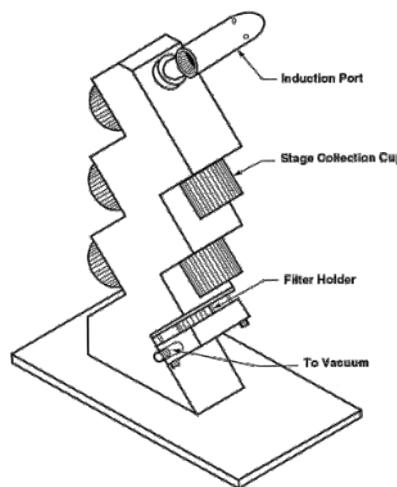


Fig. 6. Apparatus 2: Assembly of induction port, stage collector, and filter holder. (Marple-Miller impactor, Model 160 with USP induction port.)

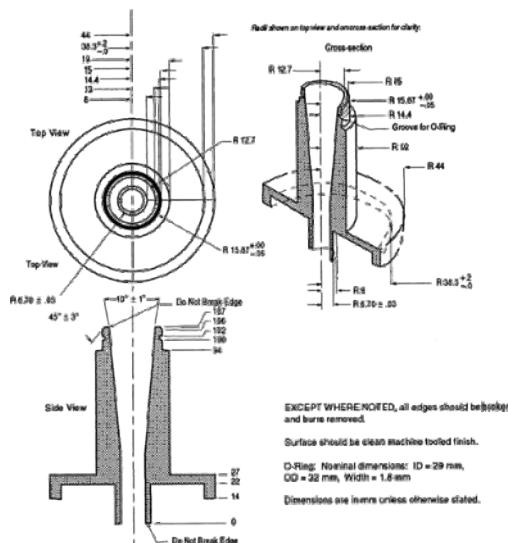


Fig. 7. Apparatus 3: Expanded views of top for the Andersen preseparator adapted to the USP induction port. Material may be aluminum, stainless steel, or other suitable material; interior bore should be polished to surface roughness ( $R_a$ ) approximately 0.4  $\mu\text{m}$ .

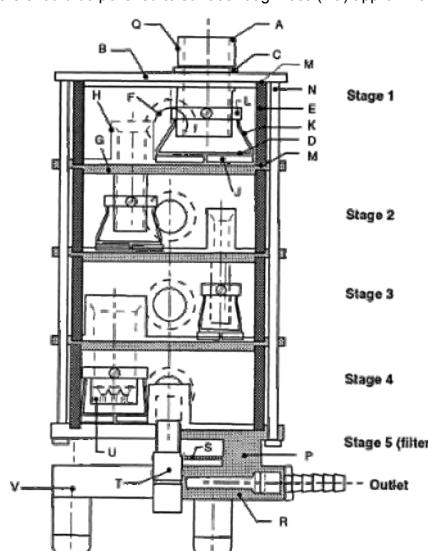


Fig. 8. Apparatus 4: Schematic of multistage liquid impinger. (See Table 4 for component specifications.)

Because the size distributions produced by different impactors are often a function of impactor design and the airflow rate through them, there is a need to standardize the instruments that are used to test inhalers (i.e., Apparatus 1 or 6 for metered-dose inhalers) or to provide guidelines on system suitability where different apparatuses may be used (i.e., Apparatus 2, 3, 4, or 5 for dry powder inhalers).

Because of the varied nature of the formulations and devices being tested, the cascade impaction system and technique selected for testing an inhaler should fulfill a number of criteria.

**Stage Mensuration**— Manufacturers of cascade impaction devices provide a definitive calibration for the separation characteristics of each impaction stage in terms of the relationship between the stage collection efficiency and the aerodynamic diameter of particles and droplets passing through it as an aerosol. Calibration is a property of the jet dimensions, the spatial arrangement of the jet and its collection surface, and the airflow rate passing through it. Because jets can corrode and wear over time, the critical dimensions of each stage,



...which define that impaction stage's calibration, must be measured on a regular basis. This process, known as stage mensuration, replaces the need for repetitive calibration (using standard aerosols) and ensures that only devices that conform to specifications are used for testing inhaler output. The process involves the measurement and adjustment of the critical dimensions of the instrument.

**Interstage Drug Loss (wall losses)—** Where method variations are possible and there is no apparatus specified in the monograph, the selected technique should ensure that not more than 5% of the inhaler's total delivered drug mass (into the impactor) is subject to loss between the impaction device's sample collection surfaces. In the event that interstage drug losses are known to be greater than 5%, either the procedure should be performed in such a way that wall losses are included along with the associated collection plate, or an alternative apparatus should be used. As an example, the following procedures described for Apparatus 1 and 3 have been written to include wall losses along with the associated collection plate. Provided, however, that such losses are known to be less than or equal to 5% of the total delivered drug mass into the impactor and that there are no instructions to the contrary in an individual monograph, the technique may be simplified by only assaying drug on the collection plates.

**Re-Entrainment—** Where method variations are possible, the selected technique should seek to minimize particle re-entrainment (from an upper to a lower impaction stage) on stages that contribute to size fractions defined in the individual monograph, especially where this may affect the amounts of drug collected. Minimizing the number of sampled doses, the use of coated particle collection surfaces, and proving that multiple-dose techniques produce statistically similar results to those from smaller numbers of doses, are all methods that can be used for this purpose. In the event that re-entrainment cannot be avoided, the number of doses collected, the time interval between doses, and the total duration of airflow through the cascade impaction device should be standardized. Under these circumstances, the presentation of impaction data should not presume the validity of the impactor's calibration (i.e., aerodynamic diameter ranges should not be assigned to drug masses collected on specific stages).

By using appropriate assay methods and a suitable mensurated impaction device, aerodynamic particle size distributions can be determined for drugs leaving the mouthpieces of metered-dose or dry powder inhalers. If temperature or humidity limits for use of the inhaler are stated on the label, it may be necessary to control the temperature and humidity of the air surrounding and passing through the device to conform to those limits. Ambient conditions are presumed, unless otherwise specified in individual monographs.

**Mass Balance—** In addition to the size distribution, good analytical practice dictates that a mass-balance be performed in order to confirm that the amount of the drug discharged from the inhaler, which is captured and measured in the induction port-cascade impactor apparatus, is within an acceptable range around the expected value. The total mass of drug collected in all of the components (material balance) divided by the total number of minimum recommended doses discharged is not less than 75% and not more than 125% of the average minimum recommended dose determined during testing for Delivered-Dose Uniformity. This is not a test of the inhaler but serves to ensure that the test results are valid.

Use one of the multistage impaction devices shown below, or an equivalent, to determine aerodynamic particle size distributions of drugs leaving the mouthpieces of metered-dose or dry powder inhalers. Apparatus 1 and 6 (Figures 4 and 9 (without preseparator), respectively) are intended for use with metered-dose inhalers at a single airflow rate. Apparatus 2, 3, 4, and 5 (Figures 6, 7, 8, and 9, respectively) are intended for use with dry powder inhalers at the appropriate airflow rate,  $Q_{out}$ , determined earlier, provided that the value of  $Q_{out}$  falls in the range 30–100 L per minute.

**note—**If  $Q_{out}$  is greater than 100 L per minute, testing should be performed with  $Q_{out}$  set at 100 L per minute; if  $Q_{out}$  is less than 30 L per minute, testing is performed with  $Q_{out}$  at 30 L per minute.

**Apparatus 1 for Metered-Dose Inhalers—** Use this apparatus, or an equivalent, at a flow rate of 28.3 L per minute ( $\pm 5\%$ ), as specified by the manufacturer of the cascade impactor.

**Design—** The design and assembly of this apparatus and the induction port to connect the device to an inhaler are shown in Figures 4, 4a, and 4b<sup>4</sup>.

Critical engineering dimensions applied by manufacturers to the stages of Apparatus 1 are provided in Table 2. During use, some occlusion and blockage of jet nozzles may occur and therefore, "in use" mensuration tolerances need to be justified.

Table 2. Critical Dimensions for the Jet Nozzles of Apparatus 1

Stage #	Number of Jets	Nozzle Diameter (mm)
0	96	$2.55 \pm 0.025$
1	96	$1.89 \pm 0.025$
2	400	$0.914 \pm 0.0127$
3	400	$0.711 \pm 0.0127$
4	400	$0.533 \pm 0.0127$
5	400	$0.343 \pm 0.0127$
6	400	$0.254 \pm 0.0127$
7	201	$0.254 \pm 0.0127$

**Procedure—** Set up the multistage cascade impactor as described in the manufacturer's literature with an after filter below the final stage to capture any fine particles that otherwise would escape from the device. To ensure efficient particle capture, coat the particle collection surface of each stage with glycerol, silicone oil, or other suitable liquid typically deposited from a volatile solvent, unless it has been demonstrated to be unnecessary. Attach the induction port and mouthpiece adapter to produce an airtight seal between the inhaler mouthpiece and the induction port as shown in Figure 4. Use a mouthpiece adapter that ensures that the tip of the inhaler mouthpiece is flush with the open end of the induction port. Ensure that the various stages of the cascade impactor are connected with airtight seals to prevent leaks. Turn on the vacuum pump to draw air through the cascade impactor, and calibrate the airflow through the system with an appropriate flowmeter attached to the open end of the induction port. Adjust the flow-control valve on the vacuum pump to achieve steady flow through the system at the required rate, and ensure that the airflow through the system is within  $\pm 5\%$  of the flow rate specified by the manufacturer. Unless otherwise prescribed in the patient instructions, shake the inhaler for 5 seconds and discharge one delivery to waste. With the vacuum pump running, insert the mouthpiece into the mouthpiece adapter and immediately fire the minimum recommended dose into the cascade impactor. Keep the valve depressed for a duration sufficient to ensure that the dose has been completely discharged. If additional sprays are required for the sample, wait for 5 seconds before removing the inhaler from the mouthpiece adapter, shake the inhaler, reinsert it into the mouthpiece adapter, and immediately fire the next minimum recommended dose. Repeat until the required number of doses have been discharged. The number of minimum recommended doses discharged must be sufficient to ensure an accurate and precise determination of Aerodynamic Size Distribution. [note—The number of minimum recommended doses is typically not greater than 10.] After the last dose has been discharged, remove the inhaler from the mouthpiece adapter. Rinse the mouthpiece adapter and induction port with a suitable solvent, and dilute quantitatively to an appropriate volume. Disassemble the cascade impactor, place each stage and its associated collection plate or filter in a separate container, and rinse the drug from each of them. [note—If it has been determined that wall losses in the impactor are less than or equal to 5%, then the collection plates only may be used.]

Dilute each quantitatively to an appropriate volume. Using the method of analysis specified in the individual monograph, determine the mass of drug collected in each of the components. To analyze the data, proceed as directed under Data Analysis.

#### Apparatus 2 for Dry Powder Inhalers—

**Design—** The design and assembly of Apparatus 2, and the induction port to connect the device to an inhaler, are shown in Figure 6.<sup>2</sup> [note—The induction port is shown in detail in Figure 4a.] The impactor has five impaction stages and an after filter. At a volumetric airflow rate of 60 L per minute (the nominal flow rate,  $Q_n$ ), the cutoff aerodynamic diameters  $D_{50,Qn}$  of Stages 1 to 5 are 10, 5, 2.5, 1.25, and 0.625  $\mu\text{m}$ , respectively. The after filter effectively retains aerosolized drug in the particle size range up to 0.625  $\mu\text{m}$ . Set up the multistage cascade impactor with the control system as specified in Figure 5. To ensure efficient particle capture, coat the particle collection surface of each stage with glycerol, silicone oil, or other suitable liquid typically deposited from a volatile solvent, unless it has been demonstrated to be unnecessary. Assemble the impactor as described in the manufacturer's literature with an after filter below the final stage to capture any fine particles that otherwise would escape from the device. Attach the induction port and mouthpiece adapter to produce an airtight seal between the inhaler mouthpiece and the induction port. Use a mouthpiece adapter that ensures that the tip of the inhaler mouthpiece is flush with the open end of the induction port. Ensure that the various stages of the cascade impactor are connected with airtight seals to prevent leaks.

Turn on the vacuum pump, open the solenoid valve, and calibrate the airflow through the system as follows. Connect a flowmeter to the induction port. Use a flowmeter calibrated for the volumetric flow leaving the meter to directly determine  $Q_{out}$ , or, if such a meter is unobtainable, calculate the volumetric flow leaving the meter ( $Q_{out}$ ) using the ideal gas law. For



example, for a meter calibrated for the entering volumetric flow ( $Q_{in}$ ), use the formula:

$$Q_{out} = Q_{in}P_0 / (P_0 - \Delta P)$$

where  $P_0$  is the atmospheric pressure and  $\Delta P$  is the pressure drop over the meter. Adjust the flow-control valve to achieve a steady flow through the system at the required rate,  $Q_{out}$ , so that  $Q_{out}$  is within  $\pm 5\%$  of the value determined during testing for Delivered-Dose Uniformity. Ensure that critical flow occurs in the flow-control valve, at the airflow rate to be used during testing, by using the following procedure. With the inhaler in place, and the intended flow running, measure the absolute pressure on both sides of the flow-control valve ( $P_2$  and  $P_3$  in [Figure 5](#)). A ratio of  $P_3/P_2 \leq 0.5$  indicates critical flow. Switch to a more powerful pump, and remeasure the test flow rate if  $P_3/P_2 > 0.5$ . Adjust the timer controlling the operation of the two-way solenoid valve so that it opens this valve for a duration of  $T$  seconds as determined during testing for Delivered-Dose Uniformity. Prime or load the dry powder inhaler with powder for inhalation according to the labeled instructions. With the vacuum pump running and the two-way solenoid valve closed, insert the inhaler mouthpiece, held horizontally, into the induction port mouthpiece adapter. Discharge the powder into the apparatus by opening the two-way solenoid valve for a duration of  $T$  seconds. After the two-way solenoid valve has closed, remove the inhaler from the mouthpiece adapter. If additional doses are required for the sample, reload the inhaler according to the labeled instructions, reinsert the mouthpiece into the mouthpiece adapter, and repeat the operation until the required number of doses have been discharged. After discharge of the last dose, switch off the vacuum pump.

Rinse the mouthpiece adapter and induction port with a suitable solvent, and quantitatively dilute to an appropriate volume. Disassemble the cascade impactor, and place the after filter in a separate container. Rinse the drug from each of the stages and the filter, and quantitatively dilute each to an appropriate volume. Using the method of analysis specified in the individual monograph, determine the mass of drug collected in each of the components. Determine the cutoff diameters of each of the individual stages of the impactor, at the value of

$$Q = Q_{out} \text{ employed in the test by the formula:}$$

$$D_{50,Q} = D_{50,Qn}(Q_n / Q_1) / 2, \text{ (Eq. 1)}$$

where  $D_{50,Q}$  is the cutoff diameter at the flow rate,  $Q$ , employed in the test, and the subscript,  $n$ , refers to the nominal values determined when  $Q_n$  equals 60 L per minute. Thus, when  $Q$  equals 40 L per minute, the cutoff diameter of Stage 2 is given by the formula:

$$D_{50,40LPM} = 5 \mu\text{m} \times [60/40]1 / 2 = 6.1 \mu\text{m}.$$

**General Procedure**— Perform the test using Apparatus 2 at the airflow rate,  $Q_{out}$  determined earlier, during testing for Delivered-Dose Uniformity, provided  $Q_{out}$  is less than or equal to 100 L per minute. [note—If  $Q_{out}$  is greater than 100 L per minute, use an airflow rate of 100 L per minute.] Connect the apparatus to a flow control system that is based upon critical (sonic) flow as specified in [Figure 5](#) (see also [Table 3](#)).

Table 3. Component Specifications for [Figure 5](#)

Code	Item	Description	Dimensions
A	Connector	(e.g., short metal coupling with low diameter branch to $P_3$ )	$\geq 8\text{-mm ID}$
B	Vacuum tubing	(e.g., silicon tubing with an outside diameter of 14 mm and an internal diameter of 8 mm)	A length of suitable tubing $\geq 8$ mm ID with an internal volume of $25 \pm 5$ mL.
C	Two-way solenoid valve <sup>a</sup>	See Fig. 5	2-way, 2-port solenoid valve having an ID $\geq 8$ mm and an opening response time of $\leq 100$ milliseconds.
D	Vacuum pump <sup>b</sup>	See Fig. 5	Pump must be capable of drawing the required flow rate through the assembled apparatus with the dry powder inhaler in the mouthpiece adapter. Connect the pump to the solenoid valve using short and wide ( $\geq 10$ -mm ID) vacuum tubing and connectors to minimize pump capacity requirements.
E P2, P3	Timer <sup>c</sup> Pressure measurements	See Fig. 5	The timer switches current directly to the solenoid valve for the required duration. Determine under steady-state flow conditions with an absolute pressure transducer.
F	Flow control valve <sup>d</sup>	See Fig. 5	Adjustable regulating valve with maximum $C_v \geq 1$ .

<sup>a</sup> An example being ASCO product number 8030G13 (Automatic Switch Company, 60 Hanover Road, Florham Park, NJ 07932) or equivalent. See also Footnote h in [Table 1](#).

<sup>b</sup> Gast product type 1023, 1423, or 2565 (Gast Manufacturing Inc., PO Box 97, Benton Harbor, MI 49022) or equivalent.

<sup>c</sup> An example being Eaton Product number 45610-400 (Eaton Corporation, Automotive Products Division, 901 South 12th Street, Watertown, WI 53094) or equivalent.

<sup>d</sup> Parker Hannifin type 8FV12LNSS, or equivalent (Parker Hannifin plc, Riverside Road, Barnstable, Devon EX31 1NP, UK). See also Footnote h in [Table 1](#).

Table 4. Component Units of Multistage Liquid Impinger (see [Figure 8](#))

Code <sup>1</sup>	Item	Description	Dimensions <sup>2</sup>
A,H	Jet tube	Metal tube screwed onto partition wall sealed by gasket (C), polished inner surface	see <a href="#">Figure 8a</a>
B,G	Partition wall	Circular metal plate, diameter	120
		Thickness	see <a href="#">Figure 8a</a>
C	Gasket	e.g., PTFE	to fit jet tube
D	Impaction plate	Porosity O sintered-glass disk,	
		Diameter	see <a href="#">Figure 8a</a>
E	Glass cylinder	Plane polished cut glass tube	
		Height, including gaskets	46
		Outer diameter	100
		Wall thickness	3.5

	Sampling port (F) diameter	18
	Stopper in sampling port	ISO 24/25
J	Metal frame	I-profiled circular frame with slit
	Inner diameter	to fit impaction plate
	Height	4
	Thickness of horizontal section	0.5
	Thickness of vertical section	2
K	Wire	Steel wire interconnecting metal frame and sleeve (two for each frame)
	Diameter	1
L	Sleeve	Metal sleeve secured on jet tube by screw
	Inner diameter	to fit jet tube
	Height	6
	Thickness	5
M	Gasket	e.g., silicone
		to fit glass cylinder
N	Bolt	Metal bolt with nut (six pairs), length
	Diameter	4
P	O-ring	Rubber O-ring, diameter $\times$ thickness
Q	O-ring	Rubber O-ring, diameter $\times$ thickness
R	Filter holder	Metal housing with stand and outlet
S	Filter support	Perforated sheet metal, diameter
	Hole diameter	65
	Distance between holes (center-points)	3
T	Snap-locks	
U	Multi-jet tube	Jet tube (H) ending in multijet arrangement
V	Outlet	Outlet and nozzle for connection to vacuum
		Internal diameter $\geq 10$ (Figure 8b)

1 See [Fig. 8](#).

2 Measurements in mm unless otherwise stated.

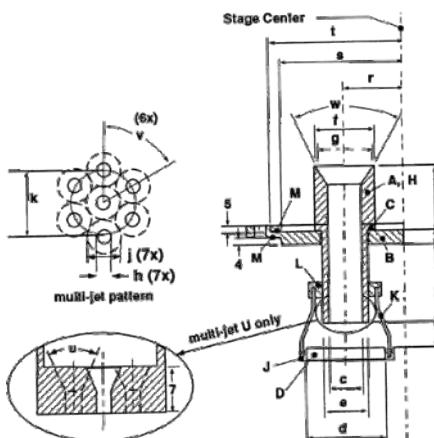


Fig. 8a. Apparatus 4: Details of jet tube and impaction plate. Inserts show end of multi-jet tube U leading to Stage 4. (See [Table 5](#) for dimension specifications.)

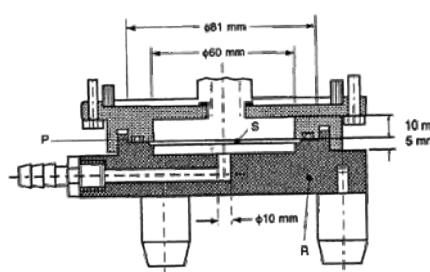


Fig. 8b. Apparatus 4: Expanded view of Stage 5. (See [Table 4](#) for component specifications.)

Table 5. Apparatus 4: Dimensions<sup>1</sup> of Jet Tube with Impaction Plate (see [Fig. 8a](#)).

Type	Code <sup>2</sup>	Stage 1	Stage 2	Stage 3	Stage 4	Filter (Stage 5)
Distance	1	9.5 (-0, +.5)	5.5 (-0, +.5)	4.0 (-0, +.5)	6.0 (-0, +.5)	n.a.
Distance	2	26	31	33	30.5	0

Distance	3	8	5	5	5	5
Distance	4	3	3	3	3	n.a.
Distance	5	0	3	3	3	3
Distance	6 <sup>3</sup>	20	25	25	25	25
Distance	7	n.a.	n.a.	n.a.	8.5	n.a.
Diameter	c	25	14	8.0(±0.1)	21	14
Diameter	d	50	30	20	30	n.a.
Diameter	e	27.9	16.5	10.5	23.9	n.a.
Diameter	f	31.75 (−.05, +.00)	22	14	31	22
Diameter	g	25.4	21	13	30	21
Diameter	h	n.a.	n.a.	n.a.	2.70 (±.05)	n.a.
Diameter	j	n.a.	n.a.	n.a.	6.3	n.a.
Diameter	k	n.a.	n.a.	n.a.	12.6	n.a.
Radius <sup>4</sup>	r	16	22	27	28.5	0
Radius <sup>4</sup>	s	46	46	46	46	n.a.
Radius <sup>4</sup>	t	n.a.	50	50	50	50
Angle	w	10°	53°	53°	53°	53°
Angle	u	n.a.	n.a.	n.a.	45°	n.a.
Angle	v	n.a.	n.a.	n.a.	60°	n.a.

1 Measurements in mm with tolerances according to ISO 2768-m, unless otherwise stated.

2 See [Fig. 8a](#).

3 Including gasket.

4 Relative centerline of stage compartment.

n.a.: not applicable.

Under steady flow conditions, at the appropriate volumetric airflow rate through the entire apparatus, ensure that critical (sonic) flow occurs in the flow control valve by determining the individual values for absolute pressure, P2 and P3, so that their ratio P3/P2 is less than or equal to 0.5. Coat the particle collection surface of each of the stages of the cascade impactor to ensure that particles that have impacted on a given stage are not re-entrained in the flowing airstream, unless this has been shown to be unnecessary. Analyze the data as directed under Data Analysis.

#### Apparatus 3 for Dry Powder Inhalers—

Design— Apparatus 3 is identical to [Apparatus 1 \(Figure 4\)](#), except that the manufacturer's preseparator is added atop Stage 0 to collect large masses of noninhalable powder prior to their entry into the impactor, and the outlet nipple, used to connect to vacuum tubing B ([Figure 5](#)), is replaced with one having an internal diameter  $\geq 8$  mm. To connect the preseparator of the impactor to the induction port ([Figure 4a](#)), a specially designed top for the preseparator must be used. This is shown in [Figure 7<sup>3</sup>](#). The impactor, therefore, has eight stages, a preseparator (to collect large particulates), and an after filter. At a volumetric airflow rate of 28.3 L per minute (the nominal flow rate, Qn), the cutoff aerodynamic diameters D50,Qn of Stages 0 to 7 are 9.0, 5.8, 4.7, 3.3, 2.1, 1.1, 0.7, and 0.4  $\mu$ m, respectively. The after filter effectively retains aerosolized drug in the particle size range up to 0.4  $\mu$ m. Connect the cascade impactor into the control system specified in [Figure 5](#). Omit Stage 6 and Stage 7 from the impactor if the test flow rate, Qout, used during testing for Delivered-Dose Uniformity was greater than or equal to 60 L per minute. To ensure efficient particle capture, coat the particle collection surface of each stage with glycerol, silicone oil, or other suitable liquid typically deposited from a volatile solvent, unless it has been demonstrated to be unnecessary. Assemble the impactor as described in the manufacturer's literature with an after filter below the final stage to capture any fine particles that otherwise would escape from the device. Place an appropriate volume (up to 10 mL) of an appropriate solvent into the preseparator, or coat the particle collection surfaces of the preseparator to prevent re-entrainment of impacted particles. [Caution—Some solvents form flammable vapor-air mixtures that may be ignited during passage through a vacuum pump. Take appropriate precautions (alternative solvents, use of vapor traps, minimal pump operating times, etc.) to ensure operator safety during testing.] Attach a molded mouthpiece adapter to the end of the induction port to produce an airtight seal between the inhaler mouthpiece and the induction port. Use a mouthpiece adapter that ensures that the tip of the inhaler mouthpiece is flush with the open end of the induction port. Ensure that the various stages of the cascade impactor are connected with airtight seals to prevent leaks.

Turn on the vacuum pump, open the two-way solenoid valve, and calibrate the airflow through the system as follows. Prime or load the dry powder inhaler with powder for inhalation according to the labeled instructions. With the vacuum pump running and the two-way solenoid valve closed, insert the inhaler mouthpiece, held horizontally, into the induction port mouthpiece adapter. Once the inhaler is positioned, discharge the powder into the apparatus by activating the timer and opening the two-way solenoid valve for the required duration, T  $\pm 5\%$ , as determined during testing for Delivered-Dose Uniformity. After the two-way solenoid valve has closed, remove the inhaler from the mouthpiece adapter. If additional doses are required for the sample, reload the inhaler according to the labeled instructions, reinsert the mouthpiece into the mouthpiece adapter, and repeat the operation until the required number of doses have been discharged. After discharge of the last dose, remove the inhaler from the mouthpiece adapter, and switch off the vacuum pump.

Carefully disassemble the apparatus. Using a suitable solvent, rinse the drug from the mouthpiece adapter, induction port, and preseparator, and quantitatively dilute to an appropriate volume. Rinse the drug from each stage, and the impaction plate immediately below, into appropriately sized flasks. Quantitatively dilute each flask to an appropriate volume. Using the method of analysis specified in the individual monograph, determine the mass of drug collected in each of the samples. The aerodynamic cutoff diameters of the individual stages of this device, in the airflow range between 30 and 100 L per minute, are currently not well established. Do not use the formula in Equation 1 to calculate cutoff diameters.

Procedure— Proceed as directed in the General Procedure under Apparatus 2, except to use Apparatus 3.

#### Apparatus 4 for Dry Powder Inhalers—

note—Apparatus 4, the multistage liquid impinger, has a small number of stages and is used extensively outside the USA. It is provided here for the benefit of users in countries other than the USA.

Design— The design and assembly of Apparatus 4 are shown in [Figs. 8, 8a, and 8b<sup>4</sup>](#). The induction port, used to connect the device to an inhaler, is shown in [Fig. 4a](#). The device is a multi-stage liquid impinger consisting of impaction Stages 1, 2, 3, and 4 and an integral after filter (Stage 5). The collection stages of the liquid impinger (see [Fig. 8](#) and [Table 4](#)) are kept moist, unlike those of traditional impactors, such as Apparatus 1, 2, 3, 5, and 6; wetting may produce an effect similar to coating the stages of Apparatus 2, 3, 5, and 6 at certain flow rates, although this should be confirmed by demonstrating control over re-entrainment as described earlier. An impaction stage comprises an upper horizontal metal partition wall (B) through which a metal inlet jet tube (A) with its impaction plate (D) is protruding; a glass cylinder (E) with sampling port (F), forming the vertical wall of the stage; and a lower horizontal metal partition wall (G) through which a jet tube (H) connects to the lower stage. The tube into Stage 4 (U) ends in a multi-jet arrangement. The impaction plate (D) is secured in a metal frame (J), which is fastened by two wires (K) to a sleeve (L) secured on the jet tube (C). For more detail of the jet tube and impaction plate, see [Fig. 8a](#). The horizontal plane of the collection plate is perpendicular to the axis of the jet tube and centrally aligned. The upper surface of the impaction plate is slightly raised above the edge of the metal frame. A recess around the perimeter of the horizontal partition wall guides the position of the glass cylinder. The glass cylinders are sealed against the horizontal partition walls with gaskets (M) and clamped together by six bolts (N). The sampling ports are sealed by stoppers. The bottom side of the lower partition wall of Stage 4 has a concentric protrusion fitted with a rubber O-ring (P) that seals against the edge of a filter placed in the filter holder. The filter holder (R) is a basin with a concentric recess in which a perforated filter support (S) is flush-fitted. The filter holder is designed for 76-mm diameter filters. The whole impaction stage assembly is clamped onto the filter holder by two snap locks (T). The impinger is equipped with an induction port ([Fig. 4a](#)) that fits onto the Stage 1 inlet jet tube. A rubber O-ring on the jet tube provides an airtight connection to the induction port. An elastomeric mouthpiece adapter to fit the inhaler being tested provides an airtight seal between the inhaler and the induction port.



At a volumetric airflow rate of 60 L per minute (the nominal flow rate,  $Q_n$ ), the cutoff aerodynamic diameters  $D_{50,Q_n}$  of Stages 1 to 4 are 13.0, 6.8, 3.1, and 1.7  $\mu\text{m}$ , respectively. The after filter effectively retains aerosolized drug in the particle size range up to 1.7  $\mu\text{m}$ . Ensure that Apparatus 4 is clean and free of drug solution from any previous tests. Place a 76-mm diameter filter in the filter stage, and assemble the apparatus. Use a low pressure filter capable of quantitatively collecting the passing drug aerosol, which also allows a quantitative recovery of the collected drug. Set up Apparatus 4 using the control system as specified in [Figure 5](#). Attach the induction port ([Figure 4a](#)) and mouthpiece adapter to produce an airtight seal between the inhaler mouthpiece and the induction port. Use a mouthpiece adapter that ensures that the tip of the inhaler mouthpiece is flush with the open end of the induction port. Ensure that the various stages of the apparatus are connected with airtight seals to prevent leaks. Turn on the vacuum pump, open the two-way solenoid valve, and calibrate the airflow through the system as follows. Connect a flowmeter, calibrated for the volumetric flow rate leaving the meter, to the induction port. Adjust the flow-control valve to achieve a steady flow through the system at the required rate,  $Q_{out}$ , so that  $Q_{out}$  is within  $\pm 5\%$  of the value determined during testing for Delivered-Dose Uniformity. Ensure that critical flow occurs in the flow-control valve, at the value of  $Q_{out}$  to be used during testing, using the following procedure. With the inhaler in place, and the intended flow running, measure the absolute pressure on both sides of the flow-control valve ( $P_2$  and  $P_3$  in [Figure 5](#)). A ratio of  $P_3/P_2 \leq 0.5$  indicates critical flow. Switch to a more powerful pump, and remeasure the test flow rate if  $P_3/P_2 > 0.5$ . Adjust the timer controlling the operation of the two-way solenoid valve so that it opens that valve for the same duration,  $T$ , as used during testing for Delivered-Dose Uniformity. Dispense 20 mL of a solvent, capable of dissolving the drug, into each of the four upper stages of Apparatus 4, and replace the stoppers. [Caution—Some solvents form flammable vapor-air mixtures that may be ignited during passage through a vacuum pump. Take appropriate precautions (alternative solvents, use of vapor traps, minimal pump operating times, etc.) to ensure operator safety during testing.] Tilt the apparatus to wet the stoppers, thereby neutralizing their electrostatic charge. Adjust the timer controlling the operation of the two-way solenoid valve so that it opens the valve for the same duration,  $T$ , as used during testing for Delivered-Dose Uniformity. Prime or load the dry powder inhaler with powder for inhalation according to the labeled instructions. With the vacuum pump running and the two-way solenoid valve closed, insert the inhaler mouthpiece, held horizontally, into the induction port mouthpiece adapter. Discharge the powder into the apparatus by activating the timer and opening the two-way solenoid valve for the required duration,  $T \pm 5\%$ . After the two-way solenoid valve has closed, remove the inhaler from the mouthpiece adapter. If additional doses are required for the sample, reload the inhaler according to the labeled instructions, reinsert the mouthpiece into the mouthpiece adapter, and repeat the operation until the required number of doses have been discharged. After discharge of the last dose, switch off the vacuum pump.

Dismantle the filter stage of Apparatus 4. Carefully remove the filter, and extract the drug with solvent. Rinse the mouthpiece adapter and induction port with a suitable solvent, and quantitatively dilute to an appropriate volume. Rinse the inside of the inlet jet tube to Stage 1 ([Figure 8](#)), allowing the solvent to flow into the stage. Rinse the drug from the inner walls and the collection plate of each of the four upper stages of the apparatus, into the solution in the respective stage, by tilting and rotating the apparatus, while ensuring that no liquid transfer occurs between the stages. Using the method of analysis specified in the individual monograph, determine the mass of drug collected in each of the six volumes of solvent. Ensure that the method corrects for possible evaporation of the solvent during the test. This may involve the use of an internal standard (of known original concentration in the solvent and assayed at the same time as the drug) or the quantitative transfer of the liquid contents from each of the stages, followed by dilution to a known volume. Determine the cutoff diameters of each of the individual stages of the impactor, at the value of  $Q = Q_{out}$  employed in the test by the formula:

$$D_{50,Q} = D_{50,Q_n} (Q_n / Q)^{1/2}$$

where  $D_{50,Q}$  is the cutoff diameter at the flow rate,  $Q$ , employed in the test, and the subscript,  $n$ , refers to the nominal values determined when  $Q_n$  equals 60 L of air per minute. Thus, when  $Q$  equals 40 L of air per minute, the cutoff diameter of Stage 2 is given by the formula:

$$D_{50,40LPM} = 6.8 \mu\text{m} \times (60/40)^{1/2} = 8.3 \mu\text{m}.$$

Procedure— Proceed as directed in the General Procedure under Apparatus 2, except to use Apparatus 4.

#### Apparatus 5 for Dry Powder Inhalers—

Design— The design and assembly of Apparatus 5<sup>5</sup> are shown in [Figures 9, 9a, 9b, 9c](#), and [9d](#). The induction port, used to connect the device to an inhaler, is shown in [Figure 4a](#). The device is a cascade impactor with seven stages and a micro-orifice collector (MOC). Over the design flow-rate range of 30 to 100 L per minute, the 50% efficiency cut-off diameters of the stages ( $D_{50}$  values) range between 0.24  $\mu\text{m}$  to 11.7  $\mu\text{m}$ , evenly spaced on a logarithmic scale. In the design flow-rate range, there are always at least five stages with  $D_{50}$  values between 0.5  $\mu\text{m}$  and 6.5  $\mu\text{m}$ . The collection efficiency curves for each stage are sharp and minimize overlap between stages. Material may be aluminum, stainless steel, or other suitable material.

The impactor layout has removable impaction cups with all the cups in one plane ([Figures 9–9c](#)). There are three main sections to the impactor: the bottom frame that holds the impaction cups, the seal body that holds the jets, and the lid that contains the interstage passageways (shown in [Figures 9–9b](#)). Multiple nozzles are used at all but the first stage ([Figure 9c](#)). The flow passes through the impactor in a saw-tooth pattern.

Stage mensuration is performed periodically together with confirmation of other dimensions critical to the effective operation of the impactor. Critical dimensions are provided below in [Table 6](#).

Table 6. Critical Dimensions for Apparatus 5 and 6

Description	Dimension (mm)
Preseparator (dimension a—see <a href="#">Figure 9d</a> )	12.80 $\pm$ 0.05
Stage 1 <sup>1</sup> Nozzle diameter	14.30 $\pm$ 0.05
Stage 2 <sup>2</sup> Nozzle diameter	4.88 $\pm$ 0.04
Stage 3 <sup>3</sup> Nozzle diameter	2.185 $\pm$ 0.02
Stage 4 <sup>4</sup> Nozzle diameter	1.207 $\pm$ 0.01
Stage 5 <sup>5</sup> Nozzle diameter	0.608 $\pm$ 0.01
Stage 6 <sup>6</sup> Nozzle diameter	0.323 $\pm$ 0.01
Stage 7 <sup>7</sup> Nozzle diameter	0.206 $\pm$ 0.01
MOC <sup>1</sup>	approximately 0.070
Cup Depth (Dimension b—see <a href="#">Figure 9b</a> )	14.625 $\pm$ 0.10
Collection cup surface roughness	0.5 to 2 $\mu\text{m}$
Stage 1 Nozzle to seal body distance <sup>2</sup> —dimension c	0 $\pm$ 1.18
Stage 2 Nozzle to seal body distance <sup>2</sup> —dimension c	5.236 $\pm$ 0.736
Stage 3 Nozzle to seal body distance <sup>2</sup> —dimension c	8.445 $\pm$ 0.410
Stage 4 Nozzle to seal body distance <sup>2</sup> —dimension c	11.379 $\pm$ 0.237
Stage 5 Nozzle to seal body distance <sup>2</sup> —dimension c	13.176 $\pm$ 0.341
Stage 6 Nozzle to seal body distance <sup>2</sup> —dimension c	13.999 $\pm$ 0.071
Stage 7 Nozzle to seal body distance <sup>2</sup> —dimension c	14.000 $\pm$ 0.071
MOC Nozzle to seal body distance <sup>2</sup> —dimension c	14.429 – 14.571

<sup>1</sup> See [Figure 9c](#).

<sup>2</sup> See [Figure 9b](#).

In routine operation, the seal body and lid are held together as a single assembly. The impaction cups are accessible when this assembly is opened at the end of an inhaler test. The cups are held in a support tray, so that all cups can be removed from the impactor simultaneously by lifting out the tray.



An induction port with internal dimensions identical to those defined in [Figure 4a](#) is connected to the impactor inlet. When necessary, with dry powder inhalers, a preseparator can be added to avoid overloading the first stage. This preseparator connects between the induction port and the impactor. A suitable mouthpiece adapter is used to provide an airtight seal between the inhaler and the induction port.

At a volumetric airflow rate of 60 L per minute (the assigned reference flow rate for cutoff-diameter calculations,  $Q_n$ ), the cutoff-aerodynamic diameters  $D_{50,Q_n}$  of Stages 1 to 7 are 8.06, 4.46, 2.82, 1.66, 0.94, 0.55 and 0.34  $\mu\text{m}$ , respectively. The apparatus contains a terminal micro-orifice collector (MOC) that for most formulations may eliminate the need for a final filter as determined by method validation. The MOC is an impactor nozzle plate and collection cup. The nozzle plate contains, nominally, 4032 jets, each approximately 70  $\mu\text{m}$  in diameter. Most particles not captured on Stage 7 of the impactor will be captured on the cup surface below the MOC. (For impactors operated at 60 L per minute, the MOC is capable of collecting 80% of 0.14- $\mu\text{m}$  particles). For formulations with a significant fraction of particles not captured by the MOC, there is an optional filter holder that can replace the MOC or be placed downstream of the MOC containing a suitable after-filter (glass fiber is often suitable).

Procedure— Assemble the apparatus with the preseparator ([Figure 9d](#)), unless experiments have shown that its omission does not result in increased interstage drug losses (>5%) or particle re-entrainment, in which case the preseparator may be omitted.

Place cups into the apertures in the cup tray. To ensure efficient particle capture, coat the particle collection surface of each stage with glycerol, silicone oil, or other suitable liquid typically deposited from a volatile solvent, unless it has been demonstrated to be unnecessary. Insert the cup tray into the bottom frame, and lower into place. Close the impactor lid with the seal body attached, and operate the handle to lock the impactor together so that the system is airtight.

The preseparator may be assembled as follows: assemble the preseparator insert into the preseparator base; fit the preseparator base to the impactor inlet; add 15 mL of the solvent used for sample recovery to the central cup of the preseparator insert; place the preseparator body on top of this assembly; and close the two catches. [Caution—Some solvents form flammable vapor-air mixtures that may be ignited during passage through a vacuum pump. Take appropriate precautions (e.g., alternative solvents, use of vapor traps, minimal pump operating times, etc.) to ensure operator safety during testing.]

Connect an induction port with internal dimensions as defined in [Figure 4a](#) either to the impactor inlet or to the preseparator inlet atop the cascade impactor ([Figure 9d](#)). Place a suitable mouthpiece adapter in position at the end of the induction port so that the mouthpiece end of the inhaler, when inserted, lines up along the horizontal axis of the induction port. The front face of the inhaler mouthpiece is flush with the front face of the induction port, producing an airtight seal. When attached to the mouthpiece adapter, the inhaler should be positioned in the same orientation as intended for use. Connect the apparatus to a flow system according to the scheme specified in [Figure 5](#).

Unless otherwise prescribed, conduct the test at the flow rate used in the test for Delivered-Dose Uniformity drawing 4 L of air from the mouthpiece of the inhaler and through the apparatus. Connect a flowmeter to the induction port. Use a flowmeter calibrated for the volumetric flow leaving the meter, or calculate the volumetric flow leaving the meter ( $Q_{out}$ ) using the ideal gas law. For a meter calibrated for the entering volumetric flow ( $Q_{in}$ ), use the formula:

$$Q_{out} = Q_{in}P_0 / (P_0 - \Delta P)$$

where  $P_0$  is the atmospheric pressure and  $\Delta P$  is the pressure drop over the meter. Adjust the flow control valve to achieve steady flow through the system at the required rate,  $Q_{out}$  ( $\pm 5\%$ ). Ensure that critical flow occurs in the flow-control valve by the procedure described for Apparatus 2. Adjust the timer controlling the operation of the two-way solenoid valve so that it opens the valve for the same duration,  $T$ , as used during testing for Delivered-Dose Uniformity.

Prime or load the dry powder inhaler with powder for inhalation according to the labeled instructions. With the vacuum pump running and the two-way solenoid valve closed, insert the inhaler mouthpiece, held horizontally, into the induction port mouthpiece adapter. Discharge the powder into the apparatus by activating the timer and opening the two-way solenoid valve for the required duration,  $T$  ( $\pm 5\%$ ). After the two-way solenoid valve has closed, remove the inhaler from the mouthpiece adapter. If additional doses are required for the sample, reload the inhaler according to the labeled instructions, reinsert the mouthpiece into the mouthpiece adapter, and repeat the operation until the required number of doses have been discharged. After discharge of the last dose, switch off the vacuum pump.

Dismantle the apparatus, and recover drug for analysis as follows: remove the induction port and mouthpiece adapter from the preseparator and extract the drug into an aliquot of solvent; if used, remove the preseparator from the impactor, without spilling the solvent into the impactor; and recover the active ingredient from all inner surfaces.

Open the impactor by releasing the handle and lifting the lid. Remove the cup tray, with the collection cups, and recover the active ingredient from each cup into an aliquot of solvent. Using the method of analysis specified in the individual monograph, determine the mass of drug contained in each of the aliquots of solvent.

Determine the cutoff diameters of each of the individual stages of the impactor, at the value of  $Q = Q_{out}$  employed in the test by the formula:

$$D_{50,Q} = D_{50,Q_n} (Q_n / Q)^X, \quad (\text{Eq. 2})$$

where  $D_{50,Q}$  is the cutoff diameter at the flow rate,  $Q$ , employed in the test, and the subscript,  $n$ , refers to the nominal or reference value for  $Q_n = 60$  L of air per minute (see [Table 7](#)). The values for the exponent,  $x$ , are listed in [Table 7](#). Thus, when  $Q = 40$  L of air per minute, the cutoff diameter of Stage 2 is given by the formula:

$$D_{50,40LPM} = 4.46 \mu\text{m} \times (60/40)^{0.52} = 5.51 \mu\text{m}.$$

Analyze the data as directed under Data Analysis.

Table 7. Cutoff Aerodynamic Diameter for Stages of Apparatus 5 and 6

Use Eq. 2 to calculate $D_{50,Q}$ for flow rates, $Q$ , in the range 30 to 100 L per minute with $Q_n = 60$ L per minute.		
Stage	$D_{50,Q_n}$	$x$
1	8.06	0.54
2	4.46	0.52
3	2.82	0.50
4	1.66	0.47
5	0.94	0.53
6	0.55	0.60
7	0.34	0.67

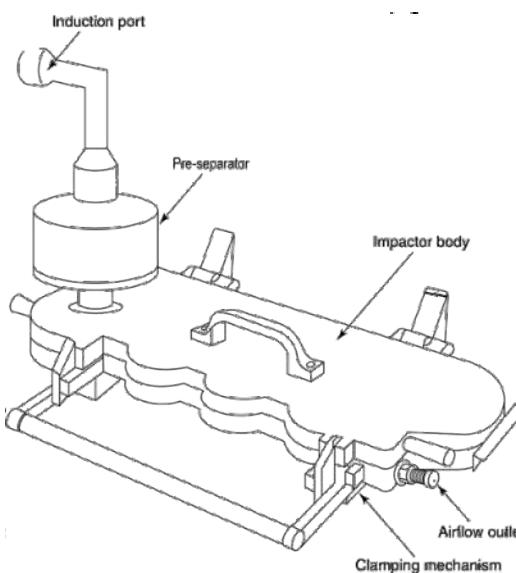


Fig. 9. Apparatus 5 (shown with the preseparator in place).

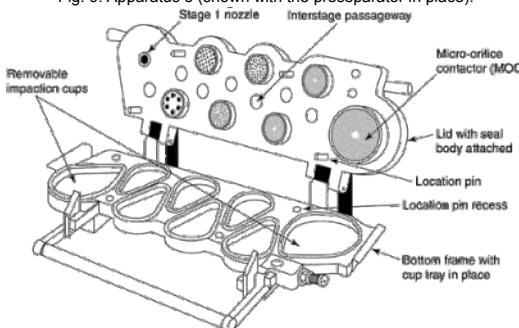


Fig. 9a. Components of Apparatus 5.

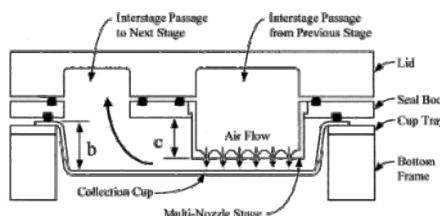


Fig. 9b. Layout of interstage passageways of Apparatus 5.

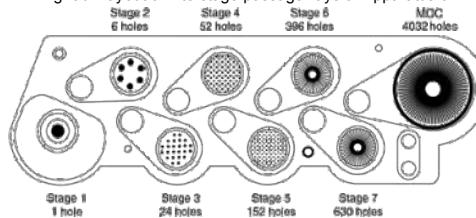


Fig. 9c. Nozzle configuration of Apparatus 5.

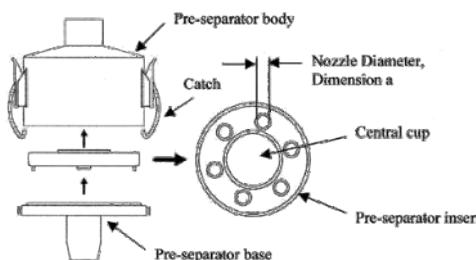


Fig. 9d. Pre-separator layout for Apparatus 5.

#### Apparatus 6 for Metered-Dose Inhalers—

Design— Apparatus 6 is identical to Apparatus 5 (Figures 9-9d), except that the preseparator is not to be used. Use this apparatus at a flow rate of 30 L per minute ( $\pm 5\%$ ), unless otherwise prescribed in the individual monograph.

Procedure— Assemble the apparatus without the preseparator. Place cups into the apertures in the cup tray. To ensure efficient particle capture, coat the particle collection surface of each stage with glycerol, silicone oil, or other suitable liquid typically deposited from a volatile solvent, unless it has been demonstrated to be unnecessary. Insert the cup tray into the



bottom frame, and lower into place. Close the impactor lid with seal body attached, and operate the handle to lock the impactor together so that the system is airtight. Connect a induction port with internal dimensions as defined in [Figure 4a](#) to the impactor inlet. Use a mouthpiece adapter that ensures that the tip of the inhaler mouthpiece is flush with the open end of the induction port. Turn on the vacuum pump to draw air through the cascade impactor, and calibrate the airflow through the system with an appropriate flowmeter attached to the open end of the induction port. Adjust the flow-control valve on the vacuum pump to achieve steady flow through the system at the required rate, and ensure that the airflow through the system is within  $\pm 5\%$  of this flow rate. Unless otherwise prescribed in the patient instructions, shake the inhaler for 5 seconds, and discharge one delivery to waste. With the vacuum pump running, insert the mouthpiece into the mouthpiece adapter, and immediately fire the minimum recommended dose into the cascade impactor. Keep the valve depressed for a duration sufficient to ensure that the dose has been completely discharged. If additional sprays are required for the sample, shake the inhaler, reinsert it into the mouthpiece adapter, and immediately fire the next minimum recommended dose.

Repeat until the required number of doses have been discharged. The number of minimum recommended doses discharged must be sufficient to ensure an accurate and precise determination of Aerodynamic Size Distribution. [note—The number of minimum recommended doses is typically not greater than 10.] After the last dose has been discharged, remove the inhaler from the mouthpiece adapter. Rinse the mouthpiece adapter and induction port with a suitable solvent, and dilute quantitatively to an appropriate volume.

Dismantle the apparatus, and recover the drug for analysis as follows: remove the induction port and mouthpiece adapter from the apparatus, and recover the deposited drug into an aliquot of solvent; open the impactor by releasing the handle and lifting the lid; remove the cup tray, with the collection cups; and extract the active ingredient in each cup into an aliquot of solvent. Using the method of analysis specified in the individual monograph, determine the quantity of active ingredient contained in each of the aliquots of solvent.

Determine the cutoff diameters of each of the individual stages of the impactor, at the value of Q employed in the test by using Eq. 2 with values obtained from [Table 7](#). Thus, when  $Q = 30 \text{ L of air per minute}$ , the cutoff diameter of Stage 2 is given by the formula:

$$D_{50,30\text{LPM}} = 4.46 \mu\text{m} \times (60/30)0.52 = 6.40 \mu\text{m}$$

To analyze the data, proceed as directed under Data Analysis.

#### Data Analysis

This section describes the data analysis required to define the Aerodynamic Size Distribution of the drug output from the test inhaler, after the use of Apparatus 1, 2, 3, 4, 5, or 6. Enter the data collected from Apparatus 1, 2, 3, 4, 5, or 6 in the table of mass summaries as shown in Table 8. Perform only those calculations specified in the individual monograph.

Table 8. Table of Mass Summaries for Analyses of Metered-Dose Inhalers and Dry Powder Inhalers

Mass	Apparatus 1		Apparatus 2		Apparatus 3 <sup>a</sup>		Apparatus 4 <sup>b</sup>		Apparatus 5 <sup>d</sup>		Apparatus 6 <sup>d</sup>		
Mouthpiece adapter	Ai	—	Ai	Ai	—	Ai	—	Ai	—	Ai	—	Ai	—
Preseparator	—	—	—	—	AP	—	—	—	AP	—	—	—	—
Stage 0 of impactor	A0	B0	—	—	A0	B0	—	—	—	—	—	—	—
Stage 1 of impactor/impinger	A1	B1	A1	—	A1	B1	A1	—	A1	B1	A1	B1	
Stage 2 of impactor/impinger	A2	B2	A2	B2	A2	B2	A2	B2	A2	B2	A2	B2	
Stage 3 of impactor/impinger	A3	B3	A3	B3	A3	B3	A3	B3	A3	B3	A3	B3	
Stage 4 of impactor/impinger	A4	B4	A4	B4	A4	B4	A4	B4	A4	B4	A4	B4	
Stage 5 of impactor/impinger	A5	B5	A5	B5	A5	B5	—	—	A5	B5	A5	B5	
Stage 6 of impactor/impinger	A6	B6	—	—	A6	B6	—	—	A6	B6	A6	B6	
Stage 7 of impactor/impinger	A7	B7	—	—	A7	B7	—	—	A7	B7	A7	B7	
Filter	AF	BF	AF	BF	AF	BF	AF	BF	AF	BF	AF	BF	
Sums of Masses	$\Sigma A^e$	$\Sigma B^e$	$\Sigma A^e$	$\Sigma B^e$	$\Sigma A^e$	$\Sigma B^e$	$\Sigma A^e$	$\Sigma B^e$	$\Sigma A^e$	$\Sigma B^e$	$\Sigma A^e$	$\Sigma B^e$	

a Stages 6 and 7 are omitted from Apparatus 3 at airflow rates  $>60 \text{ L per minute}$ .

b Stage 5 of Apparatus 4 is the filter stage (see [Figure 8](#)).

c  $\Sigma A$  is the total drug mass recovered from the apparatus;  $\Sigma B$  is the mass of drug recovered from the impactor (Apparatus 1, 3, 5 and 6) or from the impactor stages beneath the uppermost stage (Apparatus 2 and 4).

d For Apparatus 5 and 6, values for the drug masses AF and BF refer to collections from the MOC, and/or the after-filter if used.

calculations

Fine Particle Dose and Fine Particle Fraction— Calculate the total mass,  $\Sigma A$ , of drug delivered from the mouthpiece of the inhaler into the apparatus. Then calculate the total mass, R, of drug found on the stages of the apparatus and the filter that captured the drug in the fine particle size range appropriate for the particular drug being tested. The Fine Particle Dose is calculated by the formula:

$$R/n$$

where R is as stated above, and n is the number of doses discharged during the test. The Fine Particle Fraction that would be delivered from the inhaler is then calculated by the formula:

$$R/\Sigma A.$$

Cumulative Percentage (Cum%) of Drug Mass Less Than Stated Aerodynamic Diameter— Construct [Table 9](#) by dividing the mass of drug on the filter stage by  $\Sigma B$  (see Table 8).

Multiply the quotient by 100, and enter this number as a percentage opposite the effective cutoff diameter of the stage immediately above it in the impactor or impinger stack. For Apparatus 2 or 4, use Equation 1 to calculate the stage cutoff diameters,  $D_{50,Q}$ , at the airflow rate, Q, employed during the test. For Apparatus 5 and 6, use Equation 2 with [Table 7](#). For Apparatus 1, use the cutoff diameters quoted by the manufacturer. For Apparatus 3, present the data as cumulative percentages of mass on and below the stated stage, and avoid assigning values to stage cutoff diameters.

Repeat the calculation for each of the stages in the impactor or impinger stack, in reverse numerical order (largest to smallest stage number). For each stage, calculate the cumulative percentage of mass less than the stated aerodynamic diameter by adding the percentage of the mass on that stage to the total percentage from the stages below and entering the value opposite the effective cutoff diameter of the stage above it in the stack. Thus, the percentage of drug on the filter can be seen to have aerodynamic diameters less than the cutoff diameter of the stage above the filter, and the percentage on the filter plus the percentage on the stage above have diameters less than the cutoff diameter of the stage above that, and so on. Repeat the calculation for each of the remaining stages in reverse numerical order (see [Table 9](#)).

Table 9. Cumulative Percentage (Cum%) of Mass Less than the Stated Aerodynamic Diameter

	Apparatus 1		Apparatus 2		Apparatus 3 <sup>a</sup>		Apparatus 4 <sup>b</sup>		Apparatus 5		Apparatus 6	
Mass	Cum% <sup>e</sup>	$D_{50,Q}^d$	Cum% <sup>e</sup>	$D_{50,Q}^d$	Cum% <sup>e</sup>	$D_{50,Q}^d$	Cum% <sup>e</sup>	$D_{50,Q}^d$	Cum% <sup>e</sup>	$D_{50,Q}^d$	Cum% <sup>e</sup>	$D_{50,Q}^d$
Filter	0.4	0.625	0.4	1.7	0.34	0.34						
Stage 7	b	0.7	—	—	b	0.7	—	—	b	0.55	b	0.55
Stage 6	c	1.1	—	—	c	1.1	—	—	c	0.94	c	0.94
Stage 5	d	2.1	b	1.25	d	2.1	—	—	d	1.66	d	1.66
Stage 4	e	3.3	c	2.5	e	3.3	b	3.1	e	2.82	e	2.82

Stage 3	f	4.7	d	5.0	f	4.7	c	6.8	f	4.46	f	4.46
Stage 2	g	5.8	100	10.0	g	5.8	100	13.0	g	8.06	g	8.06
Stage 1	h	9.0	—	—	h	9.0	—	—	—	—	—	—
Stage 0	100	—	—	—	100	—	—	—	100	—	100	—

a Stages 6 and 7 are omitted from Apparatus 3 at flow rates >60 L per minute; thus, values for b and c should be omitted for Apparatus 3, where necessary.

b The filter stage in Apparatus 4 is Stage 5 (see [Figure 8](#)).

c [(mass on stage /  $\Sigma$ B)  $\times 100$ ] % + (total% of  $\Sigma$ B from stages below).

d The 50% cutoff diameter of the stage immediately above that indicated (e.g., for Stage 4, enter the cutoff diameter for Stage 3; for Apparatus 2 or 4, calculate as D<sub>50,Q</sub> from Eq. 1; for Apparatus 5 or 6, calculate as D<sub>50,Q</sub> from Eq. 2 using [Table 7](#)). Values entered in the Table are correct for Apparatus 1, 2, 4, 5, and 6 only when used at 28.3, 60.0, 60.0, 60.0, and 60.0 L per minute, respectively.

e The D<sub>50</sub> values are only valid at a flow rate of 28.3 L per minute.

If necessary, and where appropriate, plot the percentage of mass less than the stated aerodynamic diameters, versus the aerodynamic diameter, D<sub>50,Q</sub>, on log probability paper.

Calculate the GSD by the equation:

$$GSD = \sqrt{\frac{\text{Size } X}{\text{Size } Y}}$$

Use these data and/or plot to determine values for MMAD and GSD etc., as appropriate and when necessary (see [Figure 10](#)).

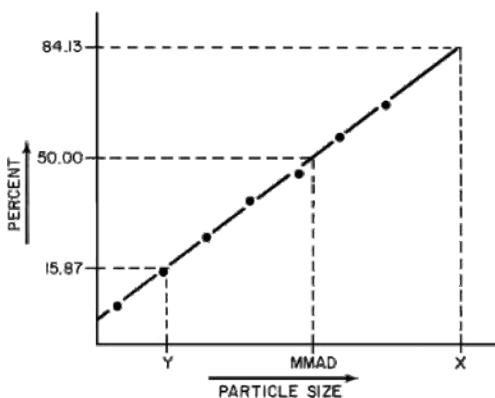


Fig. 10. Plot of cumulative percentage of mass less than stated aerodynamic diameter (probability scale) versus aerodynamic diameter (log scale).

1 A suitable cascade impactor is available as Model Mk II from Thermo-Electron, 27 Forge Parkway, Franklin, MA 02038. The impactor is used without the preseparator. The inhaler is connected to the impactor via the induction port, atop the entrance cone shown in [Figure 4](#). If an equivalent impactor is employed, the induction port in [Figure 4a](#) should be used, although the entrance cone ([Fig. 4b](#)) should be replaced with one to fit the impactor in question. Note that the internal surfaces of the induction port ([Fig. 4a](#)) are designed to fit flush with their counterparts in the entrance cone ([Fig. 4b](#)). This design avoids aerosol capture at the junction of the two pipes.

2 The cascade impactor is available as the Model 160 Marple-Miller Impactor from MSP Corporation, Minneapolis, MN. The inhaler should be connected to the impactor via the induction port, shown in [Figure 4a](#).

3 The cascade impactor is available as the Andersen 1ACFM Non-Viable Cascade Impactor (Mark II) from Thermo-Electron, 27 Forge Parkway, Franklin, MA 02038. The impactor is used with the preseparator.

4 The five-stage impinger is available from Copley Instruments, plc, Nottingham, UK. The inhaler should be connected to the impinger via the induction port, shown in [Fig. 4](#) and [Fig. 4a](#).

5 The cascade impactor is available as the Next Generation Pharmaceutical Impactor from MSP Corporation, Minneapolis, MN.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Kahkashan Zaidi, Ph.D.</a> Senior Scientist 1-301-816-8269	(AER05) Aerosols05

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611 ALCOHOL DETERMINATION

#### METHOD I—DISTILLATION METHOD

Method I is to be used for the determination of alcohol, unless otherwise specified in the individual monograph. It is suitable for examining most fluidextracts and tinctures, provided the capacity of the distilling flask is sufficient (commonly two to four times the volume of the liquid to be heated) and the rate of distillation is such that clear distillates are produced. Cloudy distillates may be clarified by agitation with talc, or with calcium carbonate, and filtered, after which the temperature of the filtrate is adjusted and the alcohol content determined from the specific gravity. During all manipulations, take precautions to minimize the loss of alcohol by evaporation.

Treat liquids that froth to a troublesome extent during distillation by rendering them strongly acidic with phosphoric, sulfuric, or tannic acid, or treat with a slight excess of calcium



chloride solution or with a small amount of paraffin or silicone oil before starting the distillation.

Prevent bumping during distillation by adding porous chips of insoluble material such as silicon carbide, or beads.

For Liquids Presumed to Contain 30% of Alcohol or Less— By means of a pipet, transfer to a suitable distilling apparatus not less than 25 mL of the liquid in which the alcohol is to be determined, and note the temperature at which the volume was measured. Add an equal volume of water, distill, and collect a volume of distillate about 2 mL less than the volume taken of the original test liquid, adjust to the temperature at which the original test liquid was measured, add sufficient water to measure exactly the original volume of the test liquid, and mix. The distillate is clear or not more than slightly cloudy, and does not contain more than traces of volatile substances other than alcohol and water. Determine the specific gravity of the liquid at 25°, as directed under [Specific Gravity](#) 841, using this result to ascertain the percentage, by volume, of C2H5OH contained in the liquid examined by reference to the Alcoholometric Table in the section Reference Tables.

For Liquids Presumed to Contain More Than 30% of Alcohol— Proceed as directed in the foregoing paragraph, except to do the following: dilute the specimen with about twice its volume of water, collect a volume of distillate about 2 mL less than twice the volume of the original test liquid, bring to the temperature at which the original liquid was measured, add sufficient water to measure exactly twice the original volume of the test liquid, mix, and determine its specific gravity. The proportion of C2H5OH, by volume, in this distillate, as ascertained from its specific gravity, equals one-half that in the liquid examined.

#### Special Treatment—

volatile acids and bases— Render preparations containing volatile bases slightly acidic with diluted sulfuric acid before distilling. If volatile acids are present, render the preparation slightly alkaline with [sodium hydroxide TS](#).

glycerin— To liquids that contain glycerin add sufficient water so that the residue, after distillation, contains not less than 50% of water.

iodine— Treat all solutions containing free iodine with powdered zinc before the distillation, or decolorize with just sufficient sodium thiosulfate solution (1 in 10), followed by a few drops of [sodium hydroxide TS](#).

other volatile substances— Spirits, elixirs, tinctures, and similar preparations that contain appreciable proportions of volatile materials other than alcohol and water, such as volatile oils, chloroform, ether, camphor, etc., require special treatment, as follows:

For Liquids Presumed to Contain 50% of Alcohol or Less— Mix 25 mL of the specimen under examination, accurately measured, with about an equal volume of water in a separator. Saturate this mixture with sodium chloride, then add 25 mL of solvent hexane, and shake the mixture to extract the interfering volatile ingredients. Draw off the separated, lower layer into a second separator, and repeat the extraction twice with two further 25-mL portions of solvent hexane. Extract the combined solvent hexane solutions with three 10-mL portions of a saturated solution of sodium chloride. Combine the saline solutions, and distill in the usual manner, collecting a volume of distillate having a simple ratio to the volume of the original specimen.

For Liquids Presumed to Contain More Than 50% of Alcohol— Adjust the specimen under examination to a concentration of approximately 25% of alcohol by diluting it with water, then proceed as directed in For Liquids Presumed to Contain 50% of Alcohol or Less, beginning with "Saturate this mixture with sodium chloride."

In preparing Collodion or Flexible Collodion for distillation, use water in place of the saturated solution of sodium chloride directed above.

If volatile oils are present in small proportions only, and a cloudy distillate is obtained, the solvent hexane treatment not having been employed, the distillate may be clarified and rendered suitable for the specific gravity determination by shaking it with about one-fifth its volume of solvent hexane, or by filtering it through a thin layer of talc.

#### Method II—Gas Chromatographic Method

Use Method IIa when Method II is specified in the individual monograph. For a discussion of the principles upon which it is based, see Gas Chromatography under [Chromatography](#) 621.

USP Reference Standards— USP Alcohol Determination—Acetonitrile RS. USP Alcohol Determination—Alcohol RS.

#### Method IIa

Apparatus— Under typical conditions, use a gas chromatograph equipped with a flame-ionization detector and a 4-mm × 1.8-m glass column packed with 100- to 120-mesh chromatographic column packing support S3, using nitrogen or helium as the carrier. Prior to use, condition the column overnight at 235° with a slow flow of carrier gas. The column temperature is maintained at 120°, and the injection port and detector temperatures are maintained at 210°. Adjust the carrier flow and temperature so that acetonitrile, the internal standard, elutes in 5 to 10 minutes.

#### Solutions—

Test Stock Preparation— Dilute the specimen under examination stepwise with water to obtain a solution containing approximately 2% (v/v) of alcohol.

Test Preparation— Pipet 5 mL each of the Test Stock Preparation and the USP Alcohol Determination—Acetonitrile RS [note—Alternatively, a 2% aqueous solution of acetonitrile of suitable quality may be used as the internal standard solution] into a 50-mL volumetric flask, dilute with water to volume, and mix.

Standard Preparation— Pipet 5 mL each of the USP Alcohol Determination—Alcohol RS and the USP Alcohol Determination—Acetonitrile RS [note—Alternatively, a 2% aqueous solution of acetonitrile of suitable quality may be used as the internal standard solution] into a 50-mL volumetric flask, dilute with water to volume, and mix.

Procedure— Inject about 5 µL each of the Test Preparation and the Standard Preparation, in duplicate, into the gas chromatograph, record the chromatograms, and determine the peak response ratios. Calculate the percentage of alcohol (v/v) in the specimen under test according to the formula:

$$CD(RU / RS)$$

in which C is the labeled concentration of USP Alcohol Determination—Alcohol RS; D is the dilution factor (the ratio of the volume of the Test Stock Preparation to the volume of the specimen taken); and RU and RS are the peak response ratios obtained from the Test Preparation and the Standard Preparation, respectively.

System Suitability Test— In a suitable chromatogram, the resolution factor, R, is not less than 2; the tailing factor of the alcohol peak is not greater than 2.0; and six replicate injections of the Standard Preparation show a relative standard deviation of not more than 2.0% in the ratio of the peak of alcohol to the peak of the internal standard.

#### Method IIb

Apparatus— The gas chromatograph is equipped with a split injection port with a split ratio of 5:1, a flame-ionization detector, and a 0.53-mm × 30-m capillary column coated with a 3.0-µm film of phase G43. Helium is used as the carrier gas at a linear velocity of 34.0 cm per second. The chromatograph is programmed to maintain the column temperature at 50° for 5 minutes, then to increase the temperature at a rate of 10° per minute to 200°, and maintain at this temperature for 4 minutes. The injection port temperature is maintained at 210° and the detector temperature at 280°.

#### Solutions—

Test Stock Preparation— Dilute the specimen under examination stepwise with water to obtain a solution containing approximately 2% (v/v) of alcohol.

Test Preparation— Pipet 5 mL each of the Test Stock Preparation and the USP Alcohol Determination—Acetonitrile RS [note—Alternatively, a 2% aqueous solution of acetonitrile of suitable quality may be used as the internal standard solution] into a 25-mL volumetric flask, dilute with water to volume, and mix.

Standard Preparation— Pipet 5 mL each of the USP Alcohol Determination—Alcohol RS and the USP Alcohol Determination—Acetonitrile RS [note—Alternatively, a 2% aqueous solution of acetonitrile of suitable quality may be used as the internal standard solution] into a 25-mL volumetric flask, dilute with water to volume, and mix.

Procedure— Inject about 0.2 to 0.5 µL each of the Test Preparation and the Standard Preparation, in duplicate, into the gas chromatograph, record the chromatograms, and determine the peak response ratios. Calculate the percentage of alcohol (v/v) in the specimen under test according to the formula:

$$CD(RU / RS)$$

... which C is the labeled concentration of USP Alcohol Determination—Alcohol RS; D is the dilution factor (the ratio of the volume of the Test Stock Preparation to the volume of the specimen taken); and RU and RS are the peak response ratios obtained from the Test Preparation and the Standard Preparation, respectively.

System Suitability Test— In a suitable chromatogram, the resolution factor, R, between alcohol and the internal standard is not less than 4; the tailing factor of the alcohol peak is not greater than 2.0; and six replicate injections of the Standard Preparation show a relative standard deviation of not more than 4.0% in the ratio of the peak of alcohol to the peak of the internal standard.

Auxiliary Information— Please [check your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Horacio N. Pappa, Ph.D.</a> Senior Scientist and Latin American Liaison 1-301-816-8319	(GC05) General Chapters 05

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#### 616 BULK DENSITY AND TAPPED DENSITY

The bulk density of a solid is often very difficult to measure since the slightest disturbance of the bed may result in a new bulk density. Moreover, it is clear that the bulking properties of a powder are dependent on the "history" of the powder (e.g., how it was handled), and that it can be packed to have a range of bulk densities. Thus, it is essential in reporting bulk density to specify how the determination was made.

Because the interparticulate interactions that influence the bulking properties of a powder are also the interactions that interfere with powder flow, a comparison of the bulk and tapped densities can give a measure of the relative importance of these interactions in a given powder. Such a comparison is often used as an index of the ability of the powder to flow. The bulk density often is the bulk density of the powder "as poured" or as passively filled into a measuring vessel. The tapped density is a limiting density attained after "tapping down," usually in a device that lifts and drops a volumetric measuring cylinder containing the powder a fixed distance.

#### BULK DENSITY

Bulk density is determined by measuring the volume of a known mass of powder sample that has been passed through a screen into a graduated cylinder (Method I) or through a volume-measuring apparatus into a cup (Method II).

##### Method I—Measurement in a Graduated Cylinder

Procedure— Unless otherwise specified, pass a quantity of material sufficient to complete the test through a 1.00-mm (No. 18) screen to break up agglomerates that may have formed during storage. Into a dry 250-mL cylinder introduce, without compacting, approximately 100 g of test sample, M, weighed with 0.1% accuracy. If it is not possible to use 100 g, the amount of the test sample and the volume of the cylinder may be modified and the test conditions specified with the results. Select a sample mass having an untapped apparent volume of 150 to 250 mL. A 100-mL cylinder is used for apparent volumes between 50 mL and 100 mL. Carefully level the powder without compacting, if necessary, and read the unsettled apparent volume,  $V_0$ , to the nearest graduated unit. Calculate the bulk density, in g per mL<sup>1</sup>, by the formula:

$$(M) / (V_0)$$

Generally replicate determinations are desirable for the determination of this property.

##### Method II—Measurement in a Volumeter

The apparatus ([Fig. 1](#)),

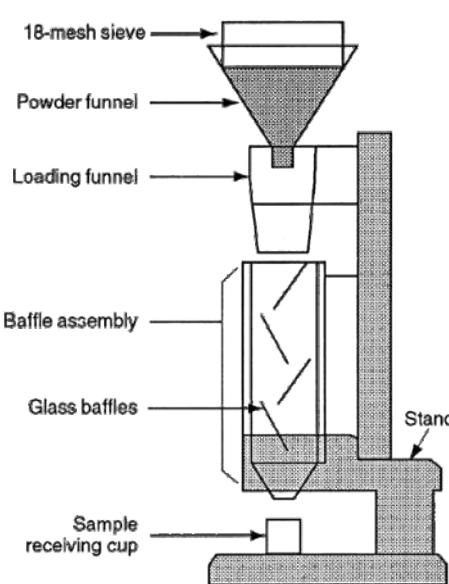


Fig. 1. Scott Volumeter.

conforming to the dimensions in ASTM B 329-90 (Scott Volumeter)<sup>2</sup>, consists of a top funnel fitted with a 1.00-mm (No. 18) screen or the screen opening specified in the individual monograph. The funnel is mounted over a baffle box containing four glass baffle plates over which the powder slides and bounces as it passes. At the bottom of the baffle box is a funnel that collects the powder and allows it to pour into a cup of specified capacity mounted directly below it. The cup may be cylindrical ( $25.00 \pm 0.05$  mL volume with an inside diameter of  $30.00 \pm 2.00$  mm) or a square ( $16.39 \pm 0.05$  mL volume with inside dimensions of  $25.4 \pm 0.076$  mm).

Procedure— Allow an excess of powder to flow through the apparatus into the sample receiving cup until it overflows, using a minimum of 25 cm<sup>3</sup> of powder with the square cup and 35 cm<sup>3</sup> of powder with the cylindrical cup. Carefully scrape excess powder from the top of the cup by smoothly moving the edge of the blade of a spatula perpendicular to and in contact with the top surface of the cup, taking care to keep the spatula perpendicular to prevent packing or removal of powder from the cup. Remove any material from the sides of the cup, and determine the weight, M, of the powder to the nearest 0.1%. Calculate the bulk density, in g per mL, by the formula:

$$(M) / (V_0)$$

in which  $V_0$  is the volume, in mL, of the cup. Generally replicate determinations are desirable for the determination of this property.

#### TAPPED DENSITY

Tapped density is achieved by mechanically tapping a measuring cylinder containing a powder sample. After observing the initial volume, the cylinder is mechanically tapped, and volume readings are taken until little further volume change is observed. The mechanical tapping is achieved by raising the cylinder and allowing it to drop under its own weight a specified distance by either of two methods as described below. Devices that rotate the cylinder during tapping may be preferred to minimize any possible separation of the mass during tapping down.

##### Method I

Procedure— Unless otherwise specified, pass a quantity of material sufficient to complete the test through a 1.00-mm (No. 18) screen to break up agglomerates that may have formed during storage. Into a dry 250-mL glass graduated cylinder (readable to 2 mL) weighing  $220 \pm 44$  g and mounted on a holder weighing  $450 \pm 10$  g introduce, without compacting, approximately 100 g of test sample, M, weighed with 0.1% accuracy. If it is not possible to use 100 g, the amount of the test sample may be reduced and the volume of the cylinder may be modified by using a suitable 100-mL graduated cylinder (readable to 1 mL) weighing  $130 \pm 16$  g and mounted on a holder weighing  $240 \pm 12$  g. The modified test conditions are specified with the results. Carefully level the powder without compacting, if necessary, and read the unsettled apparent volume,  $V_0$ , to the nearest graduated unit.

Mechanically tap the cylinder containing the sample by raising the cylinder and allowing it to drop under its own weight using a suitable mechanical tapped density tester that provides a fixed drop of  $14 \pm 2$  mm at a nominal rate of 300 drops per minute. Unless otherwise specified, tap the cylinder 500 times initially and measure the tapped volume,  $V_a$ , to the nearest graduated unit. Repeat the tapping an additional 750 times and measure the tapped volume,  $V_b$ , to the nearest graduated unit. [note—Fewer taps may be appropriate, if validated, for some powders.] If the difference between the two volumes is less than 2%,  $V_b$  is the final tapped volume,  $V_f$ . Repeat in increments of 1250 taps, as needed, until the difference between succeeding measurements is less than 2%. Calculate the tapped density, in g per mL, by the formula:

$$(M) / (V_f)$$

Generally replicate determinations are desirable for the determination of this property.

##### Method II

Proceed as directed under Method I except that a suitable mechanical tapped density tester that provides a fixed drop of 3 mm ( $\pm 10\%$ ) at a nominal rate of 250 drops per minute is used.

#### MEASURES OF POWDER COMPRESSIBILITY

The Compressibility Index and Hausner Ratio are measures of the propensity of a powder to be compressed. As such, they are measures of the relative importance of interparticulate interactions. In a free-flowing powder, such interactions are generally less significant, and the bulk and tapped densities will be closer in value. For poorer flowing materials, there are frequently greater interparticle interactions, and a greater difference between the bulk and tapped densities will be observed. These differences are reflected in the Compressibility Index and the Hausner Ratio.

Compressibility Index— Calculate by the formula:

$$\frac{100(V_o - V_f)}{V_o}$$

Hausner Ratio— Calculate by the formula:

$$\frac{V_o}{V_f}$$

1 The density of solids normally is expressed in g per cm<sup>3</sup> and that of liquids is normally expressed in g per mL; however, because powder volumes are measured in cylinders graduated in mL, bulk and tapped densities will be expressed in g per mL. By definition, the mL and the cm<sup>3</sup> are equivalent volumes.

2 Apparatus may be purchased from scientific supply companies and is usually described as a "Scott, Schaeffer and White Paint Pigment Volumeter."

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee

## INTRODUCTION

This chapter defines the terms and procedures used in chromatography and provides general information. Specific requirements for chromatographic procedures for drug substances and dosage forms, including adsorbent and developing solvents, are given in the individual monographs.

Chromatography is defined as a procedure by which solutes are separated by a dynamic differential migration process in a system consisting of two or more phases, one of which moves continuously in a given direction and in which the individual substances exhibit different mobilities by reason of differences in adsorption, partition, solubility, vapor pressure, molecular size, or ionic charge density. The individual substances thus separated can be identified or determined by analytical procedures.

The general chromatographic technique requires that a solute undergo distribution between two phases, one of them fixed (stationary phase), the other moving (mobile phase). It is the mobile phase that transfers the solute through the medium until it eventually emerges separated from other solutes that are eluted earlier or later. Generally, the solute is transported through the separation medium by means of a flowing stream of a liquid or a gaseous solvent known as the "eluent." The stationary phase may act through adsorption, as in the case of adsorbents such as activated alumina and silica gel, or it may act by dissolving the solute, thus partitioning the latter between the stationary and mobile phases. In the latter process, a liquid coated onto an inert support, or chemically bonded onto silica gel, or directly onto the wall of a fused silica capillary, serves as the stationary phase. Partitioning is the predominant mechanism of separation in gas-liquid chromatography, paper chromatography, in forms of column chromatography, and in thin-layer chromatography designated as liquid-liquid chromatography. In practice, separations frequently result from a combination of adsorption and partitioning effects. Other separation principles include ion exchange, ion-pair formation, size exclusion, hydrophobic interaction, and chiral recognition.

The types of chromatography useful in qualitative and quantitative analysis that are employed in the USP procedures are column, gas, paper, thin-layer, (including high-performance thin-layer chromatography), and pressurized liquid chromatography (commonly called high-pressure or high-performance liquid chromatography). Paper and thin-layer chromatography are ordinarily more useful for purposes of identification, because of their convenience and simplicity. Column chromatography offers a wider choice of stationary phases and is useful for the separation of individual compounds, in quantity, from mixtures. Modern high-performance thin-layer chromatography, gas chromatography, and pressurized liquid chromatography require more elaborate apparatus but usually provide high resolution and identify and quantitate very small amounts of material.

**Use of Reference Substances in Identity Tests**— In paper and thin-layer chromatography, the ratio of the distance (this distance being measured to the point of maximum intensity of the spot or zone) traveled on the medium by a given compound to the distance traveled by the front of the mobile phase, from the point of application of the test substance, is designated as the RF value of the compound. The ratio between the distances traveled by a given compound and a reference substance is the RR value. RF values vary with the experimental conditions, and thus identification is best accomplished where an authentic specimen of the compound in question is used as a reference substance on the same chromatogram.

For this purpose, chromatograms are prepared by applying on the thin-layer adsorbent or on the paper in a straight line, parallel to the edge of the chromatographic plate or paper, solutions of the substance to be identified, the authentic specimen, and a mixture of nearly equal amounts of the substance to be identified and the authentic specimen. Each sample application contains approximately the same quantity by weight of material to be chromatographed. If the substance to be identified and the authentic specimen are identical, all chromatograms agree in color and RF value and the mixed chromatogram yields a single spot; i.e., RR is 1.0.

**Location and Identification of Components**— The spots produced by paper or thin-layer chromatography may be located by: (1) direct inspection if the compounds are visible under white or either short-wavelength (254 nm) or long-wavelength (360 nm) UV light, (2) inspection in white or UV light after treatment with reagents that will make the spots visible (reagents are most conveniently applied with an atomizer), (3) use of a Geiger-Müller counter or autoradiographic techniques in the case of the presence of radioactive substances, or (4) evidence resulting from stimulation or inhibition of bacterial growth by the placing of removed portions of the adsorbent and substance on inoculated media.

In open-column chromatography, in pressurized liquid chromatography performed under conditions of constant flow rate, and in gas chromatography, the retention time, *t*, defined as the time elapsed between sample injection and appearance of the peak concentration of the eluted sample zone, may be used as a parameter of identification. Solutions of the substance to be identified or derivatives thereof, of the reference compound, and of a mixture of equal amounts of these two are chromatographed successively on the same column under the same chromatographic conditions. Only one peak should be observed for the mixture. The ratio of the retention times of the test substance, the reference compound, and a mixture of these, to the retention time of an internal standard is called the relative retention time RR and is also used frequently as a parameter of identification.

The deviations of RR, RF, or *t* values measured for the test substance from the values obtained for the reference compound and mixture should not exceed the reliability estimates determined statistically from replicate assays of the reference compound.

Chromatographic identification by these methods under given conditions strongly indicates identity but does not constitute definitive identification. Coincidence of identity parameters under three to six different sets of chromatographic conditions (temperatures, column packings, adsorbents, eluants, developing solvents, various chemical derivatives, etc.) increases the probability that the test and reference substances are identical. However, many isomeric compounds cannot be separated. Specific and pertinent chemical, spectroscopic, or physicochemical identification of the eluted component combined with chromatographic identity is the most valid criterion of identification. For this purpose, the individual components separated by chromatography may be collected for further identification.

## PAPER CHROMATOGRAPHY

In paper chromatography the adsorbent is a sheet of paper of suitable texture and thickness. Chromatographic separation may proceed through the action of a single liquid phase in a process analogous to adsorption chromatography in columns. Since the natural water content of the paper, or selective imbibition of a hydrophilic component of the liquid phase by the paper fibers, may be regarded as a stationary phase, a partitioning mechanism may contribute significantly to the separation.

Alternatively, a two-phase system may be used. The paper is impregnated with one of the phases, which then remains stationary (usually the more polar phase in the case of unmodified paper). The chromatogram is developed by slow passage of the other, mobile phase over the sheet. Development may be ascending, in which case the solvent is carried up the paper by capillary forces, or descending, in which case the solvent flow is also assisted by gravitational force.

Differences in the value of RF have been reported where chromatograms developed in the direction of the paper grain (machine direction) are compared with others developed at right angles to the grain; therefore, the orientation of paper grain with respect to solvent flow should be maintained constant in a series of chromatograms. (The machine direction is usually designated by the manufacturer on packages of chromatography paper.)

## Descending Chromatography

In descending chromatography, the mobile phase flows downward on the chromatographic sheet.

**Apparatus**— The essential equipment for descending chromatography consists of the following:

A vapor-tight chamber provided with inlets for addition of solvent or for releasing internal pressure. The chamber is constructed preferably of glass, stainless steel, or porcelain and is so designed as to permit observation of the progress of the chromatographic run without opening of the chamber. Tall glass cylinders are convenient if they are made vapor-tight with suitable covers and a sealing compound.

A rack of corrosion-resistant material about 5 cm shorter than the inside height of the chamber. The rack serves as a support for solvent troughs and for antisiphon rods which, in turn, hold up the chromatographic sheets.



One or more glass troughs capable of holding a volume of solvent greater than that needed for one chromatographic run. The troughs must also be longer than the width of the chromatographic sheets.

Heavy glass antisiphon rods to be supported by the rack and running outside of, parallel to, and slightly above the edge of the glass trough.

Chromatographic sheets of special filter paper at least 2.5 cm wide and not wider than the length of the troughs are cut to a length approximately equal to the height of the chamber. A fine pencil line is drawn horizontally across the filter paper at a distance from one end such that, when the sheet is suspended from the antisiphon rods with the upper end of the paper resting in the trough and the lower portion hanging free into the chamber, the line is located a few centimeters below the rods. Care is necessary to avoid contaminating the filter paper by excessive handling or by contact with dirty surfaces.

Procedure— The substance or substances to be analyzed are dissolved in a suitable solvent. Convenient volumes, delivered from suitable micropipets, of the resulting solution, normally containing 1 to 20 µg of the compound, are placed in 6- to 10-mm spots not less than 3 cm apart along the pencil line. If the total volume to be applied would produce spots of a diameter greater than 6 to 10 mm, it is applied in separate portions to the same spot, each portion being allowed to dry before the next is added.

The spotted chromatographic sheet is suspended in the chamber by use of the antisiphon rod, which holds the upper end of the sheet in the solvent trough. The bottom of the chamber is covered with the prescribed solvent system. Saturation of the chamber with solvent vapor is facilitated by lining the inside walls with paper that is wetted with the prescribed solvent system. It is important to ensure that the portion of the sheet hanging below the rods is freely suspended in the chamber without touching the rack or the chamber walls or the fluid in the chamber. The chamber is sealed to allow equilibration (saturation) of the chamber and the paper with the solvent vapor. Any excess pressure is released as necessary. For large chambers, equilibration overnight may be necessary.

A volume of the mobile phase in excess of the volume required for complete development of the chromatogram is saturated with the immobile phase by shaking. After equilibration of the chamber, the prepared mobile solvent is introduced into the trough through the inlet. The inlet is closed and the mobile solvent phase is allowed to travel the desired distance down the paper. Precautions must be taken against allowing the solvent to run down the sheet when opening the chamber and removing the chromatogram. The location of the solvent front is quickly marked, and the sheets are dried.

The chromatogram is observed and measured directly or after suitable development to reveal the location of the spots of the isolated drug or drugs. The paper section(s) predetermined to contain the isolated drug(s) may be cut out and eluted by an appropriate solvent, and the solutions may be made up to a known volume and quantitatively analyzed by appropriate chemical or instrumental techniques. Similar procedures should be conducted with various amounts of similarly spotted reference standard on the same paper in the concentration range appropriate to prepare a valid calibration curve.

#### Ascending Chromatography

In ascending chromatography, the lower edge of the sheet (or strip) is dipped into the mobile phase to permit the mobile phase to rise on the chromatographic sheet by capillary action.

Apparatus— The essential equipment for ascending chromatography is substantially the same as that described under Descending Chromatography.

Procedure— The test materials are applied to the chromatographic sheets as directed under Descending Chromatography, and above the level to which the paper is dipped into the developing solvent. The bottom of the developing chamber is covered with the developing solvent system. If a two-phase system is used, both phases are added. It is also desirable to line the walls of the chamber with paper and to saturate this lining with the solvent system. Empty solvent troughs are placed on the bottom of the chamber, and the chromatographic sheets are suspended so that the end on which the spots have been added hangs free inside the empty trough.

The chamber is sealed, and equilibration is allowed to proceed as described under Descending Chromatography. Then the developing solvent (mobile phase) is added through the inlet to the trough in excess of the solvent required for complete moistening of the chromatographic sheet. The chamber is resealed. When the solvent front has reached the desired height, the chamber is opened and the sheet is removed and dried.

Quantitative analyses of the spots may be conducted as described under Descending Chromatography.

#### THIN-LAYER CHROMATOGRAPHY

In thin-layer chromatography, the adsorbent is a relatively thin, uniform layer of dry, finely powdered material applied to a glass, plastic, or metal sheet or plate, glass plates being most commonly employed. The coated plate can be considered an "open chromatographic column" and the separations achieved may be based upon adsorption, partition, or a combination of both effects, depending on the particular type of stationary phase, its preparation, and its use with different solvents. Thin-layer chromatography on ion-exchange layers can be used for the fractionation of polar compounds. Presumptive identification can be effected by observation of spots or zones of identical RF value and about equal magnitude obtained, respectively, with an unknown and a reference sample chromatographed on the same plate. A visual comparison of the size or intensity of the spots or zones may serve for semiquantitative estimation. Quantitative measurements are possible by means of densitometry (absorbance or fluorescence measurements), or the spots may be carefully removed from the plate, followed by elution with a suitable solvent and spectrophotometric measurement. For two-dimensional thin-layer chromatography, the chromatographed plate is turned at a right angle and again chromatographed, usually in another chamber equilibrated with a different solvent system.

Apparatus— Acceptable apparatus and materials for thin-layer chromatography consist of the following.

A TLC or HPTLC plate. The chromatography is generally carried out using precoated plates or sheets (on glass, aluminum, or polyester support) of suitable size. It may be necessary to clean the plates prior to separation. This can be done by migration of, or immersion in, an appropriate solvent. The plates may also be impregnated by procedures such as development, immersion, or spraying. At the time of use, the plates may be activated, if necessary, by heating in an oven at 120° for 20 minutes. The stationary phase of TLC plates has an average particle size of 10–15 µm, and that of HPTLC plates an average particle size of 5 µm. Commercial plates with a preadsorbant zone can be used if they are specified in a monograph. Sample applied to the preabsorbant region develops into sharp, narrow bands at the preabsorbant-sorbent interface. Alternatively, flat glass plates of convenient size, typically 20 cm × 20 cm can be coated as described under Preparation of Chromatographic Plates.

A suitable manual, semiautomatic, or automatic application device can be used to ensure proper positioning of the plate and proper transfer of the sample, with respect to volume and position, onto the plate. Alternatively, a template can be used to guide in manually placing the test spots at definite intervals, to mark distances as needed, and to aid in labeling the plates. For the proper application of the solutions, micropipets, microsyringes, or calibrated disposable capillaries are recommended.

For ascending development, a chromatographic chamber made of inert, transparent material and having the following specifications is used: a flat bottom or twin trough, a tightly fitted lid, and a size suitable for the plates. For horizontal development, the chamber is provided with a reservoir for the mobile phase, and it also contains a device for directing the mobile phase to the stationary phase.

Devices for transfer of reagents onto the plate by spraying, immersion, or exposure to vapor and devices to facilitate any necessary heating for visualization of the separated spots or zones.

A UV light source suitable for observations under short (254 nm) and long (365 nm) wavelength UV light.

A suitable device for documentation of the visualized chromatographic result.

Procedure— Apply the prescribed volume of the test solution and the standard solution in sufficiently small portions to obtain circular spots of 2 to 5 mm in diameter (1 to 2 mm on HPTLC plates) or bands of 10 to 20 mm by 1 to 2 mm (5 to 10 mm by 0.5 to 1 mm on HPTLC plates) at an appropriate distance from the lower edge—during chromatography the application position must be at least 3 mm (HPTLC) or 5 mm (TLC) above the level of the developing solvent—and from the sides of the plate. Apply the solutions on a line parallel to the lower edge of the plate with an interval of at least 10 mm (5 mm on HPTLC plates) between the centers of spots or 4 mm (2 mm on HPTLC plates) between the edges of bands, and allow to dry.

Ascending Development— Line at least one wall of the chromatographic chamber with filter paper. Pour into the chromatographic chamber a quantity of the mobile phase sufficient for the size of the chamber to give, after impregnation of the filter paper, a level of depth appropriate to the dimension of the plate used. For saturation of the chromatographic chamber, close the lid, and allow the system to equilibrate. Unless otherwise indicated, the chromatographic separation is performed in a saturated chamber.



Place the plate in the chamber, ensuring that the plate is as vertical as possible and that the spots or bands are above the surface of the mobile phase, and close the chamber. The stationary phase faces the inside of the chamber. Remove the plate when the mobile phase has moved over the prescribed distance. Dry the plate, and visualize the chromatograms as prescribed. For two-dimensional chromatography, dry the plates after the first development, and carry out a second development in a direction perpendicular to that of the first development.

**Horizontal Development**— Introduce a sufficient quantity of the developing solvent into the reservoir of the chamber using a syringe or pipet. Place the plate horizontally in the chamber, connect the mobile phase direction device according to the manufacturer's instructions, and close the chamber. If prescribed, develop the plate starting simultaneously at both ends. Remove the plate when the mobile phase has moved over the distance prescribed in the monograph. Dry the plate, and visualize the chromatograms as prescribed.

For two-dimensional chromatography, dry the plates after the first development, and carry out a second development in a direction perpendicular to that of the first development.

**Detection**— Observe the dry plate first under short-wavelength UV light (254 nm) and then under long-wavelength UV light (365 nm) or as stated in the monograph. If further directed, spray, immerse, or expose the plate to vapors of the specified reagent, heat the plate when required, observe, and compare the test chromatogram with the standard chromatogram.

Document the plate after each observation. Measure and record the distance of each spot or zone from the point of origin, and indicate for each spot or zone the wavelength under which it was observed. Determine the RF values for the principal spots or zones (see *Glossary of Symbols*).

**Quantitative Measurement**— Using appropriate instrumentation, substances separated by TLC and responding to ultraviolet-visible (UV-Vis) irradiation prior to or after derivatization can be determined directly on the plate. While moving the plate or the measuring device, the plate is examined by measuring the reflectance of the incident light. Similarly, fluorescence may be measured using an appropriate optical system. Substances containing radionuclides can be quantified in three ways: (1) directly by moving the plate alongside a suitable counter or vice versa; (2) by cutting the plates into strips and measuring the radioactivity on each individual strip using a suitable counter; or (3) by scraping off the stationary phase, dissolving it in a suitable scintillation cocktail, and measuring the radioactivity using a liquid scintillation counter (see [Radioactivity \(821\)](#)).

The apparatus for direct quantitative measurement on the plate is a densitometer that is composed of a mechanical device to move the plate or the measuring device along the x-axis and the y-axis, a recorder, a suitable integrator or a computer; and, for substances responding to UV-Vis irradiation, a photometer with a source of light, an optical device capable of generating monochromatic light, and a photo cell of adequate sensitivity, all of which are used for the measurement of reflectance. In the case where fluorescence is measured, a suitable filter is also required to prevent the light used for excitation from reaching the photo cell while permitting the emitted light or specific portions thereof to pass. The linearity range of the counting device must be verified.

For quantitative tests, it is necessary to apply to the plate not fewer than three standard solutions of the substance to be examined, the concentrations of which span the expected value in the test solution (e.g., 80%, 100%, and 120%). Derivatize with the prescribed reagent, if necessary, and record the reflectance or fluorescence in the chromatograms obtained.

Use the measured results for the calculation of the amount of substance in the test solution.

#### Preparation of Chromatographic Plates— Apparatus—

Flat glass plates of convenient size, typically 20 cm × 20 cm.

An aligning tray or a flat surface upon which to align and rest the plates during the application of the adsorbent.

A storage rack to hold the prepared plates during drying and transportation. The rack holding the plates should be kept in a desiccator or be capable of being sealed in order to protect the plates from the environment after removal from the drying oven.

The adsorbent consists of finely divided adsorbent materials, normally 5 to 40 µm in diameter, suitable for chromatography. It can be applied directly to the glass plate or can be bonded to the plate by means of plaster of Paris [calcium sulfate hemihydrate (at a ratio of 5% to 15%)] or with starch paste or other binders. The plaster of Paris will not yield as hard a surface as will the starch, but it is not affected by strongly oxidizing spray reagents. The adsorbent may contain fluorescing material to aid in the visualization of spots that absorb UV light.

A spreader, which, when moved over the glass plate, will apply a uniform layer of adsorbent of desired thickness over the entire surface of the plate.

**Procedure**— [note—In this procedure, use Purified Water that is obtained by distillation.] Clean the glass plates scrupulously, using an appropriate cleaning solution (see [Cleaning Glass Apparatus \(1051\)](#)), rinsing them with copious quantities of water until the water runs off the plates without leaving any visible water or oily spots, then dry. It is important that the plates be completely free from lint and dust when the adsorbent is applied.

Arrange the plate or plates on the aligning tray, place a 5- × 20-cm plate adjacent to the front edge of the first square plate and another 5- × 20-cm plate adjacent to the rear edge of the last square, and secure all of the plates so that they will not slip during the application of the adsorbent. Position the spreader on the end plate opposite the raised end of the aligning tray. Mix 1 part of adsorbent with 2 parts of water (or in the ratio suggested by the supplier) by shaking vigorously for 30 seconds in a glass-stoppered conical flask, and transfer the slurry to the spreader. Usually 30 g of adsorbent and 60 mL of water are sufficient for five 20- × 20-cm plates. Complete the application of adsorbents using plaster of Paris binder within 2 minutes of the addition of the water, because thereafter the mixture begins to harden. Draw the spreader smoothly over the plates toward the raised end of the aligning tray, and remove the spreader when it is on the end plate next to the raised end of the aligning tray. (Wash away all traces of adsorbent from the spreader immediately after use.) Allow the plates to remain undisturbed for 5 minutes, then transfer the square plates, layer side up, to the storage rack, and dry at 105° for 30 minutes. Preferably place the rack at an angle in the drying oven to prevent the condensation of moisture on the back sides of plates in the rack. When the plates are dry, allow them to cool to room temperature, and inspect the uniformity of the distribution and the texture of the adsorbent layer; transmitted light will show uniformity of distribution, and reflected light will show uniformity of texture. Store the satisfactory plates over silica gel in a suitable chamber.

#### COLUMN CHROMATOGRAPHY

**Apparatus**— The apparatus required for column chromatographic procedures is simple, consisting only of the chromatographic tube itself and a tamping rod, which may be needed to pack a pledget of glass wool or cotton, if needed, in the base of the tube and compress the adsorbent or slurry uniformly within the tube. In some cases a porous glass disk is sealed at the base of the tube in order to support the contents. The tube is cylindrical and is made of glass, unless another material is specified in the individual monograph. A smaller-diameter delivery tube is fused or otherwise attached by a leakproof joint to the lower end of the main tube. Column dimensions are variable; the dimensions of those commonly used in pharmaceutical analysis range from 10 to 30 mm in uniform inside diameter and 150 to 400 mm in length, exclusive of the delivery tube. The delivery tube, usually 3 to 6 mm in inside diameter, may include a stopcock for accurate control of the flow rate of solvents through the column. The tamping rod, a cylindrical ram firmly attached to a shaft, may be constructed of plastic, glass, stainless steel, or aluminum, unless another material is specified in the individual monograph. The shaft of the rod is substantially smaller in diameter than the column and is not less than 5 cm longer than the effective length of the column. The ram has a diameter about 1 mm smaller than the inside diameter of the column.

#### Column Adsorption Chromatography

The adsorbent (such as activated alumina or silica gel, calcined diatomaceous silica, or chromatographic purified siliceous earth) as a dry solid or as a slurry is packed into a glass or quartz chromatographic tube. A solution of the drug in a small amount of solvent is added to the top of the column and allowed to flow into the adsorbent. The drug principles are quantitatively removed from the solution and are adsorbed in a narrow transverse band at the top of the column. As additional solvent is allowed to flow through the column, either by gravity or by application of air pressure, each substance progresses down the column at a characteristic rate resulting in a spatial separation to give what is known as the chromatogram. The rate of movement for a given substance is affected by several variables, including the adsorptive power of the adsorbent and its particle size and surface area; the nature and polarity of the solvent; the hydrostatic head or applied pressure; and the temperature of the chromatographic system.

If the separated compounds are colored or if they fluoresce under UV light, the adsorbent column may be extruded and, by transverse cuts, the appropriate segments may then be isolated. The desired compounds are then extracted from each segment with a suitable solvent. If the compounds are colorless, they may be located by means of painting or spraying the extruded column with color-forming reagents. Chromatographed radioactive substances may be located by means of Geiger-Müller detectors or similar sensing and recording instruments. Clear plastic tubing made of a material such as nylon, which is inert to most solvents and transparent to short-wavelength UV light, may be packed with adsorbent and used as a chromatographic column. Such a column may be sliced with a sharp knife without removing the packing from the tubing. If a fluorescent adsorbent is used, the column may



be marked under UV light in preparation for slicing.

A "flowing" chromatogram, which is extensively used, is obtained by a procedure in which solvents are allowed to flow through the column until the separated drug appears in the effluent solution, known as the "eluate." The drug may be determined in the eluate by titration or by a spectrophotometric or colorimetric method, or the solvent may be evaporated, leaving the drug in more or less pure form. If a second drug principle is involved, it is eluted by continuing the first solvent or by passing a solvent of stronger eluting power through the column. The efficiency of the separation may be checked by obtaining a thin-layer chromatogram on the individual fractions.

A modified procedure for adding the mixture to the column is sometimes employed. The drug, in a solid form, and, as in the case of a powdered tablet, without separation from the excipients, is mixed with some of the adsorbent and added to the top of a column. The subsequent flow of solvent moves the drug down the column in the manner described.

#### Column Partition Chromatography

In partition chromatography the substances to be separated are partitioned between two immiscible liquids, one of which, the immobile phase, is adsorbed on a Solid Support, thereby presenting a very large surface area to the flowing solvent or mobile phase. The exceedingly high number of successive liquid-liquid contacts allows an efficiency of separation not achieved in ordinary liquid-liquid extraction.

The Solid Support is usually polar, and the adsorbed immobile phase more polar than the mobile phase. The Solid Support that is most widely used is chromatographic siliceous earth having a particle size suitable to permit proper flow of eluant.<sup>1</sup> In reverse-phase partition chromatography the adsorbed immobile phase is less polar than the mobile phase and the solid adsorbent is rendered nonpolar by treatment with a silanizing agent, such as dichlorodimethylsilane, to give silanized chromatographic siliceous earth.

The sample to be chromatographed is usually introduced into the chromatographic system in one of two ways: (a) a solution of the sample in a small volume of the mobile phase is added to the top of the column; or, (b) a solution of the sample in a small volume of the immobile phase is mixed with the Solid Support and transferred to the column as a layer above a bed of a mixture of immobile phase with adsorbent.

Development and elution are accomplished with flowing solvent as before. The mobile solvent usually is saturated with the immobile solvent before use.

In conventional liquid-liquid partition chromatography, the degree of partition of a given compound between the two liquid phases is expressed by its partition or distribution coefficient. In the case of compounds that dissociate, distribution can be controlled by modifying the pH, dielectric constant, ionic strength, and other properties of the two phases. Selective elution of the components of a mixture can be achieved by successively changing the mobile phase to one that provides a more favorable partition coefficient, or by changing the pH of the immobile phase in situ with a mobile phase consisting of a solution of an appropriate acid or base in an organic solvent.

Unless otherwise specified in the individual monograph, assays and tests that employ column partition chromatography are performed according to the following general methods.

**Solid Support**— Use purified siliceous earth. Use silanized chromatographic siliceous earth for reverse-phase partition chromatography.

**Stationary Phase**— Use the solvent or solution specified in the individual monograph. If a mixture of liquids is to be used as the Stationary Phase, mix them prior to the introduction of the Solid Support.

**Mobile Phase**— Use the solvent or solution specified in the individual monograph. Equilibrate it with water if the Stationary Phase is an aqueous solution; if the Stationary Phase is a polar organic fluid, equilibrate with that fluid.

**Preparation of Chromatographic Column**— Unless otherwise specified in the individual monograph, the chromatographic tube is about 22 mm in inside diameter and 200 to 300 mm in length, without porous glass disk, to which is attached a delivery tube, without stopcock, about 4 mm in inside diameter and about 50 mm in length. Pack a puglet of fine glass wool in the base of the tube. Place the specified volume of Stationary Phase in a 100- to 250-mL beaker, add the specified amount of Solid Support, and mix to produce a homogeneous, fluffy mixture. Transfer this mixture to the chromatographic tube, and tamp, using gentle pressure, to obtain a uniform mass. If the specified amount of Solid Support is more than 3 g, transfer the mixture to the column in portions of approximately 2 g, and tamp each portion. If the assay or test requires a multisegment column, with a different Stationary Phase specified for each segment, tamp after the addition of each segment, and add each succeeding segment directly to the previous one.

If a solution of the analyte is incorporated in the Stationary Phase, complete the quantitative transfer to the chromatographic tube by scrubbing the beaker used for the preparation of the test mixture with a mixture of about 1 g of Solid Support and several drops of the solvent used to prepare the test solution.

Pack a puglet of fine glass wool above the completed column packing. The Mobile Phase flows through a properly packed column as a moderate stream or, if reverse-phase chromatography is applied, as a slow trickle.

**Procedure**— Transfer the Mobile Phase to the column space above the column packing, and allow it to flow through the column under the influence of gravity. Rinse the tip of the chromatographic column with about 1 mL of Mobile Phase before each change in composition of Mobile Phase and after completion of the elution. If the analyte is introduced into the column as a solution in the Mobile Phase, allow it to pass completely into the column packing, then add Mobile Phase in several small portions, allowing each to drain completely, before adding the bulk of the Mobile Phase. Where the assay or test requires the use of multiple chromatographic columns mounted in series and the addition of Mobile Phase in divided portions is specified, allow each portion to drain completely through each column, and rinse the tip of each with Mobile Phase prior to the addition of each succeeding portion.

#### GAS CHROMATOGRAPHY

The distinguishing features of gas chromatography are a gaseous mobile phase and a solid or immobilized liquid stationary phase. Liquid stationary phases are available in packed or capillary columns. In the packed columns, the liquid phase is deposited on a finely divided, inert solid support, such as diatomaceous earth, porous polymer, or graphitized carbon, which is packed into a column that is typically 2 to 4 mm in internal diameter and 1 to 3 m in length. In capillary columns, which contain no packing, the liquid phase is deposited on the inner surface of the column and may be chemically bonded to it. In gas-solid chromatography, the solid phase is an active adsorbent, such as alumina, silica, or carbon, packed into a column. Polyaromatic porous resins, which are sometimes used in packed columns, are not coated with a liquid phase.

When a vaporized compound is introduced into the carrier gas and carried into the column, it is partitioned between the gas and stationary phases by a dynamic countercurrent distribution process. The compound is carried down the column by the carrier gas, retarded to a greater or lesser extent by sorption and desorption on the stationary phase. The elution of the compound is characterized by the partition ratio,  $k'$ , a dimensionless quantity also called the capacity factor (see Glossary of Symbols for the definition of symbols). It is equivalent to the ratio of the time required for the compound to flow through the column (the retention time) to the elution time of an unretained compound. The value of the capacity factor depends on the chemical nature of the compound, the nature, amount, and surface area of the liquid phase, the column temperature, and the gas flow rate. Under a specified set of experimental conditions, a characteristic capacity factor exists for every compound. Separation by gas chromatography occurs only if the compounds concerned have different capacity factors.

**Apparatus**— A gas chromatograph consists of a carrier gas source, an injection port, column, detector, and recording device. The injection port, column, and detector are temperature-controlled. The typical carrier gas is helium, nitrogen, or hydrogen, depending on the column and detector in use. The gas is supplied from a high-pressure cylinder or high-purity gas generator and passes through suitable pressure-reducing valves and a flow meter to the injection port and column. Compounds to be chromatographed, either in solution or as gases, are injected into the gas stream at the injection port. Depending upon the configuration of the apparatus, the test mixture may be injected directly into the column or be vaporized in the injection port and mixed into the flowing carrier gas prior to entering the column.

Once in the column, compounds in the test mixture are separated by virtue of differences in their capacity factors, which in turn depend upon vapor pressure and degree of interaction with the stationary phase. The capacity factor, which governs resolution, retention times, and column efficiencies of components of the test mixture, is also temperature-dependent.

The use of temperature-programmable column ovens takes advantage of this dependence to achieve efficient separation of compounds differing widely in vapor pressure.

As resolved compounds emerge separately from the column, they pass through a differential detector, which responds to the amount of each compound present. The type of detector to be used depends upon the nature of the compounds to be analyzed and is specified in the individual monograph. Detectors are heated to prevent condensation of the eluting compounds.

Detector output is recorded as a function of time, producing a chromatogram, which consists of a series of peaks on a time axis. Each peak represents a compound in the vaporized test mixture, although some peaks may overlap. The elution time is a characteristic of an individual compound; and the instrument response, measured as peak area or peak height, is



a function of the amount present.

Injectors—Sample injection devices range from simple syringes to fully programmable automatic injectors. The amount of sample that can be injected into a capillary column without overloading is small compared to the amount that can be injected into packed columns, and may be less than the smallest amount that can be manipulated satisfactorily by syringe. Capillary columns, therefore, often are used with injectors able to split samples into two fractions, a small one that enters the column and a large one that goes to waste. Such injectors may be used in a splitless mode for analyses of trace or minor components.

Purge and trap injectors are equipped with a sparging device by which volatile compounds in solution are carried into a low-temperature trap. When sparging is complete, trapped compounds are desorbed into the carrier gas by rapid heating of the temperature-programmable trap.

Headspace injectors are equipped with a thermostatically controlled sample heating chamber. Solid or liquid samples in tightly closed containers are heated in the chamber for a fixed period of time, allowing the volatile components in the sample to reach an equilibrium between the nongaseous phase and the gaseous or headspace phase.

After this equilibrium has been established, the injector automatically introduces a fixed amount of the headspace in the sample container into the gas chromatograph.

Columns—Capillary columns, which are usually made of fused silica, are typically 0.2 to 0.53 mm in internal diameter and 5 to 60 m in length. The liquid or stationary phase, which is sometimes chemically bonded to the inner surface, is 0.1 to 1.0  $\mu$ m thick, although nonpolar stationary phases may be up to 5  $\mu$ m thick. A list of liquid phases in current use is given in the section Chromatographic Reagents.

Packed columns, made of glass or metal, are 1 to 3 m in length with internal diameters of 2 to 4 mm. Those used for analysis typically are porous polymers or solid supports with liquid phase loadings of about 5% (w/w). High-capacity columns, with liquid phase loadings of about 20% (w/w), are used for large test specimens and for the determination of low molecular weight compounds such as water. The capacity required influences the choice of solid support.

Supports for analysis of polar compounds on low-capacity, low-polarity liquid phase columns must be inert to avoid peak tailing. The reactivity of support materials can be reduced by silanizing prior to coating with liquid phase. Acid-washed, flux-calcined diatomaceous earth is often used for drug analysis. Support materials are available in various mesh sizes, with 80- to 100-mesh and 100- to 120-mesh being most commonly used with 2- to 4-mm columns. Supports and liquid phases are listed in the section Chromatographic Reagents.

Retention time and the peak efficiency depend on the carrier gas flow rate; retention time is also directly proportional to column length, while resolution is proportional to the square root of the column length. For packed columns, the carrier gas flow rate is usually expressed in mL per minute at atmospheric pressure and room temperature. It is measured at the detector outlet with a flowmeter while the column is at operating temperature. The linear flow rate through a packed column is inversely proportional to the square of the column diameter for a given flow volume. Flow rates of 60 mL per minute in a 4-mm column and 15 mL per minute in a 2-mm column give identical linear flow rates and thus similar retention times. Unless otherwise specified in the individual monograph, flow rates for packed columns are about 30 to 60 mL per minute. For capillary columns, linear flow velocity is often used instead of flow rate. This is conveniently determined from the length of the column and the retention time of a dilute methane sample, provided a flame-ionization detector is in use. At high operating temperatures there is sufficient vapor pressure to result in a gradual loss of liquid phase, a process called bleeding.

Detectors—Flame-ionization detectors are used for most pharmaceutical analyses, with lesser use made of thermal conductivity, electron-capture, nitrogen-phosphorus, and mass spectrometric detectors. For quantitative analyses, detectors must have a wide linear dynamic range: the response must be directly proportional to the amount of compound present in the detector over a wide range of concentrations. Flame-ionization detectors have a wide linear range and are sensitive to most organic compounds. Detector response depends on the structure and concentration of the compound and on the flow rates of the combustion, air, makeup, and carrier gases. Unless otherwise specified in individual monographs, flame-ionization detectors with either helium or nitrogen carrier gas are to be used for packed columns, and helium or hydrogen is used for capillary columns.

The thermal conductivity detector employs a heated wire placed in the carrier gas stream. When an analyte enters the detector with the carrier gas, the difference in thermal conductivity of the gas stream (carrier and sample components) relative to that of a reference flow of carrier gas alone is measured. In general, the thermal conductivity detector responds uniformly to volatile compounds regardless of structure; however, it is considerably less sensitive than the flame-ionization detector.

The alkali flame-ionization detector, sometimes called an NP or nitrogen-phosphorus detector, contains a thermionic source, such as an alkali-metal salt or a glass element containing rubidium or other metal, that results in the efficient ionization of organic nitrogen and phosphorus compounds. It is a selective detector that shows little response to hydrocarbons.

The electron-capture detector contains a radioactive source of ionizing radiation. It exhibits an extremely high response to compounds containing halogens and nitro groups but little response to hydrocarbons. The sensitivity increases with the number and atomic weight of the halogen atoms.

Data Collection Devices—Modern data stations receive the detector output, calculate peak areas and peak heights, and print chromatograms, complete with run parameters and peak data. Chromatographic data may be stored and reprocessed, with integration and other calculation variables being changed as required. Data stations are used also to program the chromatograph, controlling most operational variables and providing for long periods of unattended operation.

Data can also be collected for manual measurement on simple recorders or on integrators whose capabilities range from those providing a printout of peak areas to those providing chromatograms with peak areas and peak heights calculated and data stored for possible reprocessing.

Procedure—Packed and capillary columns must be conditioned before use until the baseline and other characteristics are stable. This may be done by operation at a temperature above that called for by the method or by repeated injections of the compound or mixture to be chromatographed. The column or packing material supplier provides instructions for the recommended conditioning procedure. In the case of thermally stable methyl- and phenyl-substituted polysiloxanes, a special sequence increases inertness and efficiency; maintain the column at a temperature of 250° for 1 hour, with helium flowing, to remove oxygen and solvents. Stop the flow of helium, heat at about 340° for 4 hours, then reduce the heating to a temperature of 250°, and condition with helium flowing until stable.

Most drugs are reactive polar molecules. Successful chromatography may require conversion of the drug to a less polar and more volatile derivative by treatment of reactive groups with appropriate reagents. Silylating agents are widely used for this purpose and are readily available.

Assays require quantitative comparison of one chromatogram with another. A major source of error is irreproducibility in the amount of sample injected, notably when manual injections are made with a syringe. The effects of variability can be minimized by addition of an internal standard, a noninterfering compound present at the same concentration in test and standard solutions. The ratio of peak response of the analyte to that of the internal standard is compared from one chromatogram to another. Where the internal standard is chemically similar to the substance being determined, there is also compensation for minor variations in column and detector characteristics. In some cases, the internal standard may be carried through the sample preparation procedure prior to gas chromatography to control other quantitative aspects of the assay. Automatic injectors greatly improve the reproducibility of sample injections and reduce the need for internal standards.

Many monographs require that system suitability requirements be met before samples are analyzed (see System Suitability and Interpretation of Chromatograms).

#### HIGH-PRESSURE LIQUID CHROMATOGRAPHY

High-pressure liquid chromatography (HPLC), sometimes called high-performance liquid chromatography, is a separation technique based on a solid stationary phase and a liquid mobile phase. Separations are achieved by partition, adsorption, or ion-exchange processes, depending upon the type of stationary phase used. HPLC has distinct advantages over gas chromatography for the analysis of organic compounds. Compounds to be analyzed are dissolved in a suitable solvent, and most separations take place at room temperature.

Thus, most drugs, being nonvolatile or thermally unstable compounds, can be chromatographed without decomposition or the necessity of making volatile derivatives. Most pharmaceutical analyses are based on partition chromatography and are completed within 30 minutes.

As in gas chromatography, the elution time of a compound can be described by the capacity factor,  $k'$  (see Glossary of Symbols), which depends on the chemical nature of the analyte, the composition and flow rate of the mobile phase, and the composition and surface area of the stationary phase. Column length is an important determinant of resolution. Only compounds having different capacity factors can be separated by HPLC.

Apparatus—A liquid chromatograph consists of a reservoir containing the mobile phase, a pump to force the mobile phase through the system at high pressure, an injector to introduce the sample into the mobile phase, a chromatographic column, a detector, and a data collection device such as a computer, integrator, or recorder. Short, small-bore columns containing densely packed particles of stationary phase provide for the rapid exchange of compounds between the mobile and stationary phases. In addition to receiving and reporting



detector output, computers are used to control chromatographic settings and operations, thus providing for long periods of unattended operation.

**Pumping Systems**—HPLC pumping systems deliver metered amounts of mobile phase from the solvent reservoirs to the column through high-pressure tubing and fittings. Modern systems consist of one or more computer-controlled metering pumps that can be programmed to vary the ratio of mobile phase components, as is required for gradient chromatography, or to mix isocratic mobile phases (i.e., mobile phases having a fixed ratio of solvents). However, the proportion of ingredients in premixed isocratic mobile phases can be more accurately controlled than in those delivered by most pumping systems. Operating pressures up to 5000 psi or higher, with delivery rates up to about 10 mL per minute are typical. Pumps used for quantitative analysis should be constructed of materials inert to corrosive mobile phase components and be capable of delivering the mobile phase at a constant rate with minimal fluctuations over extended periods of time.

**Injectors**—After dissolution in mobile phase or other suitable solution, compounds to be chromatographed are injected into the mobile phase, either manually by syringe or loop injectors, or automatically by autosamplers. The latter consist of a carousel or rack to hold sample vials with tops that have a pierceable septum or stopper and an injection device to transfer sample from the vials to a loop from which it is loaded into the chromatograph. Some autosamplers can be programmed to control sample volume, the number of injections and loop rinse cycles, the interval between injections, and other operating variables.

A syringe can be used for manual injection of samples through a septum when column head pressures are less than 70 atmospheres (about 1000 psi). At higher pressures an injection valve is essential. Some valve systems incorporate a calibrated loop that is filled with test solution for transfer to the column in the mobile phase. In other systems, the test solution is transferred to a cavity by syringe and then switched into the mobile phase.

**Columns**—For most pharmaceutical analyses, separation is achieved by partition of compounds in the test solution between the mobile and stationary phases. Systems consisting of polar stationary phases and nonpolar mobile phases are described as normal phase, while the opposite arrangement, polar mobile phases and nonpolar stationary phases, is called reverse-phase chromatography. Partition chromatography is almost always used for hydrocarbon-soluble compounds of molecular weight less than 1000. The affinity of a compound for the stationary phase, and thus its retention time on the column, is controlled by making the mobile phase more or less polar. Mobile phase polarity can be varied by the addition of a second, and sometimes a third or even a fourth, component.

Stationary phases for modern, reverse-phase liquid chromatography typically consist of an organic phase chemically bound to silica or other materials. Particles are usually 3 to 10  $\mu$ m in diameter, but sizes may range up to 50  $\mu$ m or more for preparative columns. Small particles thinly coated with organic phase provide for low mass transfer resistance and, hence, rapid transfer of compounds between the stationary and mobile phases. Column polarity depends on the polarity of the bound functional groups, which range from relatively nonpolar octadecyl silane to very polar nitrile groups. Liquid, nonbound stationary phases must be largely immiscible in the mobile phase. Even so, it is usually necessary to presaturate the mobile phase with stationary phase to prevent stripping of the stationary phase from the column. Polymeric stationary phases coated on the support are more durable.

Columns used for analytical separations usually have internal diameters of 2 to 5 mm; larger diameter columns are used for preparative chromatography. Columns may be heated to give more efficient separations, but only rarely are they used at temperatures above 60° because of potential stationary phase degradation or mobile phase volatility. Unless otherwise specified in the individual monograph, columns are used at ambient temperature.

**Ion-exchange chromatography** is used to separate water-soluble, ionizable compounds of molecular weight less than 1500. The stationary phases are usually synthetic organic resins; cation-exchange resins contain negatively charged active sites and are used to separate basic substances such as amines, while anion-exchange resins have positively charged active sites for separation of compounds with negatively charged groups, such as phosphate, sulfonate, or carboxylate groups. Water-soluble ionic or ionizable compounds are attracted to the resins, and differences in affinity bring about the chromatographic separation. The pH of the mobile phase, temperature, ion type, ionic concentration, and organic modifiers affect the equilibrium, and these variables can be adjusted to obtain the desired degree of separation.

In size-exclusion chromatography, columns are packed with a porous stationary phase. Molecules of the compounds being chromatographed are filtered according to size. Those too large to enter the pores pass unretained through the column. Smaller molecules enter the pores and are increasingly retained as molecular size decreases. These columns are typically used to measure aggregation and degradation of large molecules (see Size-Exclusion Chromatography section).

**Detectors**—Many compendial HPLC methods require the use of spectrophotometric detectors. Such a detector consists of a flow-through cell mounted at the end of the column. A beam of UV radiation passes through the flow cell and into the detector. As compounds elute from the column, they pass through the cell and absorb the radiation, resulting in measurable energy level changes.

Fixed, variable, and multi-wavelength detectors are widely available. Fixed wavelength detectors operate at a single wavelength, typically 254 nm, emitted by a low-pressure mercury lamp. Variable wavelength detectors contain a continuous source, such as a deuterium or high-pressure xenon lamp, and a monochromator or an interference filter to generate monochromatic radiation at a wavelength selected by the operator. The wavelength accuracy of a variable-wavelength detector equipped with a monochromator should be checked by the procedure recommended by its manufacturer; if the observed wavelengths differ by more than 3 nm from the correct values, recalibration of the instrument is indicated. Modern variable wavelength detectors can be programmed to change wavelength while an analysis is in progress. Multi-wavelength detectors measure absorbance at two or more wavelengths simultaneously. In diode array multi-wavelength detectors, continuous radiation is passed through the sample cell, then resolved into its constituent wavelengths, which are individually detected by the photodiode array. These detectors acquire absorbance data over the entire UV-visible range, thus providing the analyst with chromatograms at multiple, selectable wavelengths and spectra of the eluting peaks. Diode array detectors usually have lower signal-to-noise ratios than fixed or variable wavelength detectors, and thus are less suitable for analysis of compounds present at low concentrations.

Differential refractometer detectors measure the difference between the refractive index of the mobile phase alone and that of the mobile phase containing chromatographed compounds as it emerges from the column. Refractive index detectors are used to detect non-UV absorbing compounds, but they are less sensitive than UV detectors. They are sensitive to small changes in solvent composition, flow rate, and temperature, so that a reference column may be required to obtain a satisfactory baseline.

Fluorometric detectors are sensitive to compounds that are inherently fluorescent or that can be converted to fluorescent derivatives either by chemical transformation of the compound or by coupling with fluorescent reagents at specific functional groups. If derivatization is required, it can be done prior to chromatographic separation or, alternatively, the reagent can be introduced into the mobile phase just prior to its entering the detector.

Potentiometric, voltametric, or polarographic electrochemical detectors are useful for the quantitation of species that can be oxidized or reduced at a working electrode. These detectors are selective, sensitive, and reliable, but require conducting mobile phases free of dissolved oxygen and reducible metal ions. A pulseless pump must be used, and care must be taken to ensure that the pH, ionic strength, and temperature of the mobile phase remain constant. Working electrodes are prone to contamination by reaction products with consequent variable responses.

Electrochemical detectors with carbon-paste electrodes may be used advantageously to measure nanogram quantities of easily oxidized compounds, notably phenols and catechols. New detectors continue to be developed in attempts to overcome the deficiencies of those being used.

**Data Collection Devices**—Modern data stations receive and store detector output and print out chromatograms complete with peak heights, peak areas, sample identification, and method variables. They are also used to program the liquid chromatograph, controlling most variables and providing for long periods of unattended operation.

Data also may be collected on simple recorders for manual measurement or on stand-alone integrators, which range in complexity from those providing a printout of peak areas to those providing chromatograms with peak areas and peak heights calculated and data stored for possible subsequent reprocessing.

**Procedure**—The mobile phase composition significantly influences chromatographic performance and the resolution of compounds in the mixture being chromatographed. For accurate quantitative work, high-purity reagents and "HPLC grade" organic solvents must be used. Water of suitable quality should have low conductivity and low UV absorption, appropriate to the intended use.

Reagents used with special types of detectors (e.g., electrochemical, mass spectrometer) may require the establishment of additional tolerances for potential interfering species. Composition has a much greater effect than temperature on the capacity factor,  $k'$ .

In partition chromatography, the partition coefficient, and hence the separation, can be changed by addition of another component to the mobile phase. In ion-exchange chromatography, pH and ionic strength, as well as changes in the composition of the mobile phase, affect capacity factors. The technique of continuously changing the solvent composition during the chromatographic run is called gradient elution or solvent programming. It is sometimes used to chromatograph complex mixtures of components differing greatly

in their capacity factors. Detectors that are sensitive to change in solvent composition, such as the differential refractometer, are more difficult to use with the gradient elution technique.

The detector must have a broad linear dynamic range, and compounds to be measured must be resolved from any interfering substances. The linear dynamic range of a compound is the range over which the detector signal response is directly proportional to the amount of the compound. For maximum flexibility in quantitative work, this range should be about three orders of magnitude. HPLC systems are calibrated by plotting peak responses in comparison with known concentrations of a reference standard, using either an external or an internal standardization procedure.

Reliable quantitative results are obtained by external calibration if automatic injectors or autosamplers are used. This method involves direct comparison of the peak responses obtained by separately chromatographing the test and reference standard solutions. If syringe injection, which is irreproducible at the high pressures involved, must be used, better quantitative results are obtained by the internal calibration procedure where a known amount of a noninterfering compound, the internal standard, is added to the test and reference standard solutions, and the ratios of peak responses of drug and internal standard are compared.

Because of normal variations in equipment, supplies, and techniques, a system suitability test is required to ensure that a given operating system may be generally applicable. The main features of system suitability tests are described below.

For information on the interpretation of results, see the section Interpretation of Chromatograms.

#### Size-Exclusion Chromatography

Size-exclusion chromatography is a high-pressure liquid chromatographic technique that separates molecules in solution according to their size. Methods for size-exclusion chromatography are divided into gel permeation chromatographic methods, which utilize nonpolar organic mobile phases and hydrophilic packings, and gel filtration chromatographic methods, which utilize aqueous mobile phases and hydrophobic packings. The sample is introduced into a column, which is filled with a gel or a porous particle packing material and is carried by the mobile phase through the column. The size separation takes place by repeated exchange of the solute molecules between the solvent of the mobile phase and the same solvent in the stationary liquid phase within the pores of the packing material. The pore-size range of the packing material determines the molecular-size range within which separation can occur.

Molecules small enough to penetrate all the pore spaces elute at the total permeation volume,  $VT$ . On the other hand, molecules apparently larger than the maximum pore size of the packing material migrate along the column only through the spaces between the particles of the packing material without being retained and elute at the exclusion volume,  $VO$  (void volume). Separation according to molecular size occurs between the exclusion volume and the total permeation volume, useful separation usually occurring in the first two-thirds of this range.

Apparatus— The components of the chromatograph are described under High-Pressure Liquid Chromatography.

Column—If necessary, the column is temperature-controlled. It is packed with a separation material that is capable of fractionation in the appropriate range of molecular sizes and through which the eluent is passed at a constant rate. One end of the column is usually fitted with a suitable device for applying the sample, such as a flow adaptor, a syringe through a septum or an injection valve, and it may also be connected to a suitable pump for controlling the flow of the eluent. Alternatively, the sample may be applied directly to the drained bed surface, or, where the sample is denser than the eluent, it may be layered beneath the eluent. The packing material may be a soft support such as a swollen gel or a rigid support composed of a material such as glass, silica, or a solvent-compatible, cross-linked organic polymer. Rigid supports usually require pressurized systems giving faster separations. The mobile phase is chosen according to sample type, separation medium, and method of detection.

Detector—The outlet of the column is usually connected to a suitable detector fitted with an automatic recorder that enables the monitoring of the relative concentrations of separated components of the sample. Detectors are usually based on photometric, refractometric, or luminescent properties (see Detectors under High-Pressure Liquid Chromatography). An automatic fraction collector may be attached, if necessary.

Procedure— Before carrying out the separation, the packing material is treated and the column is packed, as described in the individual monograph or according to the manufacturer's instructions. Where necessary, procedures for verifying the suitability of the system are described in the individual monograph. The column efficiency may be evaluated from the number of theoretical plates,  $N$  (see the section Interpretation of Chromatograms). The elution characteristics of a compound in a particular column may be described by the distribution coefficient,  $KD$ , which is calculated by the formula:

$$(VI - VO) / (VT - VO)$$

in which  $VO$ ,  $VT$ , and  $VI$  are the retention volumes for the nonretained component, the component that has full access to all the pores in the support, and the compound under test, respectively. Each retention volume is measured from the time of application to the time of the peak maximum.

Determination of Relative Component Composition of Mixture—Carry out the separation as directed in the individual monograph. Monitor the elution of the components continuously, and measure the corresponding peak areas. If all the components under test exhibit equivalent responses to the physicochemical property being monitored (for example, if they exhibit corresponding absorptivities), calculate the relative amount of each component by dividing the respective peak area by the sum of the peak areas of all the components under test. If the responses to the property used for detection of the components under test are not equivalent, calculate the content using calibration curves obtained from the calibration procedure specified in the individual monograph.

Determination of Molecular Weights—Size-exclusion chromatography is used to determine molecular weights of components under test by comparison to calibration standards specified in the individual monograph. Plot the retention volumes of the calibration standards versus the logarithm of their molecular weights. Draw the line that best fits the plotted points within the exclusion and total permeation limits for the particular separation medium. From the calibration curve, molecular weights of components under test are estimated. This calibration is valid only for the particular macromolecular solute-solvent system used under the specified experimental conditions.

Determination of Molecular Weight Distribution of Polymers—The material used for calibration and the methods for determination of the distribution of molecular weights of polymers are specified in the individual monograph. However, sample comparison is valid only for results obtained under identical experimental conditions.

#### INTERPRETATION OF CHROMATOGRAMS

[Figure 1](#) represents a typical chromatographic separation of two substances, 1 and 2, where  $t_1$  and  $t_2$  are the respective retention times; and  $h$ ,  $h/2$ , and  $Wh/2$  are the height, the half-height, and the width at half-height, respectively, for peak 1.  $W_1$  and  $W_2$  are the respective widths of peaks 1 and 2 at the baseline. Air peaks are a feature of gas chromatograms and correspond to the solvent front in liquid chromatography. The retention time of these unretained components is designated as  $tM$ .

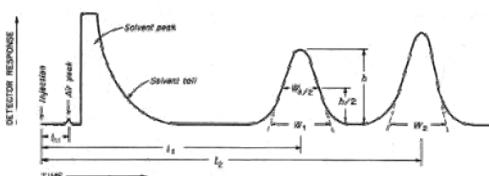


Figure 1. Chromatographic separation of two substances

Chromatographic retention times are characteristic of the compounds they represent but are not unique. Coincidence of retention times of a test and a reference substance can be used as a feature in construction of an identity profile but is insufficient on its own to establish identity. Absolute retention times of a given compound vary from one chromatogram to the next.

Because in most procedures there is no need to identify an unretained peak, comparisons are normally made in terms of relative retention times,  $Rr$ :

$$R_r = \frac{t_2}{t_1}$$

where  $t_2$  and  $t_1$  are the retention times, measured from the point of injection, of the test and the reference substances, respectively, determined under identical experimental conditions on the same column.

Other procedures may identify the peak position using the relative retention,  $r$ :

$$r = \frac{t_2 - t_M}{t_1 - t_M}$$

where  $t_M$  is the retention time of a non-retained marker, which needs to be defined in the procedure.

The number of theoretical plates,  $N$ , is a measure of column efficiency. For Gaussian peaks, it is calculated by the equation:

$$N = 16 \left( \frac{t}{W} \right)^2$$

where  $t$  is the retention time of the substance and  $W$  is the width of the peak at its base, obtained by extrapolating the relatively straight sides of the peak to the baseline. The value of  $N$  depends upon the substance being chromatographed as well as the operating conditions such as mobile phase or carrier gas flow rates and temperature, the quality of the packing, the uniformity of the packing within the column and, for capillary columns, the thickness of the stationary phase film, and the internal diameter and length of the column.

The separation of two components in a mixture, the resolution,  $R$ , is determined by the equation:

$$R = \frac{2(t_2 - t_1)}{W_2 + W_1}$$

in which  $t_2$  and  $t_1$  are the retention times of the two components, and  $W_2$  and  $W_1$  are the corresponding widths at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline.

Where electronic integrators are used, it may be convenient to determine the resolution,  $R$ , by the equation:

$$R = \frac{2(t_2 - t_1)}{1.70(W_{1,h/2} + W_{2,h/2})}$$

and to determine the number of theoretical plates,  $N$ , by the equation:

$$N = 5.54(t/W_{h/2})^2$$

where  $W_{h/2}$  is the peak width at half-height, obtained directly by electronic integrators. However, in the event of dispute, only equations based on peak width at baseline are to be used.

Peak areas and peak heights are usually proportional to the quantity of compound eluting. These are commonly measured by electronic integrators but may be determined by more classical approaches. Peak areas are generally used but may be less accurate if peak interference occurs. For manual measurements, the chart should be run faster than usual, or a comparator should be used to measure the width at half-height and the width at the base of the peak, to minimize error in these measurements. For accurate quantitative work, the components to be measured should be separated from any interfering components. Peak tailing and fronting and the measurement of peaks on solvent tails are to be avoided.

Chromatographic purity tests for drug raw materials are sometimes based on the determination of peaks due to impurities, expressed as a percentage of the area due to the drug peak. It is preferable, however, to compare impurity peaks with those in the chromatogram of a standard at a similar concentration. The standard may be the drug itself at a level corresponding to, for example, 0.5% impurity, or in the case of toxic or signal impurities, a standard of the impurity itself.

#### SYSTEM SUITABILITY

System suitability tests are an integral part of gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such.

The resolution,  $R$ , [note—All terms and symbols are defined in the Glossary of Symbols] is a function of column efficiency,  $N$ , and is specified to ensure that closely eluting compounds are resolved from each other, to establish the general resolving power of the system, and to ensure that internal standards are resolved from the drug. Column efficiency may be specified also as a system suitability requirement, especially if there is only one peak of interest in the chromatogram; however, it is a less reliable means to ensure resolution than direct measurement. Column efficiency is a measure of peak sharpness, which is important for the detection of trace components.

Replicate injections of a standard preparation used in the assay or other standard solution are compared to ascertain whether requirements for precision are met. Unless otherwise specified in the individual monograph, data from five replicate injections of the analyte are used to calculate the relative standard deviation,  $SR$ , if the requirement is 2.0% or less; data from six replicate injections are used if the relative standard deviation requirement is more than 2.0%.

The tailing factor,  $T$ , a measure of peak symmetry, is unity for perfectly symmetrical peaks and its value increases as tailing becomes more pronounced (see [Figure 2](#)). In some cases,

values less than unity may be observed. As peak asymmetry increases, integration, and hence precision, becomes less reliable.

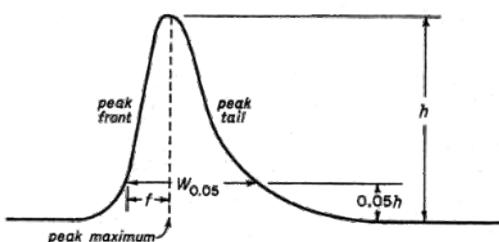


Figure 2. Asymmetrical chromatographic peak

These tests are performed by collecting data from replicate injections of standard or other solutions as specified in the individual monograph. The specification of definitive parameters in a monograph does not preclude the use of other suitable operating conditions (see Procedures under Tests and Assays in the General Notices). If adjustments of operating conditions to meet system suitability requirements are necessary, each of the following is the maximum variation that can be considered, unless otherwise directed in the monograph. Adjustments are permitted only when suitable standards (including Reference Standards) are available for all compounds used in the suitability test and only when those standards are used to show that the adjustments have improved the quality of the chromatography in meeting system suitability requirements. Adjustments to chromatographic systems performed in order to comply with system suitability requirements are not to be made to compensate for column failure or system malfunction. The changes described below may require additional validation data. The user should verify the suitability of the method under the new conditions by assessing the relevant analytical performance characteristics potentially affected by the change. Multiple adjustments can have a cumulative effect in the performance of the system and should be considered carefully before implementation.

pH of Mobile Phase (HPLC)—The pH of the aqueous buffer used in the preparation of the mobile phase can be adjusted to within  $\pm 0.2$  units of the value or range specified.

Concentration of Salts in Buffer (HPLC)—The concentration of the salts used in the preparation of the aqueous buffer used in the mobile phase can be adjusted to within  $\pm 10\%$ , provided the permitted pH variation (see above) is met.

Ratio of Components in Mobile Phase (HPLC)—The following adjustment limits apply to minor components of the mobile phase (specified at 50% or less). The amount(s) of these component(s) can be adjusted by  $\pm 30\%$  relative. However, the change in any component cannot exceed  $\pm 10\%$  absolute (i.e., in relation to the total mobile phase). Adjustment can be made to one minor component in a ternary mixture. Examples of adjustments for binary and ternary mixtures are given below.

#### Binary Mixtures—

specified ratio of 50:50—Thirty percent of 50 is 15% absolute, but this exceeds the maximum permitted change of  $\pm 10\%$  absolute in either component. Therefore, the mobile phase ratio may be adjusted only within the range of 40:60 to 60:40.

specified ratio of 2:98—Thirty percent of 2 is 0.6% absolute. Therefore the maximum allowed adjustment is within the range of 1.4:98.6 to 2.6:97.4.

#### Ternary Mixtures—

specified ratio of 60:35:5—For the second component, 30% of 35 is 10.5% absolute, which exceeds the maximum permitted change of  $\pm 10\%$  absolute in any component. Therefore the second component may be adjusted only within the range of 25% to 45% absolute. For the third component, 30% of 5 is 1.5% absolute. In all cases, a sufficient quantity of the first component is used to give a total of 100%. Therefore, mixture ranges of 50:45:5 to 70:25:5 or 58.5:35:6.5 to 61.5:35:3.5 would meet the requirement.

Wavelength of UV-Visible Detector (HPLC)—Deviations from the wavelengths specified in the method are not permitted. The procedure specified by the detector manufacturer, or another validated procedure, is to be used to verify that error in the detector wavelength is, at most,  $\pm 3$  nm.

Column Length (GC, HPLC): can be adjusted by as much as  $\pm 70\%$ .

Column Inner Diameter (GC, HPLC): can be adjusted by as much as  $\pm 25\%$  for HPLC and  $\pm 50\%$  for GC.

Film Thickness (Capillary GC): can be adjusted by as much as  $-50\%$  to  $100\%$ .

Particle Size (HPLC): can be reduced by as much as 50%.

Particle Size (GC): going from a larger to a smaller or a smaller to a larger (if it is the same "Range Ratio", which is the diameter of the largest particle divided by the diameter of the smallest particle) particle size GC mesh support is acceptable, provided the chromatography meets the requirements of the system suitability.

Flow Rate (GC, HPLC): can be adjusted by as much as  $\pm 50\%$ .

Injection Volume (GC, HPLC): can be reduced as far as is consistent with accepted precision and detection limits.

Column Temperature (HPLC): can be adjusted by as much as  $\pm 10^\circ$ . Column thermostating is recommended to improve control and reproducibility of retention time.

Oven Temperature (GC): can be adjusted by as much as  $\pm 10\%$ .

Oven Temperature Program (GC)—Adjustment of temperatures is permitted as stated above. For the times specified for the temperature to be maintained or for the temperature to be changed from one value to another, an adjustment of up to  $\pm 20\%$  is permitted.

Unless otherwise directed in the monograph, system suitability parameters are determined from the analyte peak.

Relative retention times may be provided in monographs for informational purposes only, to aid in peak identification. There are no acceptance criteria applied to relative retention times.

To ascertain the effectiveness of the final operating system, it should be subjected to suitability testing. Replicate injections of the standard preparation required to demonstrate adequate system precision may be made before the injection of samples or may be interspersed among sample injections. System suitability must be demonstrated throughout the run by injection of an appropriate control preparation at appropriate intervals, including at the end of the analysis. The control preparation can be a standard preparation or a solution containing a known amount of analyte and any additional materials useful in the control of the analytical system, such as excipients or impurities. Whenever there is a significant change in equipment or in a critical reagent, suitability testing should be performed before the injection of samples. No sample analysis is acceptable unless the requirements of system suitability have been met. Sample analyses obtained while the system fails system suitability requirements are unacceptable.

Change to read:

#### GLOSSARY OF SYMBOLS

To promote uniformity of interpretation, the following symbols and definitions are employed where applicable in presenting formulas in the individual monographs. Where a different symbol or definition is used in an individual monograph, the monograph text takes precedence (see General Notices). [note—Where the terms W and t both appear in the same equation they must be expressed in the same units.]

f distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline.  
k' capacity factor.▲2▲USP32

$$k' = \frac{\text{amount of substance in stationary phase}}{\text{amount of substance in mobile phase}}$$

N

number of theoretical plates in a chromatographic column.<sup>▲2▲USP32</sup>

$$k' = \frac{\text{time spent by substance in stationary phase}}{\text{time spent by substance in mobile phase}} = \frac{t}{t_0} - 1$$

$$N = 16 \left( \frac{t}{W} \right)^2 \text{ or } N = 5.54 \left( \frac{t}{W_{1/2}} \right)^2$$

r

relative retention.<sup>▲2▲USP32</sup>

$$r = \frac{t_2 - t_M}{t_1 - t_M}$$

ri

peak response of an impurity obtained from a chromatogram.

rIS

peak response of the internal standard obtained from a chromatogram.

rS

peak response of the Reference Standard obtained from a chromatogram.

rU

peak response of the analyte obtained from a chromatogram.

R

resolution between two chromatographic peaks,

$$R = \frac{2(t_2 - t_1)}{W_1 + W_2}$$

$$\text{or } R = \frac{2(t_2 - t_1)}{1.70(W_{1,b/2} + W_{2,b/2})}$$

RF

chromatographic retardation factor equal to the ratio of the distance from the origin to the center of a zone divided by the distance from the origin to the solvent front.

Rr

relative retention time.<sup>▲2▲USP32</sup>

$$R_r = \frac{t_2}{t_1}$$

Rrel

relative retardation

$$R_{rel} = \frac{\text{distance traveled by test substance}}{\text{distance traveled by standard}}$$

RS

peak response ratio for a Standard preparation containing Reference Standard and internal standard,

$$R_S = \frac{r_s}{r_{IS}}$$

RU

peak response ratio for Assay preparation containing the analyte and internal standard,

$$R_U = \frac{r_U}{r_{IS}}$$

▲%RSD▲USP32

percent relative standard deviation,

$$\text{▲ } \%RSD = \frac{100}{\bar{X}} \left[ \sqrt{\frac{\sum_{i=1}^N (X_i - \bar{X})^2}{N-1}} \right] \text{ ▲USP32}$$

where  $X_i$  is an individual measurement in a set of  $N$  measurements and  $\bar{X}$  is the arithmetic mean of the set.

T

tailing factor.<sup>▲3▲USP32</sup>

$$T = \frac{W_{0.05}}{2f}$$

t

retention time measured from time of injection to time of elution of peak maximum.

tM

retention time of nonretarded component, air with thermal conductivity detection.

W

width of peak measured by extrapolating the relatively straight sides to the baseline.

Wh/2

width of peak at half height.

W0.05

width of peak at 5% height.

<sup>▲2</sup> The parameters  $k'$ ,  $N$ ,  $r$ , and  $Rr$  were developed for isothermal GC separations and isocratic HPLC separations. Because these terms are thermodynamic parameters, they are valid only for separations made at constant temperature, mobile phase composition, and flow rate. However, for separations made with a temperature program or solvent gradient, these parameters may be used simply as comparative means to ensure that adequate chromatographic conditions exist to perform the methods as intended in the monographs.

<sup>▲USP32</sup>

<sup>▲3</sup> It is also a common practice to measure the Asymmetry factor as the ratio of the distance between the vertical line connecting the peak apex with the interpolated baseline and the peak front, and the distance between that line and the peak back measured at 10% of the peak height (in Figure 2), it would be  $(W_{0.10} - f_{0.10}) / f_{0.10}$ . However, for the purposes of USP, only the formula presented in the Glossary of Symbols is valid.

<sup>▲USP32</sup>

#### CHROMATOGRAPHIC REAGENTS

A complete list of Packings (L), Phases (G), and Supports (S) used in USP–NF tests and assays is located under Chromatographic Reagents in the Reagents, Indicators, and Solutions section. This list is intended to be a convenient reference for the chromatographer to identify the pertinent chromatographic reagent specified in the individual monograph.

[note—Particle sizes given in the listing are those generally provided. Where other, usually finer, sizes are required, the individual monograph specifies the desired particle size. Within any category of packings or phases listed there may be a wide range of columns available. Where it is necessary to define more specifically the chromatographic conditions, the individual monograph so indicates.]

1 A suitable grade is acid-washed Celite 545, available from Johns-Manville Corp., 22 East 40th St., New York, NY 10016.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	Horacio N. Pappa, Ph.D. Senior Scientist and Latin American Liaison 1-301-816-8319	(GC05) General Chapters 05

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## 631 COLOR AND ACHROMICITY

**Definition**— For the purposes of this chapter, color may be defined as the perception or subjective response by an observer to the objective stimulus of radiant energy in the visible spectrum extending over the range 400 nm to 700 nm in wavelength. Perceived color is a function of three variables: spectral properties of the object, both absorptive and reflective; spectral properties of the source of illumination; and visual characteristics of the observer.

Two objects are said to have a color match for a particular source of illumination when an observer cannot detect a color difference. Where a pair of objects exhibit a color match for one source of illumination and not another, they constitute a metameric pair. Color matches of two objects occur for all sources of illumination if the absorption and reflectance spectra of the two objects are identical.

Achromicity or colorlessness is one extreme of any color scale for transmission of light. It implies the complete absence of color, and therefore the visible spectrum of the object lacks absorbances. For practical purposes, the observer in this case perceives little if any absorption taking place in the visible spectrum.

**Color Attributes**— Because the sensation of color has both a subjective and an objective part, color cannot be described solely in spectrophotometric terms. The common attributes of color therefore cannot be given a one-to-one correspondence with spectral terminology.

Three attributes are commonly used to identify a color: (1) hue, or the quality by which one color family is distinguished from another, such as red, yellow, blue, green, and intermediate terms; (2) value, or the quality that distinguishes a light color from a dark one; and (3) chroma, or the quality that distinguishes a strong color from a weak one, or the extent to which a color differs from a gray of the same value.

The three attributes of color may be used to define a three-dimensional color space in which any color is located by its coordinates. The color space chosen is a visually uniform one if the geometric distance between two colors in the color space is directly a measure of the color distance between them. Cylindrical coordinates are often conveniently chosen. Points along the long axis represent value from dark to light or black to white and have indeterminate hue and no chroma. Focusing on a cross-section perpendicular to the value axis, hue is determined by the angle about the long axis and chroma is determined by the distance from the long axis. Red, yellow, green, blue, purple, and intermediate hues are given by different angles. Colors along a radius of a cross-section have the same hue, which become more intense farther out. For example, colorless or achromatic water has indeterminate hue, high value, and no chroma. If a colored solute is added, the water takes on a particular hue. As more is added, the color becomes darker, more intense, or deeper; i.e., the chroma generally increases and value decreases. If, however, the solute is a neutral color, i.e., gray, the value decreases, no increase in chroma is observed, and the hue remains indeterminate.

Laboratory spectroscopic measurements can be converted to measurements of the three color attributes. Spectroscopic results for three chosen lights or stimuli are weighted by three distribution functions to yield the tristimulus values, X, Y, Z (see [1061 Color—Instrumental Measurement](#)). The distribution functions were determined in color matching experiments with human subjects.

The tristimulus values are not coordinates in a visually uniform color space; however, several transformations have been proposed that are close to being uniform, one of which is given in the chapter cited ([1061 Color—Instrumental Measurement](#)). The value is often a function of only the Y value. Obtaining uniformity in the chroma-hue subspace has been less satisfactory. In a practical sense, this means in visual color comparison that if two objects differ significantly in hue, deciding which has a higher chroma becomes difficult. This points out the importance of matching standard to sample color as closely as possible, especially for the attributes of hue and chroma.

**Color Determination and Standards**— The perception of color and color matches is dependent on conditions of viewing and illumination. Determinations should be made using diffuse, uniform illumination under conditions that reduce shadows and nonspectral reflectance to a minimum. The surface of powders should be smoothed with gentle pressure so that a planar surface free from irregularities is presented. Liquids should be compared in matched color-comparison tubes, against a white background. If results are found to vary with illumination, those obtained in natural or artificial daylight are to be considered correct. Instead of visual determination, a suitable instrumental method may be used.

Colors of standards should be as close as possible to those of test specimens for quantifying color differences. Standards for opaque materials are available as sets of color chips that are arranged in a visually uniform space.<sup>2</sup> Standards identified by a letter for matching the colors of fluids can be prepared according to the accompanying table. To prepare the matching fluid required, pipet the prescribed volumes of the colorimetric test solutions [see under Colorimetric Solutions (CS) in the section Reagents, Indicators, and Solutions] and water into one of the matching containers, and mix the solution in the container. Make the comparison as directed in the individual monograph, under the viewing conditions previously described. The matching fluids, or other combinations of the colorimetric solutions, may be used in very low concentrations to measure deviation from achromicity.

Matching Fluids

Matching Fluid	Parts of Cobaltous Chloride CS	Parts of Ferric Chloride CS	Parts of Cupric Sulfate CS	Parts of Water
A	0.1	0.4	0.1	4.4
B	0.3	0.9	0.3	3.5
C	0.1	0.6	0.1	4.2
D	0.3	0.6	0.4	3.7
E	0.4	1.2	0.3	3.1
F	0.3	1.2	0.0	3.5
G	0.5	1.2	0.2	3.1
H	0.2	1.5	0.0	3.3
I	0.4	2.2	0.1	2.3
J	0.4	3.5	0.1	1.0
K	0.5	4.5	0.0	0.0
L	0.8	3.8	0.1	0.3
M	0.1	2.0	0.1	2.8
N	0.0	4.9	0.1	0.0
O	0.1	4.8	0.1	0.0
P	0.2	0.4	0.1	4.3
Q	0.2	0.3	0.1	4.4



R	0.3	0.4	0.2	4.1
S	0.2	0.1	0.0	4.7
T	0.5	0.5	0.4	3.6

\* Collections of color chips, arranged according to hue, value, and chroma in a visually uniform space and suitable for use in color designation of specimens by visual matching are available from GretagMacbeth LLC, 617 Little Britain Road, New Windsor, NY 12553-6148.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Antonio Hernandez-Cardoso, B.S.</a> Scientist, Latin American Specialist 1-301-816-8308	(GC05) General Chapters 05

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#### 641 COMPLETENESS OF SOLUTION

Place the quantity of the substance specified in the individual monograph in a meticulously cleansed, glass-stoppered, 10-mL glass cylinder approximately 13 mm × 125 mm in size. Using the solvent that is specified in the monograph or on the label of the product, fill the cylinder almost to the constriction at the neck. Shake gently to effect solution: the solution is not less clear than an equal volume of the same solvent contained in a similar vessel and examined similarly.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Antonio Hernandez-Cardoso, B.S.</a> Scientist, Latin American Specialist 1-301-816-8308	(GC05) General Chapters 05

USP32-NF27 Page 239

#### 643 TOTAL ORGANIC CARBON

Total organic carbon (TOC) is an indirect measure of organic molecules present in pharmaceutical waters measured as carbon. Organic molecules are introduced into the water from the source water, from purification and distribution system materials, and from biofilm growing in the system. TOC can also be used as a process control attribute to monitor the performance of unit operations comprising the purification and distribution system.

A number of acceptable methods exist for analyzing TOC. This chapter does not limit or prevent alternative technologies from being used, but provides guidance on how to qualify these analytical technologies for use as well as guidance on how to interpret instrument results for use as a limit test. The Standard Solution is a theoretically easy-to-oxidize solution that gives an instrument response at the attribute limit. The analytical technology is qualified by challenging the capability of the instrument using a theoretically difficult to oxidize solution in the system suitability portion of the method.

Analytical technologies utilized to measure TOC share the objective of completely oxidizing the organic molecules in an aliquot of sample water to carbon dioxide (CO<sub>2</sub>), measuring the resultant CO<sub>2</sub> levels, and expressing this response as carbon concentration. All technologies must discriminate between the inorganic carbon, which may be present in the water from sources such as dissolved CO<sub>2</sub> and bicarbonate, and the CO<sub>2</sub> generated from the oxidation of organic molecules in the sample.

Two general approaches are used to measure TOC. One approach determines TOC by subtracting the measured inorganic carbon (IC) from the measured total carbon (TC), which is the sum of organic carbon and inorganic carbon:

$$TOC = TC - IC.$$

The other approach first purges the IC from the sample before any carbon measurement is performed. However, this IC purging step also purges some of the organic molecules, which can be re trapped, oxidized to CO<sub>2</sub>, and quantitated as purgeable organic carbon (POC). The remaining organic matter in the sample is also oxidized to CO<sub>2</sub> and quantitated as nonpurgeable organic carbon (NPOC). In this approach, TOC is the sum of POC and NPOC:

$$TOC = POC + NPOC.$$

In pharmaceutical waters, the amount of POC is negligible and can be discounted. Therefore, for the purpose of this methodology, NPOC is equivalent to TOC.

Apparatus Requirements— This test method is performed either as an on-line test or as an off-line laboratory test using a calibrated instrument. The suitability of the apparatus must be periodically demonstrated as described below. In addition, it must have a manufacturer's specified limit of detection of 0.05 mg of carbon per L (0.05 ppm of carbon) or lower.

#### USP Reference Standards (11)—USP 1,4-Benzquinone RS, USP Sucrose RS.

Reagent Water— Use water having a TOC level of not more than 0.10 mg per L. [note—A conductivity requirement may be necessary to ensure method reliability.]

Glassware Preparation— Organic contamination of glassware results in higher TOC values. Therefore, use glassware and sample containers that have been scrupulously cleaned of organic residues. Any method that is effective in removing organic matter can be used (see [Cleaning Glass Apparatus \(1051\)](#)). Use Reagent Water for the final rinse.

Standard Solution— Unless otherwise directed in the individual monograph, dissolve in the Reagent Water an accurately weighed quantity of USP Sucrose RS, to obtain a solution having a concentration of about 1.2 mg of sucrose per L (0.50 mg of carbon per liter).

Test Solution— [note—Use extreme caution when obtaining samples for TOC analysis. Water samples can be easily contaminated during the process of sampling and transportation to a testing facility.] Collect the Test Solution in a tight container with minimal head space, and test in a timely manner to minimize the impact of organic contamination from the closure and container.

System Suitability Solution— Dissolve in Reagent Water an accurately weighed quantity of USP 1,4-Benzquinone RS to obtain a solution having a concentration of 0.75 mg per L (0.50 mg of carbon per liter).

Reagent Water Control— Use a suitable quantity of Reagent Water obtained at the same time as that used in the preparation of the Standard Solution and the System Suitability Solution.



Other Control Solutions— Prepare appropriate reagent blank solutions or other specified solutions needed for establishing the apparatus baseline or for calibration adjustments following the manufacturer's instructions, and run the appropriate blanks to zero the instrument.

System Suitability— Test the Reagent Water Control in the apparatus, and record the response,  $r_w$ . Repeat the test using the Standard Solution, and record the response,  $r_S$ . Calculate the corrected Standard Solution response, which is also the limit response, by subtracting the Reagent Water Control response from the response of the Standard Solution. The theoretical limit of 0.50 mg of carbon per L is equal to the corrected Standard Solution response,  $r_S - r_w$ . Test the System Suitability Solution in the apparatus, and record the response,  $r_{SS}$ . Calculate the corrected System Suitability Solution response by subtracting the Reagent Water Control response from the response of the System Suitability Solution,  $r_{SS} - r_w$ . Calculate the response efficiency for the System Suitability Solution by the formula:

$$100[(r_{SS} - r_w) / (r_S - r_w)].$$

The system is suitable if the response efficiency is not less than 85% and not more than 115% of the theoretical response.

Procedure— Perform the test on the Test Solution, and record the response,  $r_U$ . The Test Solution meets the requirements if  $r_U$  is not more than the limit response,  $r_S - r_w$ . This method also can be performed alternatively using on-line instrumentation that has been appropriately calibrated, standardized, and has demonstrated acceptable system suitability. The acceptability of such on-line instrumentation for quality attribute testing is dependent on its location(s) in the water system. These instrument location(s) and responses must reflect the quality of the water used.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Gary E. Ritchie, M.Sc.</a> Scientific Fellow 1-301-816-8353	(PW05) Pharmaceutical Waters 05
Reference Standards	Lili Wang, Technical Services Scientist 1-301-816-8129 <a href="mailto:RSTech@usp.org">RSTech@usp.org</a>	

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Pharmacopeial Forum: Volume No. 34(5) Page 1241

#### 645 WATER CONDUCTIVITY

Electrical conductivity in water is a measure of the ion-facilitated electron flow through it. Water molecules dissociate into ions as a function of pH and temperature and result in a very predictable conductivity. Some gases, most notably carbon dioxide, readily dissolve in water and interact to form ions, which predictably affect conductivity as well as pH. For the purpose of this discussion, these ions and their resulting conductivity can be considered intrinsic to the water.

Water conductivity is also affected by the presence of extraneous ions. The extraneous ions used in modeling the conductivity specifications described below are the chloride and sodium ions. The conductivity of the ubiquitous chloride ion (at the theoretical endpoint concentration of 0.47 ppm when it was a required attribute test in USP XXII and earlier revisions) and the ammonium ion (at the limit of 0.3 ppm) represent a major portion of the allowed water impurity level. A balancing quantity of cations, such as sodium ions, is included in this allowed impurity level to maintain electroneutrality. Extraneous ions such as these may have significant impact on the water's chemical purity and suitability for use in pharmaceutical applications. The procedure described in the section Bulk Water is designed for measuring the conductivity of waters such as Purified Water, Water for Injection, Water for Hemodialysis, and the condensate of Pure Steam produced in bulk. For water packaged in bulk but manufactured elsewhere or for Sterile Purified Water, Sterile Water for Injection, Sterile Water for Inhalation, and Sterile Water for Irrigation, some additional conductivity tests may be required. Such tests are described in the section Packaged Water.

Online conductivity testing provides real-time measurements and opportunities for real-time process control, decision, and intervention. Precaution should be taken while collecting water samples for off-line conductivity measurements. The sample may be affected by the sampling method, the sampling container, and environmental factors such as ambient carbon dioxide concentration and organic vapors.

#### INSTRUMENT SPECIFICATIONS AND OPERATING PARAMETERS

Water conductivity must be measured accurately using calibrated instrumentation. The conductivity cell constant, a factor that represents the geometrical properties of the conductivity sensor, must be known within  $\pm 2\%$ . The cell constant can be verified directly by using a solution of known or traceable conductivity, or indirectly by comparing the instrument reading taken with the conductivity sensor in question to readings from a conductivity sensor of known or traceable cell constant.

Meter calibration is accomplished by replacing the conductivity sensor with NIST (or equivalent local national authority) -traceable precision resistors (accurate to  $\pm 0.1\%$  of the stated value) or an equivalently accurate adjustable resistance device, such as a Wheatstone Bridge, to give a predicted instrument response. Each scale on the meter may require separate calibration prior to use. The frequency of recalibration is a function of instrument design, degree of use, etc. However, because some multiple-scale instruments have a single calibration adjustment, recalibration may be required between each use of a different scale. Excluding the conductivity sensor cell constant accuracy, the instrument accuracy must be  $\pm 0.1 \mu\text{S}/\text{cm}$ .

In order to increase the measurement accuracy on the conductivity ranges used, which can be large, and to ensure a complete equipment calibration, it is suggested that periodic verification of the entire equipment be performed. This could be done by comparing the conductivity/resistivity values displayed by the measuring equipment with those of an external calibrated conductivity-measuring device. The two nontemperature-compensated conductivity or resistivity values must be equivalent to within  $\pm 20\%$  of each other, or at a difference that is acceptable on the basis of product water criticality and/or the water conductivity ranges in which the measurements are taken. The two conductivity sensors should be positioned close enough together to measure the same water sample in the same environmental conditions.

In addition to the verification method performed in non-temperature-compensated mode, a similar verification performed in temperature-compensated mode could be performed to ensure an appropriate accuracy of the equipment when such a mode is used for trending or other purposes.

Because temperature has a substantial impact on conductivity readings of specimens at high and low temperatures, many instruments automatically correct the actual reading to display the value that theoretically would be observed at the nominal temperature of  $25^\circ\text{C}$ . This is typically done using a temperature sensor embedded in the conductivity sensor and an algorithm in the instrument's circuitry. This temperature compensation algorithm may not be accurate. Conductivity values used in this method are nontemperature-compensated measurements. Temperature measurement is required for the performance of the Stage 1 test. It may be made using the temperature sensor embedded in the conductivity cell sensor.

An external temperature sensor positioned near the conductivity sensor is also acceptable. Accuracy of the temperature measurement must be  $\pm 2^\circ\text{C}$ .

#### BULK WATER

The procedure below shall be performed using instrumentation that has been calibrated, has conductivity sensor cell constants that have been accurately determined, and has temperature compensation function that has been disabled. For both online and offline measurements, the suitability of instrumentation for quality control testing is also dependent on the sampling location(s) in the water system. The selected sampling instrument location(s) must reflect the quality of the water used.

The combined conductivities of the intrinsic and extraneous ions vary as a function of pH and are the basis for the conductivity specifications described in the Stage 3—pH and Conductivity Requirements table and used when performing Stage 3 of the test method. Two preliminary stages are included in the test method. If the test conditions and conductivity limits are met at either of these preliminary stages, the water meets the requirements of this test. Proceeding to the third stage of the test in these circumstances is unnecessary. Only in the event of failure at the final test stage is the sample judged noncompliant with the requirements of the test.



## Procedure

## Stage 1

Stage 1 is intended for online measurement or may be performed offline in a suitable container.

1. Determine the temperature of the water and the conductivity of the water using a nontemperature-compensated conductivity reading.
2. Using the [Stage 1—Temperature and Conductivity Requirements](#) table, find the temperature value that is not greater than the measured temperature, i.e., the next lower temperature. The corresponding conductivity value on this table is the limit. [note—Do not interpolate.]
3. If the measured conductivity is not greater than the table value, the water meets the requirements of the test for conductivity. If the conductivity is higher than the table value, proceed with Stage 2.

Stage 1—Temperature and Conductivity Requirements  
(for nontemperature-compensated conductivity measurements only)

Temperature	Conductivity Requirement ( $\mu\text{S}/\text{cm}$ )
0	0.6
5	0.8
10	0.9
15	1.0
20	1.1
25	1.3
30	1.4
35	1.5
40	1.7
45	1.8
50	1.9
55	2.1
60	2.2
65	2.4
70	2.5
75	2.7
80	2.7
85	2.7
90	2.7
95	2.9
100	3.1

## Stage 2

4. Transfer a sufficient amount of water (100 mL or more) to a suitable container, and stir the test specimen. Adjust the temperature, if necessary, and, while maintaining it at  $25 \pm 1^\circ$ , begin vigorously agitating the test specimen while periodically observing the conductivity. When the change in conductivity (due to uptake of atmospheric carbon dioxide) is less than a net of 0.1  $\mu\text{S}/\text{cm}$  per 5 minutes, note the conductivity.

5. If the conductivity is not greater than 2.1  $\mu\text{S}/\text{cm}$ , the water meets the requirements of the test for conductivity. If the conductivity is greater than 2.1  $\mu\text{S}/\text{cm}$ , proceed with Stage 3.

## Stage 3

6. Perform this test within approximately 5 minutes of the conductivity determination in Step 5, while maintaining the sample temperature at  $25 \pm 1^\circ$ . Add a saturated potassium chloride solution to the same water sample (0.3 mL per 100 mL of the test specimen), and determine the pH to the nearest 0.1 pH unit, as directed under [pH \(791\)](#).
7. Referring to the [Stage 3—pH and Conductivity Requirements](#) table, determine the conductivity limit at the measured pH value. If the measured conductivity in Step 4 is not greater than the conductivity requirements for the pH determined in Step 6, the water meets the requirements of the test for conductivity. If either the measured conductivity is greater than this value or the pH is outside the range of 5.0 to 7.0, the water does not meet the requirements of the test for conductivity.

Stage 3—pH and Conductivity Requirements  
(for atmosphere- and temperature-equilibrated samples only)

pH	Conductivity Requirement ( $\mu\text{S}/\text{cm}$ )
5.0	4.7
5.1	4.1
5.2	3.6
5.3	3.3
5.4	3.0
5.5	2.8
5.6	2.6
5.7	2.5
5.8	2.4
5.9	2.4
6.0	2.4
6.1	2.4
6.2	2.5
6.3	2.4
6.4	2.3
6.5	2.2

6.6	2.1
6.7	2.6
6.8	3.1
6.9	3.8
7.0	4.6

## PACKAGED WATER

The procedure and test limits are intended for water packaged in bulk but manufactured elsewhere or for Sterile Purified Water, Sterile Water for Injection, Sterile Water for Inhalation, and Sterile Water for Irrigation. All these waters are derived from Purified Water or Water for Injection, and therefore have been determined to be compliant with the Bulk Water requirements before being stored in the container. The specification provided represents the maximum allowable conductivity value, taking into consideration the limitation of the measurement method and reasonable container leaching. Such specification and the sampling volume choices should be defined and validated on the basis of the intended purpose of the water.

## Procedure

Transfer a sufficient amount of water to a suitable container, and stir the test specimen. Adjust the temperature, if necessary, and, while maintaining it at  $25 \pm 1^\circ$ , begin vigorously agitating the test specimen while periodically observing the conductivity. When the change in conductivity (due to uptake of ambient carbon dioxide) is less than a net of  $0.1 \mu\text{S}/\text{cm}$  per 5 minutes, note the conductivity.

For containers with a nominal volume of 10 mL or less, if the conductivity is not greater than  $25 \mu\text{S}/\text{cm}$ , the water meets the requirements. For containers with a nominal volume greater than 10 mL, if the conductivity is not greater than  $5 \mu\text{S}/\text{cm}$ , the water meets the requirements.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

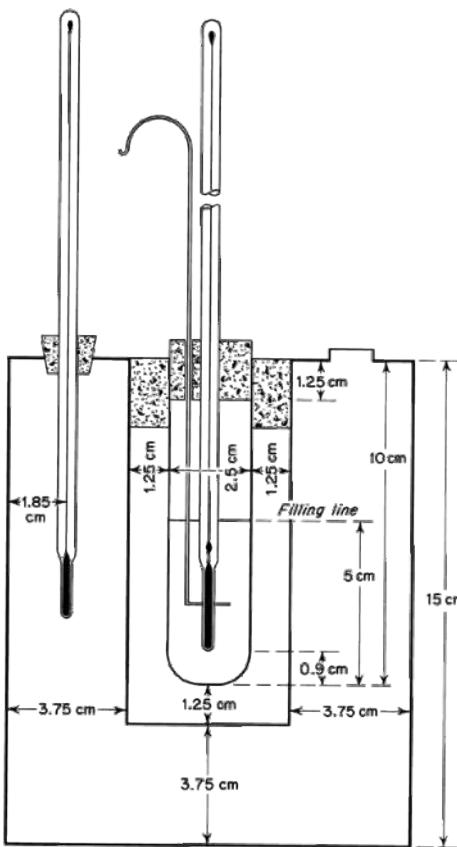
Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Gary E. Ritchie, M.Sc.</a> Scientific Fellow 1-301-816-8353	(PW05) Pharmaceutical Waters 05

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## 651 CONGEALING TEMPERATURE

The temperature at which a substance passes from the liquid to the solid state upon cooling is a useful index to purity if heat is liberated when the solidification takes place, provided that any impurities present dissolve in the liquid only, and not in the solid. Pure substances have a well-defined freezing point, but mixtures generally freeze over a range of temperatures. For many mixtures, the congealing temperature, as determined by strict adherence to the following empirical methods, is a useful index of purity. The method for determining congealing temperatures set forth here is applicable to substances that melt between  $-20^\circ$  and  $150^\circ$ , the range of the thermometer used in the bath. The congealing temperature is the maximum point (or lacking a maximum, the point of inflection) in the temperature-time curve.

Apparatus— Assemble an apparatus similar to that [illustrated](#).



Congealing Temperature Apparatus

in which the container for the substance is a 25- x 100-mm test tube. This is provided with a suitable, short-range thermometer suspended in the center, and a wire stirrer, about 30 cm long, bent at its lower end into a horizontal loop around the thermometer. Use a thermometer having a range not exceeding  $30^\circ$ , graduated in  $0.1^\circ$  divisions, and calibrated for, but not



used at, 76-mm immersion. A suitable series of thermometers, covering a range from  $-20^{\circ}$  to  $+150^{\circ}$ , is available as the ASTM E1 series 89C through 96C. Other temperature-measuring devices may be used if they are validated for this procedure (see [Thermometers \(21\)](#)). Dimensions should be within  $\pm 20\%$  of those given in the illustration.

The specimen container is supported, by means of a cork, in a suitable water-tight cylinder about 50 mm in internal diameter and 11 cm in length. The cylinder, in turn, is supported in a suitable bath sufficient to provide not less than a 37-mm layer surrounding the sides and bottom of the cylinder. The outside bath is provided with a suitable thermometer.

**Procedure**— Melt the substance, if a solid, at a temperature not exceeding  $20^{\circ}$  above its expected congealing point, and pour it into the test tube to a height of 50 to 57 mm. Assemble the apparatus with the bulb of the test tube thermometer immersed halfway between the top and bottom of the specimen in the test tube. Fill the bath to about 12 mm from the top of the tube with suitable fluid at a temperature  $4^{\circ}$  to  $5^{\circ}$  below the expected congealing point.

In case the substance is a liquid at room temperature, carry out the determination using a bath temperature about  $15^{\circ}$  below the expected congealing point.

When the test specimen has cooled to about  $5^{\circ}$  above its expected congealing point, adjust the bath to a temperature  $7^{\circ}$  to  $8^{\circ}$  below the expected congealing point. Stir the specimen continuously during the remainder of the test by moving the loop up and down between the top and bottom of the specimen, at a regular rate of 20 complete cycles per minute.

Congelation frequently may be induced by rubbing the inner walls of the test tube with the thermometer, or by introducing a small fragment of the previously congealed substance. Pronounced supercooling may cause deviation from the normal pattern of temperature changes. If the latter occurs, repeat the test, introducing small particles of the material under test in solid form at  $1^{\circ}$  intervals as the temperature approaches the expected congealing point.

Record the reading of the test tube thermometer every 30 seconds. Continue stirring only so long as the temperature is gradually falling, stopping when the temperature becomes constant or starts to rise slightly. Continue recording the temperature in the test tube every 30 seconds for at least 3 minutes after the temperature again begins to fall after remaining constant.

The average of not less than four consecutive readings that lie within a range of  $0.2^{\circ}$  constitutes the congealing temperature. These readings lie about a point of inflection or a maximum, in the temperature-time curve, that occurs after the temperature becomes constant or starts to rise and before it again begins to fall. The average to the nearest  $0.1^{\circ}$  is the congealing temperature.

**Auxiliary Information**— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Hong Wang, Ph.D.</a> Scientist 1-301-816-8351	(EGC05) Excipient General Chapters

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## 660 CONTAINERS—GLASS

Glass containers for pharmaceutical use are intended to come into direct contact with pharmaceutical preparations. Glass used for pharmaceutical containers is either a borosilicate (neutral) glass or a soda-lime glass. Borosilicate glass contains a significant amount of boric oxide, aluminum oxide, and alkali and/or alkaline earth oxides. Borosilicate glass has a high hydrolytic resistance due to the chemical composition of the glass itself; it is classified as Type I glass. Soda-lime glass is a silica glass containing alkali metal oxides. Soda-lime glass has a moderate hydrolytic resistance due to the chemical composition of the glass itself; it is classified as Type III glass. The inner surface of glass containers may be treated, for example, to improve hydrolytic resistance. The treatment of Type III soda-lime glass containers will raise their hydrolytic resistance from a moderate to a high level, changing the classification of the glass to Type II.

The outer surface of glass containers may be treated to reduce friction or for protection against abrasion or breakage. The treatment of the outer surface does not come into contact with the inner surface of the container. Glass may be colored to provide protection from light or may have a coating applied to the outer surface. Such containers will meet the requirements for Light Transmission under [Containers—Permeation \(671\)](#). A clear and colorless or a translucent container that is made light-resistant by means of an opaque enclosure (see Light-Resistant Container in Preservation, Packaging, Storage, and Labeling under the General Notices) is exempt from the requirements for Light Transmission.

The quality of glass containers is defined by measuring their resistance to chemical attack. In addition, Type I containers for aqueous parenteral preparations are tested for arsenic release, and colored glass containers are tested for light transmission.

### CHEMICAL RESISTANCE

The following tests are designed to determine the resistance to water attack of new (not previously used) glass containers. The degree of attack is determined by the amount of alkali released from the glass under the influence of the attacking medium under the conditions specified. This quantity of alkali is extremely small in the case of the more resistant glasses, thus calling for particular attention to all details of the tests and the use of apparatus of high quality and precision. The tests should be conducted in an area relatively free from fumes and excessive dust.

**Glass Types**— Glass containers suitable for packaging Pharmacopeial preparations may be classified as in [Table 1](#) on the basis of the tests set forth in this section. Containers of Type I borosilicate glass are generally used for preparations that are intended for parenteral administration. Containers of Type I glass, or of Type II glass (i.e., soda-lime glass that is suitably dealkalized) are usually used for packaging acidic and neutral parenteral preparations. Type I glass containers, or Type II glass containers (where stability data demonstrate their suitability), are used for alkaline parenteral preparations. Type III soda-lime glass containers usually are not used for parenteral preparations, except where suitable stability test data indicate that Type III glass is satisfactory for the parenteral preparations that are packaged therein.

Table 1. Glass Types

Type	General Description	Type of Test
I	Highly resistant, borosilicate glass	Powdered Glass
II	Treated soda-lime glass	Water Attack
III	Soda-lime glass	Powdered Glass

### Apparatus—

**Autoclave**— For these tests, use an autoclave capable of maintaining a temperature of  $121 \pm 2.0^{\circ}$ , equipped with a thermometer, a pressure gauge, a vent cock, and a rack adequate to accommodate at least 12 test containers above the water level.

**Mortar and Pestle**— Use a hardened-steel mortar and pestle, made according to the specifications in [Figure 1](#).

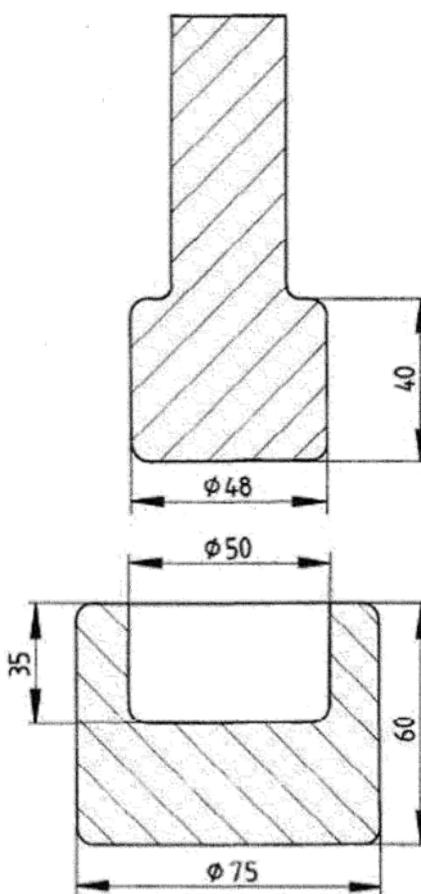


Figure 1. Mortar and Pestle for Pulverizing Glass<sup>1</sup>

Other Equipment— Also required are 20.3-cm (8-inch) sieves made of stainless steel, including the Nos. 20, 40, and 50 sieves, along with the pan and cover (see Sizes of Standard Sieve Series in Range of Interest under [Particle Size Distribution Estimation by Analytical Sieving \(786\)](#)); 250-mL conical flasks made of resistant glass aged as specified; a 900-g (2-lb) hammer; a permanent magnet; a desiccator; and an adequate volumetric apparatus.

#### Reagents—

High-Purity Water— The water used in these tests has a conductivity at 25°, as measured in an in-line cell just prior to dispensing, of not greater than 0.15  $\mu$ S per cm (6.67 Megohm-cm). There must also be an assurance that this water is not contaminated by copper or its products (e.g., copper pipes, stills, or receivers). The water may be prepared by passing distilled water through a deionizer cartridge packed with a mixed bed of nuclear-grade resin, then through a cellulose ester membrane having openings not exceeding 0.45  $\mu$ m.<sup>2</sup> Do not use copper tubing. Flush the discharge lines before water is dispensed into test vessels. When the low conductivity specification can no longer be met, replace the deionizer cartridge.

Carbon Dioxide-Free Water— This is [Purified Water](#) that has been boiled vigorously for 5 minutes or more and allowed to cool while protected from absorption of carbon dioxide from the atmosphere, or [Purified Water](#) that has a resistivity of not less than 18 Mohm-cm.

Methyl Red Solution (Powdered Glass Test and Water Attack at 121°)— Dissolve 24 mg of methyl red sodium in [Purified Water](#) to make 100 mL. If necessary, neutralize the solution with 0.02 N sodium hydroxide, or acidify it with 0.02 N sulfuric acid so that the titration of 100 mL of High-Purity Water, containing 5 drops of indicator, does not require more than 0.020 mL of 0.020 N sodium hydroxide to effect the color change of the indicator, which should occur at a pH of 5.6.

Methyl Red Solution (Surface Glass Test)— Dissolve 50 mg of methyl red solution in 1.86 mL of 0.1 M sodium hydroxide and 50 mL of ethanol (96%) and dilute to 100 mL with [Purified Water](#). To test for sensitivity, add 100 mL of Carbon Dioxide-Free Water and 0.05 mL of 0.02 M hydrochloric acid to 0.1 mL of the methyl red solution (the solution should be red). Not more than 0.1 mL of 0.02 M sodium hydroxide is required to change the color to yellow. Color change: pH 4.4 (red) to pH 6.0 (yellow).

#### Powdered Glass Test

Rinse thoroughly with [Purified Water](#) six or more containers selected at random, and dry them with a current of clean, dry air. Crush the containers into fragments about 25 mm in size, divide about 100 g of the coarsely crushed glass into three approximately equal portions, and place one of the portions in the special mortar. With the pestle in place, crush the glass further by striking 3 or 4 blows with the hammer. Nest the sieves, and empty the mortar into the No. 20 sieve. Repeat the operation on each of the two remaining portions of glass, emptying the mortar each time into the No. 20 sieve. Shake the sieves for a short time, then remove the glass from the Nos. 20 and 40 sieves, and again crush and sieve as before. Repeat again this crushing and sieving operation. Empty the receiving pan, reassemble the nest of sieves, and shake by mechanical means for 5 minutes or by hand for an equivalent length of time. Transfer the portion retained on the No. 50 sieve, which should weigh in excess of 10 g, to a closed container, and store in a desiccator until used for the test.

Spread the specimen on a piece of glazed paper, and pass a magnet through it to remove particles of iron that may be introduced during the crushing. Transfer the specimen to a 250-mL conical flask of resistant glass, and wash it with six 30-mL portions of acetone, swirling each time for about 30 seconds, and carefully decanting the acetone. After washing, the specimen should be free from agglomerations of glass powder, and the surface of the grains should be practically free from adhering fine particles. Dry the flask and contents for 20 minutes at 140°, transfer the grains to a weighing bottle, and cool in a desiccator. Use the test specimen within 48 hours after drying.

Procedure— Transfer 10.00 g of the prepared specimen, accurately weighed, to a 250-mL conical flask that has been digested (aged) previously with High-Purity Water in a bath at 90° for at least 24 hours or at 121° for 1 hour. Add 50.0 mL of High-Purity Water to this flask and to one similarly prepared to provide a blank. Cap all flasks with borosilicate glass beakers that previously have been treated as described for the flasks and that are of such size that the bottoms of the beakers fit snugly down on the top rims of the containers. Place the containers in the autoclave, and close it securely, leaving the vent cock open. Heat until steam issues vigorously from the vent cock, and continue heating for 10 minutes. Close the vent cock, and adjust the temperature to 121°, taking 19 to 23 minutes to reach the desired temperature. Hold the temperature at 121 ± 2.0° for 30 minutes, counting from the time this temperature is reached. Reduce the heat so that the autoclave cools and comes to atmospheric pressure in 38 to 46 minutes, being vented as necessary to prevent the formation of a vacuum. Cool the flask at once in running water, decant the water from the flask into a suitably cleansed vessel, and wash the residual powdered glass with four 15-mL portions of High-Purity Water, adding the decanted washings to the main portion. Add 5 drops of Methyl Red Solution, and titrate immediately with 0.020 N sulfuric acid. If the volume of titrating solution is expected to be less than 10 mL, use a microburet. Record the volume of 0.020 N sulfuric acid used to neutralize the extract from 10 g of the prepared specimen of glass,

corrected for a blank. The volume does not exceed that indicated in [Table 2](#) for the type of glass concerned.

Table 2. Test Limits for Powdered Glass Test

Type	General Description	Type of Test	Limits	
			Size, mL of 0.020 N Acid	
I	Highly resistant, borosilicate glass	Powdered Glass	All	1.0
III	Soda-lime glass	Powdered Glass	All	8.5

a The description applies to containers of this type of glass usually available.

b Size indicates the overflow capacity of the container.

#### Surface Glass Test

Determination of the Filling Volume— The filling volume is the volume to be filled with [Purified Water](#) in the container for the purpose of the test. For vials and bottles the filling volume is 90% of the brimful capacity. For ampules it is the volume up to the height of the shoulder.

Vials and Bottle— Select, at random, 6 containers from the sample lot, or 3 if their capacity exceeds 100 mL, and remove any dirt or debris. Weigh the empty containers with an accuracy of 0.1 g. Place the containers on a horizontal surface, and fill them with [Purified Water](#) to about the rim edge, avoiding overflow and introduction of air bubbles. Adjust the liquid levels to the brimful line. Weigh the filled containers to obtain the mass of the water, expressed to 2 decimal places, for containers having a nominal volume less or equal to 30 mL, and expressed to 1 decimal place for containers having a nominal volume greater than 30 mL. Calculate the mean value of the brimful capacity in mL, and multiply it by 0.9. This volume, expressed to 1 decimal place, is the filling volume for the particular container lot.

Ampules— Place at least 6 dry ampules on a flat, horizontal surface, and fill them with [Purified Water](#) from a buret until the water reaches point A, where the body of the ampule decreases to the shoulder of the ampule (see [Figure 2](#)). Read the capacities, expressed to 2 decimal places, and calculate the mean value. This volume, expressed to 1 decimal place, is the filling volume for the particular ampule lot. The filling volume may also be determined by weighing.

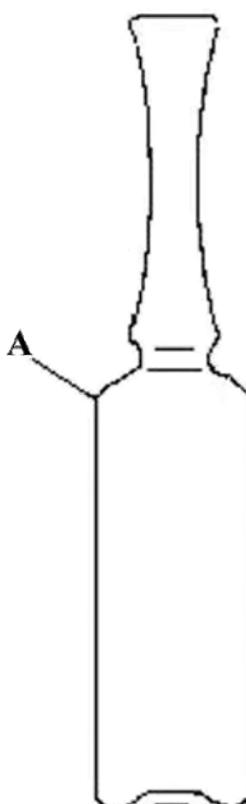


Figure 2. Filling Volumes of Ampules (up to point A)

Test— The determination is carried out on unused containers. The volumes of the test liquid necessary for the final determination are indicated in [Table 3](#).

Table 3. Volume of Test Liquid and Number of Titrations

Filling Volume (mL)	Volume of Test Liquid for One Titration (mL)	Number of Titrations
Up to 3	25.0	1
Above 3 and up to 30	50.0	2
Above 30 and up to 100	100.0	2
Above 100	100.0	3

Cleaning— Remove any debris or dust. Shortly before the test, rinse each container carefully at least twice with [Purified Water](#), and allow to stand. Immediately before testing, empty



the containers, rinse once with [Purified Water](#), then with Carbon Dioxide-Free Water and allow to drain. Complete the cleaning procedure from the first rinsing in not less than 20 minutes and not more than 25 minutes. Heat closed ampules in a water bath or in an air-oven at about 50° for approximately 2 minutes before opening. Do not rinse before testing.

**Filling and Heating**— The containers are filled with Carbon Dioxide-Free Water up to the filling volume. Containers in the form of cartridges or prefilled syringes are closed in a suitable manner with material that does not interfere with the test. Each container, including ampules, shall be loosely capped with an inert material such as a dish of neutral glass or aluminum foil previously rinsed with [Purified Water](#). Place the containers on the tray of the autoclave.

Place the tray in the autoclave containing a quantity of water such that the tray remains clear of the water. Close the autoclave, and carry out the following operations:

1. heat the autoclave to 100° and allow the steam to issue from the vent cock for 10 minutes;
2. close the vent cock and raise the temperature from 100° to 121° at a rate of 1° per minute;
3. maintain the temperature at 121 ± 1° for 60 ± 1 minutes;
4. lower the temperature from 121° to 100° at a rate of 0.5° per minute, venting to prevent a vacuum;
5. do not open the autoclave before it has cooled down to 95°;
6. remove the containers from the autoclave using normal precautions, place them in a water bath at 80°, and run cold tap water, taking care that the water does not contact the loose foil caps to avoid contamination of the extraction solution;
7. cooling time does not exceed 30 minutes.

The extraction solutions are analyzed by titration according to the method described below.

**Method**— Carry out the titration within 1 hour of removal of the containers from the autoclave.

Combine the liquids obtained from the containers, and mix. Introduce the prescribed volume indicated in [Table 3](#) into a conical flask. Place the same volume of Carbon Dioxide-Free Water into a second similar flask as a blank. Add 0.05 mL of Methyl Red Solution to each flask for each 25 mL of liquid. Titrate the blank with 0.01 M hydrochloric acid. Titrate the test liquid with the same acid until the color of the resulting solution is the same as that obtained for the blank. Subtract the value found for the blank titration from that found for the test liquid, and express the results in mL of 0.01 M hydrochloric acid per 100 mL. Express titration values of less than 1.0 mL to 2 decimal places and titration values of more than or equal to 1.0 mL to 1 decimal place.

**Limits**— The results, or the average of the results if more than one titration is performed, are not greater than the values stated in [Table 4](#).

Table 4. Test Limits for Surface Glass Test

Filling Volume (mL)	Maximum Volume of 0.01 M HCl per 100 mL of Test Liquid (mL)	
	Types I and II	Type III
Up to 1	2.0	20.0
Above 1 and Up to 2	1.8	17.6
Above 2 and Up to 5	1.3	13.2
Above 5 and Up to 10	1.0	10.2
Above 10 and Up to 20	0.80	8.1
Above 20 and Up to 50	0.60	6.1
Above 50 and Up to 100	0.50	4.8
Above 100 and Up to 200	0.40	3.8
Above 200 and Up to 500	0.30	2.9
Above 500	0.20	2.2

Water Attack at 121°

**Option**— The Water Attack at 121° test can be used to qualify Type II glass.

Rinse thoroughly 3 or more containers, selected at random, twice with High-Purity Water.

**Procedure**— Fill each container to 90% of its overflow capacity with High-Purity Water, and proceed as directed for Procedure under Powdered Glass Test, beginning with “Cap all flasks,” except that the time of autoclaving shall be 60 minutes instead of 30 minutes, and ending with “to prevent the formation of a vacuum.” Empty the contents from 1 or more containers into a 100-mL graduated cylinder, combining, in the case of smaller containers, the contents of several containers to obtain a volume of 100 mL. Place the pooled specimen

in a 250-mL conical flask of resistant glass, add 5 drops of Methyl Red Solution, and titrate, while warm, with 0.020 N sulfuric acid. Complete the titration within 60 minutes after opening the autoclave. Record the volume of 0.020 N sulfuric acid used, corrected for a blank obtained by titrating 100 mL of High-Purity Water at the same temperature and with the same amount of indicator. The volume does not exceed that indicated in [Table 5](#).

Table 5. Test Limit for Water Attack at 121°

Type	General Description <sup>a</sup>	Type of Test	Limits	
			Size, <sup>b</sup> mL	mL of 0.020 N Acid
II	Treated soda-lime glass	Water Attack	100 or less	0.7
			Over 100	0.2

<sup>a</sup> The description applies to containers of this type of glass usually available.

<sup>b</sup> Size indicates the overflow capacity of the container.

#### Arsenic

[Arsenic](#) (211) — Use as the Test Preparation 35 mL of the water from one Type I glass container or, in the case of smaller containers, 35 mL of the combined contents of several Type I glass containers, prepared as directed for Procedure under Water Attack at 121° or Surface Glass Test: the limit is 0.1 µg per g.

1 A suitable mortar and pestle is available (catalog No. H-17280) from Humboldt Manufacturing Co., 7300 West Agatite Avenue, Norridge, IL, 60706, [www.humboldtmfg.com](#)

2 A suitable nuclear-grade resin mixture of the strong acid cation exchanger in the hydrogen form and the strong base anion exchanger in the hydroxide form, with a one-to-one cation to anion equivalence ratio, is available from the Millipore Corp, 290 Concord Road Billerica, MA, 01821, [www.millipore.com](#); Barnstead International, 2555 Kerper Boulevard Dubuque, IA, 52004, [www.barnsteadthermolyne.com](#); GE Water, 4636 Somerton Road Trevose, PA, 19053, [www.gewater.com](#); Pall, 2200 Northern Boulevard East Hills, NY 11548, [www.pall.com](#); Whatman, 200 Park Avenue Florham Park, NJ, 07932, [www.whatman.com](#); Siemens Water Technologies, 14950 Heathrow Forest Pa, Houston, TX 77032, [www.usfilter.com](#)

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Desmond G. Hunt, Ph.D.</a>	(PS05) Packaging and Storage 05

## INTRODUCTION

It is the purpose of this chapter to provide standards for plastic materials and components used to package medical articles (pharmaceuticals, biologics, dietary supplements, and devices). Definitions that apply to this chapter are provided in the Preservation, Packaging, Storage, and Labeling section of the General Notices and Requirements. Standards and tests for the functional properties of containers and their components are provided in general chapter [Containers—Performance Testing \(671\)](#).

In addition to the standards provided herein, the ingredients added to the polymers, and those used in the fabrication of the containers, must conform to the requirements in the applicable sections of the Code of Federal Regulations, Title 21, Indirect Food Additives, or have been evaluated by the FDA and determined to be acceptable substances for the listed use.

Plastic articles are identified and characterized by IR spectroscopy and differential scanning calorimetry. Standards are provided in this chapter for the identification and characterization of the different types of plastic, and the test procedures are provided at the end of the chapter. The degree of testing is based on whether or not the container has direct contact with the drug product, and the risk is based on the route of administration.

Plastics are composed of a mixture of homologous polymers, having a range of molecular weights. Plastics may contain other substances such as residues from the polymerization process, plasticizers, stabilizers, antioxidants, pigments, and lubricants. These materials meet the requirements for food contact as provided in the Code of Federal Regulations, Title 21. Factors such as plastic composition, processing and cleaning procedures, surface treatment, contacting media, inks, adhesives, absorption and permeability of preservatives, and conditions of storage may also affect the suitability of a plastic for a specific use. Extraction tests are designed to characterize the extracted components and identify possible migrants.

The degree or extent of testing for extractables of the component is dependent on the intended use and the degree of risk to adversely impact the efficacy of the compendial article (drug, biologic, dietary supplement, or device). Resin-specific extraction tests are provided in this chapter for polyethylene, polypropylene, polypropylene terephthalate, and polypropylene terephthalate G. Test all other plastics as directed under Physicochemical Tests in the section Test Methods. Conduct the Buffering Capacity test only when the containers are intended to hold a liquid product.

Plastic components used for products of high risk, such as those intended for inhalation, parenteral preparation, and ophthalmics are tested using the Biological Tests in the section Test Methods.

Plastic containers intended for packaging products prepared for parenteral use meet the requirements under Biological Tests and Physicochemical Tests in the section Test Methods. Standards are also provided for polyethylene containers used to package dry oral dosage forms that are not meant for constitution into solution.

## POLYETHYLENE CONTAINERS

## Scope

The standards and tests provided in this section characterize containers and components produced from either low-density polyethylene or high-density polyethylene of either homopolymer or copolymer resin. All polyethylene components are subject to testing by IR spectroscopy and differential scanning calorimetry. Where stability studies have been performed to establish the expiration date of a particular dosage form in the appropriate polyethylene container, then any other polyethylene container meeting these requirements may be similarly used to package such a dosage form, provided that the appropriate stability programs are expanded to include the alternative container, in order to ensure that the identity, strength, quality, and purity of the dosage form are maintained throughout the expiration period.

## Background

High-density and low-density polyethylene are long-chain polymers synthesized under controlled conditions of heat and pressure, with the aid of catalysts from not less than 85.0% ethylene and not less than 95.0% total olefins. Other olefin ingredients that are most frequently used are butene, hexene, and propylene. High-density polyethylene and low-density polyethylene both have an IR absorption spectrum that is distinctive for polyethylene, and each possesses characteristic thermal properties. High-density polyethylene has a density between 0.941 and 0.965 g per cm<sup>3</sup>. Low-density polyethylene has a density between 0.850 and 0.940 g per cm<sup>3</sup>. Other properties that may affect the suitability of polyethylene include modulus of elasticity, melt index, environmental stress crack resistance, and degree of crystallinity after molding.

## High-Density Polyethylene

Infrared Spectroscopy— Proceed as directed under Multiple Internal Reflectance in the section Test Methods. The corrected spectrum of the specimen exhibits major absorption bands only at the same wavelengths as the spectrum of [USP High-Density Polyethylene RS](#).

Differential Scanning Calorimetry— Proceed as directed under Thermal Analysis in the section Test Methods. The thermogram of the specimen is similar to the thermogram of [USP High-Density Polyethylene RS](#), similarly determined, and the temperature of the endotherm (melt) in the thermogram of the specimen does not differ from that of the USP Reference Standard by more than 6.0°.

Heavy Metals and Nonvolatile Residue— Prepare extracts of specimens for these tests as directed for under Physicochemical Tests in the section Test Methods, except Sample Preparation that for each 20.0 mL of Extracting Medium the portion shall be 60 cm<sup>2</sup>, regardless of thickness.

HEAVY METALS— Containers meet the requirements for Heavy Metals under Physicochemical Tests in the section Test Methods.

NONVOLATILE RESIDUE— Proceed as directed for Nonvolatile Residue under Physicochemical Tests, except that the Blank shall be the same solvent used in each of the following test conditions: the difference between the amounts obtained from the Sample Preparation and the Blank does not exceed 12.0 mg when water maintained at a temperature of 70° is used as the Extracting Medium; does not exceed 75.0 mg when alcohol maintained at a temperature of 70° is used as the Extracting Medium; and does not exceed 100.0 mg when hexanes maintained at a temperature of 50° is used as the Extracting Medium.

Components Used in Contact with Oral Liquids— Proceed as directed under Buffering Capacity under Physicochemical Tests in the section Test Methods.

## Low-Density Polyethylene

Infrared Spectroscopy— Proceed as directed under Multiple Internal Reflectance in the section Test Methods. The corrected spectrum of the specimen exhibits major absorption bands only at the same wavelengths as the spectrum of [USP Low-Density Polyethylene RS](#).

Differential Scanning Calorimetry— Proceed as directed under Thermal Analysis in the section Test Methods. The thermogram of the specimen is similar to the thermogram of [USP Low-Density Polyethylene RS](#), similarly determined, and the temperature of the endotherm (melt) in the thermogram of the specimen does not differ from that of the USP Reference Standard by more than 8.0°.

Heavy Metals and Nonvolatile Residue— Prepare extracts of specimens for these tests as directed for Sample Preparation under Physicochemical Tests in the section Test Methods, except that for each 20.0 mL of Extracting Medium the portion shall be 60 cm<sup>2</sup>, regardless of thickness.

HEAVY METALS— Containers meet the requirements for Heavy Metals under Physicochemical Tests in the section Test Methods.

NONVOLATILE RESIDUE— Proceed as directed for Nonvolatile Residue under Physicochemical Tests in the section Test Methods, except that the Blank shall be the same solvent used in each of the following test conditions: the difference between the amounts obtained from the Sample Preparation and the Blank does not exceed 12.0 mg when water maintained at a temperature of 70° is used as the Extracting Medium; does not exceed 75.0 mg when alcohol maintained at a temperature of 70° is used as the Extracting Medium; and does not



exceed 350.0 mg when hexanes maintained at a temperature of 50° is used as the Extracting Medium.

Components Used in Contact with Oral Liquids— Proceed as directed under Buffering Capacity under Physicochemical Tests in the section Test Methods.

## POLYPROPYLENE CONTAINERS

### Scope

The standards and tests provided in this section characterize polypropylene containers, produced from either homopolymers or copolymers, that are interchangeably suitable for packaging dry solid and liquid oral dosage forms. Where suitable stability studies have been performed to establish the expiration date of a particular dosage form in the appropriate polypropylene container, then any other polypropylene container meeting these requirements may be similarly used to package such a dosage form, provided that the appropriate stability programs are expanded to include the alternative container, in order to ensure that the identity, strength, quality, and purity of the dosage form are maintained throughout the expiration period.

### Background

Propylene polymers are long-chain polymers synthesized from propylene or propylene and other olefins under controlled conditions of heat and pressure, with the aid of catalysts.

Examples of other olefins most commonly used include ethylene and butene. The propylene polymers, the ingredients used to manufacture the propylene polymers, and the ingredients used in the fabrication of the containers conform to the applicable sections of the Code of Federal Regulations, Title 21.

Factors such as plastic composition, processing and cleaning procedures, contacting media, inks, adhesives, absorption, adsorption and permeability of preservatives, and conditions of storage may also affect the suitability of a plastic for a specific use. The suitability of a specific polypropylene must be established by appropriate testing.

Polypropylene has a distinctive IR spectrum and possesses characteristic thermal properties. It has a density between 0.880 and 0.913 g per cm<sup>3</sup>. The permeation properties of molded polypropylene containers may be altered when reground polymer is incorporated, depending on the proportion of reground material in the final product. Other properties that may affect the suitability of polypropylene used in containers for packaging drugs are the following: oxygen and moisture permeability, modulus of elasticity, melt flow index, environmental stress crack resistance, and degree of crystallinity after molding. The requirements in this section are to be met when dry solid and liquid oral dosage forms are to be packaged in a container defined by this section.

Infrared Spectroscopy— Proceed as directed under Multiple Internal Reflectance in the section Test Methods. The corrected spectrum of the specimen exhibits major absorption bands only at the same wavelengths as the spectrum of the respective [USP Homopolymer Polypropylene RS](#) or USP Copolymer Polypropylene RS, similarly determined.

Differential Scanning Calorimetry— Proceed as directed under Thermal Analysis in the section Test Methods. The temperature of the endotherm (melt) in the thermogram does not differ from that of the USP Reference Standard for homopolymers by more than 6.0°. The temperature of the endotherm obtained from the thermogram of the copolymer polypropylene specimen does not differ from that of the copolymer polypropylene standard by more than 12.0°.

Heavy Metals and Nonvolatile Residue— Prepare extracts of specimens for these tests as directed for Sample Preparation under Physicochemical Tests in the section Test Methods, except that for each 20 mL of Extracting Medium the portion shall be 60 cm<sup>2</sup>, regardless of thickness.

HEAVY METALS— Containers meet the requirements for Heavy Metals under Physicochemical Tests in the section Test Methods.

NONVOLATILE RESIDUE— Proceed as directed for Nonvolatile Residue under Physicochemical Tests in the section Test Methods, except that the Blank shall be the same solvent used in each of the following test conditions: the difference between the amounts obtained from the Sample Preparation and the Blank does not exceed 10.0 mg when water maintained at a temperature of 70° is used as the Extracting Medium; does not exceed 60.0 mg when alcohol maintained at a temperature of 70° is used as the Extracting Medium; and does not exceed 225.0 mg when hexanes maintained at a temperature of 50° is used as the Extracting Medium. Containers meet these requirements for Nonvolatile Residue for all of the above extracting media. [note—Hexanes and alcohol are flammable. When evaporating these solvents, use a current of air with the water bath; when drying the residue, use an explosion-proof oven.]

Components Used in Contact with Oral Liquids— Proceed as directed in Buffering Capacity under Physicochemical Tests in the section Test Methods.

## POLYETHYLENE TEREPHTHALATE BOTTLES AND POLYETHYLENE TEREPHTHALATE G CONTAINERS

### Scope

The standards and tests provided in this section characterize polyethylene terephthalate (PET) and polyethylene terephthalate G (PETG) bottles that are interchangeably suitable for packaging liquid oral dosage forms. Where stability studies have been performed to establish the expiration date of a particular liquid oral dosage form in a bottle meeting the requirements set forth herein for either PET or PETG bottles, any other PET or PETG bottle meeting these requirements may be similarly used to package such a dosage form, provided that the appropriate stability programs are expanded to include the alternative bottle in order to ensure that the identity, strength, quality, and purity of the dosage form are maintained throughout the expiration period. The suitability of a specific PET or PETG bottle for use in the dispensing of a particular pharmaceutical liquid oral dosage form must be established by appropriate testing.

### Background

PET resins are long-chain crystalline polymers prepared by the condensation of ethylene glycol with dimethyl terephthalate or terephthalic acid. PET copolymer resins are prepared in a similar way, except that they may also contain a small amount of either isophthalic acid (not more than 3 mole percent) or 1,4-cyclohexanedimethanol (not more than 5 mole percent). Polymerization is conducted under controlled conditions of heat and vacuum, with the aid of catalysts and stabilizers.

PET copolymer resins have physical and spectral properties similar to PET and for practical purposes are treated as PET. The tests and specifications provided in this section to characterize PET resins and bottles apply also to PET copolymer resins and to bottles fabricated from them.

PET and PET copolymer resins generally exhibit a large degree of order in their molecular structure. As a result, they exhibit characteristic composition-dependent thermal behavior, including a glass transition temperature of about 76° and a melting temperature of about 250°. These resins have a distinctive IR absorption spectrum that allows them to be distinguished from other plastic materials (e.g., polycarbonate, polystyrene, polyethylene, and PETG resins). PET and PET copolymer resins have a density between 1.3 and 1.4 g per cm<sup>3</sup> and a minimum intrinsic viscosity of 0.7 dL per g, which corresponds to a number average molecular weight of about 23,000 Da.

PETG resins are high molecular weight polymers prepared by the condensation of ethylene glycol with dimethyl terephthalate or terephthalic acid and 15 to 34 mole percent of 1,4-cyclohexanedimethanol. PETG resins are clear, amorphous polymers, having a glass transition temperature of about 81° and no crystalline melting point, as determined by differential scanning calorimetry. PETG resins have a distinctive IR absorption spectrum that allows them to be distinguished from other plastic materials, including PET. PETG resins have a density of approximately 1.27 g per cm<sup>3</sup> and a minimum intrinsic viscosity of 0.65 dL per g, which corresponds to a number average molecular weight of about 16,000 Da.

PET and PETG resins, and other ingredients used in the fabrication of these bottles, conform to the requirements in the applicable sections of the Code of Federal Regulations, Title 21, regarding use in contact with food and alcoholic beverages. PET and PETG resins do not contain any plasticizers, processing aids, or antioxidants. Colorants, if used in the manufacture of PET and PETG bottles, do not migrate into the contained liquid.

Infrared Spectroscopy— Proceed as directed under Multiple Internal Reflectance in the section Test Methods. The corrected spectrum of the specimen exhibits major absorption bands only at the same wavelengths as the spectrum of [USP Polyethylene Terephthalate RS](#), or [USP Polyethylene Terephthalate G RS](#), similarly determined.

Differential Scanning Calorimetry— Proceed as directed under Thermal Analysis in the section Test Methods. For polyethylene terephthalate, the thermogram of the specimen is similar to the thermogram of [USP Polyethylene Terephthalate RS](#), similarly determined: the melting point (T<sub>m</sub>) of the specimen does not differ from that of the USP Reference Standard by more than 9.0°, and the glass transition temperature (T<sub>g</sub>) of the specimen does not differ from that of the USP Reference Standard by more than 4.0°. For polyethylene terephthalate G, the thermogram of the specimen is similar to the thermogram of USP Polyethylene Terephthalate G RS, similarly determined: the glass transition temperature (T<sub>g</sub>) of



the specimen does not differ from that of the USP Reference Standard by more than 6.0°.

Colorant Extraction— Select three test bottles. Cut a relatively flat portion from the side wall of one bottle, and trim it as necessary to fit the sample holder of the spectrophotometer.

Obtain the visible spectrum of the side wall by scanning the portion of the visible spectrum from 350 to 700 nm. Determine, to the nearest 2 nm, the wavelength of maximum absorbance. Fill the remaining two test bottles, using 50% alcohol for PET bottles and 25% alcohol for PETG bottles. Fit the bottles with impervious seals, such as aluminum foil, and apply closures. Fill a glass bottle having the same capacity as that of the test bottles with the corresponding solvent, fit the bottle with an impervious seal, such as aluminum foil, and apply a closure. Incubate the test bottles and the glass bottle at 49° for 10 days. Remove the bottles, and allow them to equilibrate to room temperature. Concomitantly determine the absorbances of the test solutions in 5-cm cells at the wavelength of maximum absorbance (see [Spectrophotometry and Light-Scattering \(851\)](#)), using the corresponding solvent from the glass bottle as the blank. The absorbance values so obtained are less than 0.01 for both test solutions.

#### Heavy Metals, Total Terephthaloyl Moieties, and Ethylene Glycol—

##### EXTRACTING MEDIA—

[Purified Water](#)— (see monograph).

50 Percent Alcohol— Dilute 125 mL of alcohol with water to 238 mL, and mix.

25 Percent Alcohol— Dilute 125 mL of 50 Percent Alcohol with water to 250 mL, and mix.

n-Heptane.

GENERAL PROCEDURE— [note—Use an Extracting Medium of 50 Percent Alcohol for PET bottles and 25 Percent Alcohol for PETG bottles.] For each Extracting Medium, fill a sufficient number of test bottles to 90% of their nominal capacity to obtain not less than 30 mL. Fill a corresponding number of glass bottles with Purified Water, a corresponding number of glass bottles with 50 Percent Alcohol or 25 Percent Alcohol, and a corresponding number of glass bottles with n-Heptane for use as Extracting Media blanks. Fit the bottles with impervious seals, such as aluminum foil, and apply closures. Incubate the test bottles and the glass bottles at 49° for 10 days. Remove the test bottles with the Extracting Media samples and the glass bottles with the Extracting Media blanks, and store them at room temperature. Do not transfer the Extracting Media samples to alternative storage vessels.

HEAVY METALS— Pipet 20 mL of the Purified Water extract of the test bottles, filtered if necessary, into one of two matched 50-mL color-comparison tubes, and retain the remaining Purified Water extract in the test bottles for use in the test for Ethylene Glycol. Adjust the extract with 1 N acetic acid or 6 N ammonium hydroxide to a pH between 3.0 and 4.0, using short-range pH paper as an external indicator. Dilute with water to about 35 mL, and mix.

Into the second color-comparison tube, pipet 2 mL of freshly prepared (on day of use) Standard Lead Solution (see [Heavy Metals \(231\)](#)), and add 20 mL of Purified Water. Adjust with 1 N acetic acid or 6 N ammonium hydroxide to a pH between 3.0 and 4.0, using short-range pH paper as an external indicator. Dilute with water to about 35 mL, and mix.

To each tube add 1.2 mL of thioacetamide–glycerin base TS and 2 mL of pH 3.5 Acetate Buffer (see [Heavy Metals \(231\)](#)), dilute with water to 50 mL, and mix: any color produced within 10 minutes in the tube containing the Purified Water extract of the test bottles does not exceed that in the tube containing the Standard Lead Solution, both tubes being viewed downward over a white surface (1 ppm in extract).

TOTAL TEREPHTHALOYL MOIETIES— Determine the absorbance of the 50 Percent Alcohol or 25 Percent Alcohol extract in a 1-cm cell at the wavelength of maximum absorbance at about 244 nm (see [Spectrophotometry and Light-Scattering \(851\)](#)), using as the blank the corresponding Extracting Medium blank: the absorbance of the extract does not exceed 0.150, corresponding to not more than 1 ppm of total terephthaloyl moieties.

Determine the absorbance of the n-Heptane extract in a 1-cm cell at the wavelength of maximum absorbance at about 240 nm (see [Spectrophotometry and Light-Scattering \(851\)](#)), using as the blank the n-Heptane Extracting Medium: the absorbance of the extract does not exceed 0.150, corresponding to not more than 1 ppm of total terephthaloyl moieties.

##### ETHYLENE GLYCOL—

Periodic Acid Solution— Dissolve 125 mg of periodic acid in 10 mL of water.

Dilute Sulfuric Acid— To 50 mL of water add slowly and with constant stirring 50 mL of sulfuric acid, and allow to cool to room temperature.

Sodium Bisulfite Solution— Dissolve 0.1 g of sodium bisulfite in 10 mL of water. Use this solution within 7 days.

Disodium Chromotropate Solution— Dissolve 100 mg of disodium chromotropate in 100 mL of sulfuric acid. Protect this solution from light, and use within 7 days.

Standard Solution— Dissolve an accurately weighed quantity of ethylene glycol in water, and dilute quantitatively, and stepwise if necessary, to obtain a solution having a known concentration of about 1 µg per mL.

Test Solution— Use the Purified Water extract.

Procedure— Transfer 1.0 mL of the Standard Solution to a 10-mL volumetric flask. Transfer 1.0 mL of the Test Solution to a second 10-mL volumetric flask. Transfer 1.0 mL of the Purified Water Extracting Medium to a third 10-mL volumetric flask. To each of the three flasks, add 100 µL of Periodic Acid Solution, swirl to mix, and allow to stand for 60 minutes. Add 1.0 mL of Sodium Bisulfite Solution to each flask, and mix. Add 100 µL of Disodium Chromotropate Solution to each flask, and mix. [note—All solutions should be analyzed within 1 hour after addition of the Disodium Chromotropate Solution.] Cautiously add 6 mL of sulfuric acid to each flask, mix, and allow the solutions to cool to room temperature. [Caution—Dilution of sulfuric acid produces substantial heat and can cause the solution to boil. Perform this addition carefully. Sulfur dioxide gas will be evolved. Use of a fume hood is recommended.] Dilute each solution with Dilute Sulfuric Acid to volume, and mix. Concomitantly determine the absorbances of the solutions from the Standard Solution and the Test

Solution in 1-cm cells at the wavelength of maximum absorbance at about 575 nm (see [Spectrophotometry and Light-Scattering \(851\)](#)), using as the blank the solution from the Purified Water Extracting Medium: the absorbance of the solution from the Test Solution does not exceed that of the solution from the Standard Solution, corresponding to not more than 1 ppm of ethylene glycol.

## TEST METHODS

### Multiple Internal Reflectance

Apparatus— Use an IR spectrophotometer capable of correcting for the blank spectrum and equipped with a multiple internal reflectance accessory and a KRS-5 internal reflection plate.<sup>1</sup> A KRS-5 crystal 2-mm thick having an angle of incidence of 45° provides a sufficient number of reflections.

Specimen Preparation— Cut two flat sections representative of the average wall thickness of the container, and trim them as necessary to obtain segments that are convenient for mounting in the multiple internal reflectance accessory. Taking care to avoid scratching the surfaces, wipe the specimens with dry paper or, if necessary, clean them with a soft cloth dampened with methanol, and permit them to dry. Securely mount the specimens on both sides of the KRS-5 internal reflection plate, ensuring adequate surface contact. Prior to mounting the specimens on the plate, they may be compressed to thin uniform films by exposing them to temperatures of about 177° under high pressures (15,000 psi or more).

General Procedure— Place the mounted specimen sections within the multiple internal reflectance accessory, and place the assembly in the specimen beam of the IR spectrophotometer. Adjust the specimen position and mirrors within the accessory to permit maximum light transmission of the unattenuated reference beam. (For a double-beam instrument, upon completing the adjustments in the accessory, attenuate the reference beam to permit full-scale deflection during the scanning of the specimen.) Determine the IR spectrum from 3500 to 600 cm<sup>-1</sup> for polyethylene and polypropylene and from 4000 to 400 cm<sup>-1</sup> for PET and PETG.

### Thermal Analysis

General Procedure— Cut a section weighing about 12 mg, and place it in the test specimen pan. [note—Intimate contact between the pan and the thermocouple is essential for reproducible results.] Determine the thermogram under nitrogen, using the heating and cooling conditions as specified for the resin type and using equipment capable of performing the determinations as specified under [Thermal Analysis \(891\)](#).

For Polyethylene— Determine the thermogram under nitrogen at temperatures between 40° and 200° at a heating rate between 2° and 10° per minute followed by cooling at a rate



between 2° and 10° per minute to 40°.

For Polypropylene— Determine the thermogram under nitrogen at temperatures ranging from ambient to 30° above the melting point. Maintain the temperature for 10 minutes, then cool to 50° below the peak crystallization temperature at a rate of 10° to 20° per minute.

For Polyethylene Terephthalate— Heat the specimen from room temperature to 280° at a heating rate of about 20° per minute. Hold the specimen at 280° for 1 minute. Quickly cool the specimen to room temperature, and reheat it to 280° at a heating rate of about 5° per minute.

For Polyethylene Terephthalate G— Heat the specimen from room temperature to 120° at a heating rate of about 20° per minute. Hold the specimen at 120° for 1 minute. Quickly cool the specimen to room temperature, and reheat it to 120° at a heating rate of about 10° per minute.

#### Biological Tests

The in vitro biological tests are performed according to the procedures set forth under [Biological Reactivity Test, In Vitro](#) (87). Components that meet the requirements of the in vitro tests are not required to undergo further testing. No plastic class designation is assigned to these materials. Materials that do not meet the requirements of the in vitro tests are not suitable for containers for drug products.

If a plastic class designation is needed for plastics and other polymers that meet the requirements under [Biological Reactivity Test, In Vitro](#) (87), perform the appropriate in vivo test specified for Classification of Plastics under [Biological Reactivity Test, In Vivo](#) (88).

#### Physicochemical Tests

The following tests, designed to determine physical and chemical properties of plastics and their extracts, are based on the extraction of the plastic material, and it is essential that the designated amount of the plastic be used. Also, the specified surface area must be available for extraction at the designated temperature.

#### Testing Parameters—

Extracting Medium— Unless otherwise directed in a specific test below, use [Purified Water](#) (see monograph) as the Extracting Medium, maintained at a temperature of 70° during the extraction of the Sample Preparation.

Blank— Use [Purified Water](#) where a blank is specified in the tests that follow.

Apparatus— Use a water bath and the Extraction Containers as described under [Biological Reactivity Tests, In Vivo](#) (88). Proceed as directed in the first paragraph of Preparation of Apparatus under [Biological Reactivity Tests, In Vivo](#) (88). [note—The containers and equipment need not be sterile.]

Sample Preparation— From a homogeneous plastic specimen, use a portion, for each 20.0 mL of Extracting Medium, equivalent to 120 cm<sup>2</sup> total surface area (both sides combined), and subdivide into strips approximately 3 mm in width and as near to 5 cm in length as is practical. Transfer the subdivided sample to a glass-stoppered, 250-mL graduated cylinder of Type I glass, and add about 150 mL of Purified Water. Agitate for about 30 seconds, drain off and discard the liquid, and repeat with a second washing.

Sample Preparation Extract— Transfer the prepared Sample Preparation to a suitable extraction flask, and add the required amount of Extracting Medium. Extract by heating in a water bath at the temperature specified for the Extracting Medium for 24 hours. Cool, but not below 20°. Pipet 20 mL of the prepared extract into a suitable container. [note—Use this portion in the test for Buffering Capacity.] Immediately decant the remaining extract into a suitably cleansed container, and seal.

Nonvolatile Residue— Transfer, in suitable portions, 50.0 mL of the Sample Preparation Extract to a suitable, tared crucible (preferably a fused-silica crucible that has been acid-cleaned), and evaporate the volatile matter on a steam bath. Similarly evaporate 50.0 mL of the Blank in a second crucible. [note—If an oily residue is expected, inspect the crucible repeatedly during the evaporation and drying period, and reduce the amount of heat if the oil tends to creep along the walls of the crucible.] Dry at 105° for 1 hour: the difference between the amounts obtained from the Sample Preparation Extract and the Blank does not exceed 15 mg.

[Residue on Ignition](#) (281)— [note—It is not necessary to perform this test when the Nonvolatile Residue test result does not exceed 5 mg.] Proceed with the residues obtained from the Sample Preparation Extract and from the Blank in the test for Nonvolatile Residue above, using, if necessary, additional sulfuric acid but adding the same amount of sulfuric acid to each crucible: the difference between the amounts of residue on ignition obtained from the Sample Preparation Extract and the Blank does not exceed 5 mg.

Heavy Metals— Pipet 20 mL of the Sample Preparation Extract, filtered if necessary, into one of two matched 50-mL color-comparison tubes. Adjust with 1 N acetic acid or 6 N ammonium hydroxide to a pH between 3.0 and 4.0, using short-range pH paper as an external indicator, dilute with water to about 35 mL, and mix.

Into the second color-comparison tube pipet 2 mL of Standard Lead Solution (see [Heavy Metals](#) (231)), and add 20 mL of the Blank. Adjust with 1 N acetic acid or 6 N ammonium hydroxide to a pH between 3.0 and 4.0, using short-range pH paper as an external indicator, dilute with water to about 35 mL, and mix. To each tube add 1.2 mL of thioacetamide-glycerin base TS and 2 mL of pH 3.5 Acetate Buffer (see [Heavy Metals](#) (231)), dilute with water to 50 mL, and mix: any brown color produced within 10 minutes in the tube containing the Sample Preparation Extract does not exceed that in the tube containing the Standard Lead Solution, both tubes being viewed downward over a white surface (1 ppm in extract).

Buffering Capacity— Titrate the previously collected 20-mL portion of the Sample Preparation Extract potentiometrically to a pH of 7.0, using either 0.010 N hydrochloric acid or 0.010 N sodium hydroxide, as required. Treat a 20.0-mL portion of the Blank similarly: if the same titrant was required for both the Sample Preparation Extract and the Blank, the difference between the two volumes is not greater than 10.0 mL; and if acid was required for either the Sample Preparation Extract or the Blank and alkali for the other, the total of the two volumes required is not greater than 10.0 mL.

1 The multiple internal reflectance accessory and KRS-5 plate are available from several sources, including Beckman Instruments, Inc., 2500 Harbor Blvd., Fullerton, CA 92634, and from Perkin Elmer Corp., Main Ave., Norwalk, CT 06856.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Desmond G. Hunt, Ph.D.</a> Scientist 1-301-816-8341	(PS05) Packaging and Storage 05

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#### 671 CONTAINERS—PERFORMANCE TESTING

It is the purpose of this chapter to provide standards for the functional properties of plastic containers and their components used to package regulated articles (pharmaceuticals, biologics, dietary supplements, and devices). Definitions that apply to this chapter are provided in the Preservation, Packaging, Storage, and Labeling section of the General Notices and Requirements. The tests that follow are provided to determine the moisture permeability and light transmission of plastic containers utilized for regulated articles. The section Multiple-Unit Containers for Capsules and Tablets applies to multiple-unit containers. The section Single-Unit Containers and Unit-Dose Containers for Capsules and Tablets applies to single-unit and unit-dose containers. The section Multiple-Unit Containers for Capsules and Tablets (Without Closure) applies to polyethylene and polypropylene containers that have no closures. The section Multiple-Unit and Single-Unit Containers for Liquids applies to multiple-unit and single-unit containers.

A container intended to provide protection from light or offered as a light-resistant container meets the requirements for Light Transmission, where such protection or resistance is by virtue of the specific properties of the material of which the container is composed, including any coating applied thereto. A clear and colorless or a translucent container that is made



...resistant by means of an opaque enclosure (see General Notices and Requirements) is exempt from the requirements for Light Transmission. As used herein, the term "container" refers to the entire system comprising, usually, the container itself, the liner (if used), the closure in the case of multiple-unit containers, and the lidding and blister in the case of unit-dose containers.

#### MULTIPLE-UNIT CONTAINERS FOR CAPSULES AND TABLETS

Desiccant— Place a quantity of 4- to 8-mesh, anhydrous calcium chloride<sup>1</sup> in a shallow container, taking care to exclude any fine powder, then dry at 110° for 1 hour, and cool in a desiccator.

Procedure— Select 12 containers of a uniform size and type, clean the sealing surfaces with a lint-free cloth, and close and open each container 30 times. Apply the closure firmly and uniformly each time the container is closed. Close screw-capped containers with a torque that is within the range of tightness specified in the accompanying table. Add Desiccant to 10 of the containers, designated test containers, filling each to within 13 mm of the closure if the container volume is 20 mL or more, or filling each to two-thirds of capacity if the container volume is less than 20 mL. If the interior of the container is more than 63 mm in depth, an inert filler or spacer may be placed in the bottom to minimize the total weight of the container and Desiccant; the layer of Desiccant in such a container shall be not less than 5 cm in depth. Close each immediately after adding Desiccant, applying the torque designated in the accompanying table when closing screw-capped containers. To each of the remaining 2 containers, designated controls, add a sufficient number of glass beads to attain a weight approximately equal to that of each of the test containers, and close, applying the torque designated in the accompanying table when closing screw-capped containers. Record the weight of the individual containers so prepared to the nearest 0.1 mg if the container volume is less than 20 mL; to the nearest mg if the container volume is 20 mL or more but less than 200 mL; or to the nearest centigram (10 mg) if the container volume is 200 mL or more; and store at 75 ± 3% relative humidity and a temperature of 23 ± 2°. [note—A saturated system of 35 g of sodium chloride with each 100 mL of water placed in the bottom of a desiccator maintains the specified humidity. Other methods may be employed to maintain these conditions.] After 336 ± 1 hours (14 days), record the weight of the individual containers in the same manner. Completely fill 5 empty containers of the same size and type as the containers under test with water or a noncompressible, free-flowing solid such as well-tamped fine glass beads, to the level indicated by the closure surface when in place. Transfer the contents of each to a graduated cylinder, and determine the average container volume, in mL. Calculate the rate of moisture permeability, in mg per day per L, by the formula:

$$(1000 / 14V)[(TF - TI) - (CF - CI)]$$

in which V is the volume, in mL, of the container, (TF - TI) is the difference, in mg, between the final and initial weights of each test container, and (CF - CI) is the difference, in mg, between the average final and average initial weights of the 2 controls. For containers used for drugs being dispensed on prescription, the containers so tested are tight containers if not more than one of the 10 test containers exceeds 100 mg per day per L in moisture permeability, and none exceeds 200 mg per day per L.

For containers used for drugs being dispensed on prescription, the containers are well-closed containers if not more than one of the 10 test containers exceeds 2000 mg per day per L in moisture permeability, and none exceeds 3000 mg per day per L.

#### Torque Applicable to Screw-Type Container

Closure Diameter <sup>a</sup> (mm)	Suggested Tightness Range with Manually Applied Torque, <sup>b</sup> (inch-pounds)
8	5
10	6
13	8
15	5–9
18	7–10
20	8–12
22	9–14
24	10–18
28	12–21
30	13–23
33	15–25
38	17–26
43	17–27
48	19–30
53	21–36
58	23–40
63	25–43
66	26–45
70	28–50
83	32–65
86	40–65
89	40–70
100	45–70
110	45–70
120	55–95
132	60–95

a The torque designated for the next larger closure diameter is to be applied in testing containers having a closure diameter intermediate to the diameters listed.

b A suitable apparatus is available from Owens-Illinois, Toledo, OH 43666. (Model 25 torque tester is used for testing between 0 and 25; Model 50 for testing between 0 and 50; and Model 100 for testing between 0 and 100 inch-pounds of torque.) The torque values refer to application, not removal, of the closure. For further detail regarding instructions, reference may be made to "Standard Test Method for Application and Removal Torque of Threaded or Lug-Style Closures" ASTM Method D3198-97, published by the American Society for Testing and Materials, 100 Barr Harbor Drive, P. O. Box C700, West Conshohocken, PA 19428-2959.

#### MULTIPLE-UNIT CONTAINERS FOR CAPSULES AND TABLETS

##### (Without Closure)

Polyethylene Container— Fit the containers with impervious seals obtained by heat-sealing the bottles with an aluminum foil–polyethylene laminate or other suitable seal.<sup>2</sup> Test the containers as specified under Multiple-Unit Containers for Capsules and Tablets: the high-density polyethylene containers so tested meet the requirements if the moisture permeability exceeds 10 mg per day per L in not more than 1 of the 10 test containers and exceeds 25 mg per day per L in none of them. The low-density polyethylene containers so tested meet the requirements if the moisture permeability exceeds 20 mg per day per L in not more than 1 of the 10 test containers and exceeds 30 mg per day per L in none of them.



—Polypropylene Containers— Fit the containers with impervious seals obtained by heat-sealing the bottles with an aluminum foil-polyethylene laminate or other suitable seal. Test the containers as described under Multiple-Unit Containers for Capsules and Tablets. The containers meet the requirements if the moisture permeability exceeds 15 mg per day per L in not more than 1 of the 10 test containers and exceeds 25 mg per day per L in none of them.

#### SINGLE-UNIT CONTAINERS AND UNIT-DOSE CONTAINERS FOR CAPSULES AND TABLETS

To permit an informed judgment regarding the suitability of the packaging for a particular type of product, the following procedure and classification scheme are provided for evaluating the moisture-permeation characteristics of single-unit and unit-dose containers. Inasmuch as equipment and operator performance may affect the moisture permeation of a container formed or closed, the moisture-permeation characteristics of the packaging system being utilized shall be determined.

Desiccant— Dry suitable desiccant pellets<sup>3</sup> at 110° for 1 hour prior to use. Use pellets weighing approximately 400 mg each and having a diameter of approximately 8 mm. [note—If necessary due to limited unit-dose container size, pellets weighing less than 400 mg each and having a diameter of less than 8 mm may be used.]

##### Procedure—

Method I— Seal not fewer than 10 unit-dose containers with 1 pellet in each, and seal 10 additional, empty unit-dose containers to provide the controls, using finger cots or padded forceps to handle the sealed containers. Number the containers, and record the individual weights<sup>4</sup> to the nearest mg. Weigh the controls as a unit, and divide the total weight by the number of controls to obtain the average. Store all of the containers at 75 ± 3% relative humidity and at a temperature of 23 ± 2°. [note—A saturated system of 35 g of sodium chloride with each 100 mL of water placed in the bottom of a desiccator maintains the specified humidity. Other methods may be employed to maintain these conditions.] After a 24-hour interval, and at each multiple thereof (see Results), remove the containers from the chamber, and allow them to equilibrate for 15 to 60 minutes in the weighing area. Again record the weight of the individual containers and the combined controls in the same manner. [note—If any indicating pellets turn pink during this procedure, or if the pellet weight increase exceeds 10%, terminate the test, and regard only earlier determinations as valid.] Return the containers to the humidity chamber. Calculate the rate of moisture permeation, in mg per day, of each container taken by the formula:

$$(1 / N)[(WF - WI) - (CF - CI)]$$

in which N is the number of days expired in the test period (beginning after the initial 24-hour equilibration period); (WF - WI) is the difference, in mg, between the final and initial weights of each test container; and (CF - CI) is the difference, in mg, between the average final and average initial weights of the controls, the data being calculated to two significant figures. [note—Where the permeations measured are less than 5 mg per day, and where the controls are observed to reach equilibrium within 7 days, the individual permeations may be determined more accurately by using the 7-day test container and control container weights as WI and CI, respectively, in the calculation. In this case, a suitable test interval for Class A (see Results) would be not less than 28 days following the initial 7-day equilibration period (a total of 35 days).]

Method II— Use this procedure for packs (e.g., punch-out cards) that incorporate a number of separately sealed unit-dose containers or blisters. Seal a sufficient number of packs, such that not fewer than 4 packs and a total of not fewer than 10 unit-dose containers or blisters filled with 1 pellet in each unit are tested. Seal a corresponding number of empty packs, each pack containing the same number of unit-dose containers or blisters as used in the test packs, to provide the controls. Store all of the containers at 75 ± 3% relative humidity and at a temperature of 23 ± 2°. [note—A saturated system of 35 g of sodium chloride with each 100 mL of water placed in the bottom of a desiccator maintains the specified humidity. Other methods may be employed to maintain these conditions.] After 24 hours, and at each multiple thereof (see Results), remove the packs from the chamber, and allow them to equilibrate for about 45 minutes. Record the weights of the individual packs, and return them to the chamber. Weigh the control packs as a unit, and divide the total weight by the number of control packs to obtain the average empty pack weight. [note—If any indicating pellets turn pink during the procedure, or if the average pellet weight increase in any pack exceeds 10%, terminate the test, and regard only earlier determinations as valid.] Calculate the average rate of moisture permeation, in mg per day, for each unit-dose container or blister in each pack taken by the formula:

$$(1 / NX)[(WF - WI) - (CF - CI)]$$

in which N is the number of days expired in the test period (beginning after the initial 24-hour equilibration period); X is the number of separately sealed units per pack; (WF - WI) is the difference, in mg, between the final and initial weights of each test pack; and (CF - CI) is the difference, in mg, between the average final and average initial weights of the control packs, the rates being calculated to two significant figures.

Results— The individual unit-dose containers as tested in Method I are designated Class A if not more than 1 of 10 containers tested exceeds 0.5 mg per day in moisture permeation rate and none exceeds 1 mg per day; they are designated Class B if not more than 1 of 10 containers tested exceeds 5 mg per day and none exceeds 10 mg per day; they are designated Class C if not more than 1 of 10 containers tested exceeds 20 mg per day and none exceeds 40 mg per day; and they are designated Class D if the containers tested meet none of the moisture permeation rate requirements.

The packs as tested in Method II are designated Class A if no pack tested exceeds 0.5 mg per day in average blister moisture permeation rate; they are designated Class B if no pack tested exceeds 5 mg per day in average blister moisture permeation rate; they are designated Class C if no pack tested exceeds 20 mg per day in average blister moisture permeation rate; and they are designated Class D if the packs tested meet none of the above average blister moisture permeation rate requirements.

With the use of the Desiccant described herein, as stated for Method I and Method II, after every 24 hours, the test and control containers or packs are weighed; and suitable test intervals for the final weighings, WF and CF, are as follows: 24 hours for Class D; 48 hours for Class C; 7 days for Class B; and not less than 28 days for Class A.

#### MULTIPLE-UNIT CONTAINERS AND UNIT-DOSE CONTAINERS FOR LIQUIDS

The standards and tests provided in this section measure the functional and performance characteristics of plastic containers used to package aqueous products by measuring the liquid water weight loss as a percent of the contents. This test can also be used to demonstrate performance or functional comparability. [note—Throughout the following procedure, determine the weights of individual container-closure systems (bottle, innerseal if used, and closure) both as tare weights and fill weights, to the nearest 0.1 mg if the bottle overflow capacity is less than 200 mL; to the nearest mg if the bottle overflow capacity is 200 mL or more but less than 1000 mL; or to the nearest centigram (10 mg) if the bottle overflow capacity is 1000 mL or more.]

Procedure for Testing Unopened Market Containers (cap liner [if applicable], innerseal, and cap)— Select 10 bottles of a uniform size and type, and clean the sealing surfaces with a lint-free cloth. Fit each bottle with a closure liner (if applicable) and closure. Number each container closure system, and record the tare weight.

Remove the closures and, using a pipette, fill the bottles with water to the overflow capacity. Fit the bottles with seals and apply the closures. If using screw closures, apply a torque that is within the range specified in [Table 1](#), and store the sealed containers at a temperature of 25 ± 2° and a relative humidity of 50 ± 2%. After 168 ± 1 hours (7 days), record the weight of the individual containers. Return the containers to storage for another 168 ± 1 hours. After the second 168 ± 1 hours, remove the containers, record the weights of each of the individual container systems, and calculate the water vapor permeation rate, in percent water weight loss, for each bottle taken by the formula:

$$(W7 - W14)365 \times 100/(W7 - WT)7 = \text{Percent per year}$$

in which W7 is the weight, in mg, of the container at 7 days; W14 is the weight, in mg, of the container at 14 days; WT is the tare weight in g; and 7 is the test time, in days, after the 7-day equilibration period. The containers so tested meet the requirements and are tight containers if the percentage of water weight loss exceeds 2.5% per year in not more than 1 of the 10 test containers and exceeds 5.0% per year in none of them.

Unit-dose containers for liquids meet the requirement of a tight container if the average water weight loss is less than or equal to 2.5% (w/w) loss per year (5% at the end of 2 years).

Procedure for Testing Multiple-Unit Containers Under Conditions of Use— Select 10 bottles of a uniform size and type. If an innerseal is used, carefully open the individual containers and remove the innerseal from each container. Fit each bottle with a closure liner (if applicable) and closure. Number each container-closure system, and record the tare weight. Open and close the containers 30 times being careful not to lose liquid in the process. Close screw-capped bottles with a torque that is within the range of tightness provided in [Table 1](#), and

store the sealed containers at a temperature of 25 ± 2° and a relative humidity of 50 ± 2%. After 168 ± 1 hours (7 days), record the weight of the individual containers. Return the containers to storage for another 168 ± 1 hours. After the second 168 ± 1 hours, remove the containers, record the weights of each of the individual container systems, and calculate



the water vapor permeation rate, in percent water weight loss, for each bottle taken by the formula:

$$(W7 - W14)365 \times 100 / (W7 - WT)7 = \text{Percent per year}$$

in which W7 is the weight, in mg, of the container at 7 days; W14 is the weight, in mg, of the container at 14 days; WT is the tare weight, in g; and 7 is the test time, in days, after the 7-day equilibration period. The containers so tested meet the requirements and are tight containers if the percentage of water weight loss exceeds 2.5% per year in not more than 1 of the 10 test containers and exceeds 5.0% per year in none of them.

#### LIGHT TRANSMISSION TEST

**Apparatus**—Use a spectrophotometer of suitable sensitivity and accuracy, adapted for measuring the amount of light transmitted by either transparent or translucent glass or plastic materials used for pharmaceutical containers. In addition, the spectrophotometer is capable of measuring and recording light transmitted in diffused as well as parallel rays.

**Procedure**—Select sections to represent the average wall thickness. Cut circular sections from two or more areas of the container and trim them as necessary to give segments of a size convenient for mounting in the spectrophotometer. After cutting, wash and dry each specimen, taking care to avoid scratching the surfaces. If the specimen is too small to cover the opening in the specimen holder, mask the uncovered portion of the opening with opaque paper or masking tape, provided that the length of the specimen is greater than that of the slit in the spectrophotometer. Immediately before mounting in the specimen holder, wipe the specimen with lens tissue. Mount the specimen with the aid of a tacky wax, or by other convenient means, taking care to avoid leaving fingerprints or other marks on the surfaces through which light must pass. Place the section in the spectrophotometer with its cylindrical axis parallel to the plane of the slit and approximately centered with respect to the slit. When properly placed, the light beam is normal to the surface of the section and reflection losses are at a minimum.

Continuously measure the transmittance of the section with reference to air in the spectral region of interest with a recording instrument or at intervals of about 20 nm with a manual instrument, in the region of 290 to 450 nm.

**Limits**—The observed light transmission does not exceed the limits given in [Table 2](#) for containers intended for parenteral use.

Table 2. Limits for Plastic Classes I–VI

Nominal Size (in mL)	Maximum Percentage of Light Transmission at Any Wavelength Between 290 and 450 nm	
	Flame-sealed Containers	Closure-sealed Containers
1	50	25
2	45	20
5	40	15
10	35	13
20	30	12
50	15	10

[note—Any container of a size intermediate to those listed above exhibits a transmission not greater than that of the next larger size container listed in the table. For containers larger than 50 mL, the limits for 50 mL apply.]

The observed light transmission for plastic containers for products intended for oral or topical administration does not exceed 10% at any wavelength in the range from 290 to 450 nm.

1 Suitable 4- to 8-mesh, anhydrous calcium chloride is available commercially as Item JT1313-1 from VWR Scientific. Consult the VWR Scientific catalog for ordering information or call 1-800-234-9300.

2 A suitable laminate for sealing has as the container layer polyethylene of not less than 0.025 mm (0.001 inch) and a second layer of aluminum foil of not less than 0.018 mm (0.0007 inch), with additional layers of suitable backing materials. A suitable seal can be obtained also by using glass plates and a sealing wax consisting of 60% of refined amorphous wax and 40% of refined crystalline paraffin wax.

3 Suitable moisture-indicating desiccant pellets are available commercially from sources such as Medical Packaging, Inc., 470 Route 31, Ringoes, NJ 08551-1409 [Telephone 800-257-5282; in NJ, 609-466-8991; FAX 609-466-3775], as Indicating Desiccant Pellets, Item No. TK-1002.

4 Accurate comparisons of Class A containers may require test periods in excess of 28 days if weighings are performed on a Class A prescription balance (see [Prescription Balances and Volumetric Apparatus](#) (1176)). The use of an analytical balance on which weights can be recorded to 4 or 5 decimal places may permit more precise characterization between containers and/or shorter test periods.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Desmond G. Hunt, Ph.D.</a> Scientist 1-301-816-8341	(PS05) Packaging and Storage 05

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## 681 REPACKAGING INTO SINGLE-UNIT CONTAINERS AND UNIT-DOSE CONTAINERS FOR NONSTERILE SOLID AND LIQUID DOSAGE FORMS

This chapter is intended to provide guidance to those engaged in pharmaceutical dispensing, not commercial repackaging. An official dosage form is required to bear on its label an expiration date assigned for the particular formulation and package of the article. This date limits the time during which the product may be dispensed or used. Because the expiration date stated on the original manufacturer's container–closure system has been determined for the drug in that particular system and is not intended to be applicable to the product where it has been repackaged in a different container, repackaged drugs dispensed pursuant to a prescription are exempt from using the expiration date from the original manufacturer's package. However, under no circumstance should the repackaged pharmaceutical preparation's expiration date exceed the original manufacturer's expiration date. It is necessary, therefore, that other precautions be taken by the dispenser to preserve the strength, quality, and purity of drugs that are repackaged for ultimate distribution or sale to patients.

The following guidelines and requirements are applicable where official dosage forms are repackaged into single-unit or unit-dose containers or mnemonic packs for dispensing pursuant to prescription.

**Labeling**—It is the responsibility of the dispenser, taking into account the nature of the drug repackaged, any packaging and expiration dating information in the manufacturer's product labeling, the characteristics of the containers, and the storage conditions to which the article may be subjected, to place a suitable expiration date on the label. Repackaged dosage forms must bear on their labels expiration dates as determined from information in the product labeling (see [Preservation, Packaging, Storage, and Labeling](#) section of the General Notices and Requirements). Each single-unit or unit-dose container bears a separate label, unless the device holding the unit-dose form does not allow for the removal or separation of the intact single-unit or unit-dose container therefrom.

**Storage**—Store the repackaged article in a humidity-controlled environment and at the temperature specified in the individual monograph or in the product labeling. Where no



temperature or humidity is specified in the monograph or in the labeling of the product, controlled room temperature and a relative humidity corresponding to 60% are not to be exceeded during repackaging or storage.

A refrigerator or freezer shall not be considered to be a humidity-controlled environment. Drugs that are to be stored at a cold temperature in a refrigerator or freezer must be protected during storage in the refrigerator or freezer. An outer container may be necessary for such protection; it is recommended that the drug monograph be referenced for storage.

**Reprocessing**— Reprocessing of repackaged unit-dose containers (i.e., removing dosage unit from one unit-dose container and placing dosage unit into another unit-dose container) shall not be done. However, reprocessing of the secondary package (e.g., removing the blister card from the cardboard carrier and placing the blister card into another cardboard carrier) is allowed provided that the original expiration date is maintained.

#### CUSTOMIZED PATIENT MEDICATION PACKAGES

In lieu of dispensing two or more prescribed drug products in separate containers, a pharmacist may, with the consent of the patient, the patient's caregiver, or a prescriber, provide a customized patient medication package (patient med pak).<sup>1</sup>

A patient med pak is a package prepared by a pharmacist for a specific patient comprising a series of containers and containing two or more prescribed solid oral dosage forms. The patient med pak is so designed or each container is so labeled as to indicate the day and time, or period of time, that the contents within each container are to be taken.

It is the responsibility of the dispenser to instruct the patient or caregiver on the use of the patient med pak.

**Label**— The patient med pak shall bear a label stating:

1. the name of the patient;
2. a serial number for the patient med pak itself and a separate identifying serial number for each of the prescription orders for each of the drug products contained therein;
3. the name, strength, physical description or identification, and total quantity of each drug product contained therein;
4. the directions for use and cautionary statements, if any, contained in the prescription order for each drug product therein;
5. any storage instructions or cautionary statements required by the official compendia;
6. the name of the prescriber of each drug product;
7. the date of preparation of the patient med pak and the beyond-use date or period of time assigned to the patient med pak (such beyond-use date or period of time shall be not longer than the shortest recommended beyond-use date for any dosage form included therein or not longer than 60 days from the date of preparation of the patient med pak and shall not exceed the shortest expiration date on the original manufacturer's bulk containers for the dosage forms included therein); alternatively, the package label shall state the date of the prescription(s) or the date of preparation of the patient med pak, provided the package is accompanied by a record indicating the start date and the beyond-use date;
8. the name, address, and telephone number of the dispenser (and the dispenser's registration number where necessary); and
9. any other information, statements, or warnings required for any of the drug products contained therein.

If the patient med pak allows for the removal or separation of the intact containers therefrom, each individual container shall bear a label identifying each of the drug products contained therein.

**Labeling**— The patient med pak shall be accompanied by a patient package insert, in the event that any medication therein is required to be dispensed with such insert as accompanying labeling. Alternatively, such required information may be incorporated into a single, overall educational insert provided by the pharmacist for the total patient med pak.

**Packaging**— In the absence of more stringent packaging requirements for any of the drug products contained therein, each container of the patient med pak shall comply with the moisture permeation requirements for a Class B single-unit or unit-dose container (see [Containers—Permeation \(671\)](#)). Each container shall be either not reclosable or so designed as to show evidence of having been opened.

**Guidelines**— It is the responsibility of the dispenser, when preparing a patient med pak, to take into account any applicable compendial requirements or guidelines and the physical and chemical compatibility of the dosage forms placed within each container, as well as any therapeutic incompatibilities that may attend the simultaneous administration of the medications. In this regard, pharmacists are encouraged to report to USP headquarters any observed or reported incompatibilities. Once a medication has been placed in a patient med pak with another solid dosage form, it may not be returned to stock, redistributed, or resold if unused.

**Recordkeeping**— In addition to any individual prescription filing requirements, a record of each patient med pak shall be made and filed. Each record shall contain, as a minimum:

1. the name and address of the patient;
2. the serial number of the prescription order for each drug product contained therein;
3. the name of the manufacturer or labeler and lot number for each drug product contained therein;
4. information identifying or describing the design, characteristics, or specifications of the patient med pak sufficient to allow subsequent preparation of an identical patient med pak for the patient;
5. the date of preparation of the patient med pak and the beyond-use date that was assigned;
6. any special labeling instructions; and
7. the name or initials of the pharmacist who prepared the patient med pak.

<sup>1</sup> It should be noted that there is no special exemption for patient med paks from the requirements of the Poison Prevention Packaging Act. Thus the patient med pak, if it does not meet child-resistant standards, shall be placed in an outer package that does comply, or the necessary consent of the purchaser or physician, to dispense in a container not intended to be child-resistant, shall be obtained.

**Auxiliary Information**— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Desmond G. Hunt, Ph.D.</a> Scientist 1-301-816-8341	(PS05) Packaging and Storage 05

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691 COTTON

Preparatory to the determination of absorbency and of fiber length, remove the Cotton from its wrappings, and condition it for not less than 4 hours in a standard atmosphere of  $65 \pm 2\%$  relative humidity at  $21 \pm 1.1^{\circ}\text{F}$  ( $70 \pm 2^{\circ}\text{F}$ ).

#### Absorbency Test

**Procedure**— Prepare a test basket, weighing not more than 3 g, from copper wire approximately 0.4 mm in diameter (No. 26 B. & S.) in the form of a cylinder approximately 5 cm in diameter and 8 cm deep, with spaces of about 2 cm between the wires. Take portions of purified cotton weighing  $1 \pm 0.05$  g from five different parts of the package by pulling, not cutting, the specimens, place the combined portions in the basket, and weigh. Hold the basket on its side approximately 12 mm above the surface of water at  $25 \pm 1^{\circ}\text{F}$ , and drop it into the water. Determine, preferably by use of a stop watch, the time in seconds required for complete submersion.

Remove the basket from the water, allow it to drain for 10 seconds in the same horizontal position, then place it immediately in a tared, covered vessel, and weigh, deducting the weight of the test basket and of the purified cotton to find the weight of water absorbed.

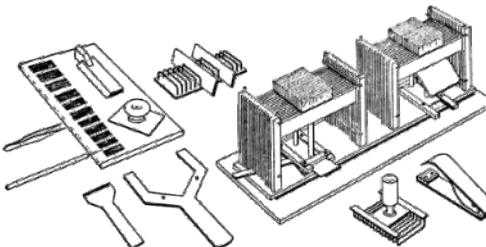
## Fiber Length

For the determination of the length and of the length distribution of cotton fibers in purified cotton use the following method:

Carry out all operations associated with the determination of fiber length of purified cotton in an atmosphere maintained at  $65 \pm 2\%$  relative humidity at  $21 \pm 1.1^{\circ}$  ( $70 \pm 2^{\circ}$  F).

These directions describe the mode of procedure that is well adapted to the sorter\* most extensively used in the United States at the present time.

Apparatus— The sorter (see [Illustration](#))



Duplex Cotton Fiber Sorter

consists of two banks of combs rigidly mounted side by side on a common base. Each bank of combs consists of at least 12 individual combs spaced 3.2 mm apart, one behind the other, and mounted in grooves so that as they are approached during the fractionating process and no longer needed, they may be dropped below the working plane. Each individual comb has a single row of accurately aligned and sharply pointed teeth, 12 mm long, consisting of needles 0.38 mm in diameter. The teeth are spaced 62 to 25 mm over an extent of approximately 50 mm.

Accessory equipment consists of fiber-sorter forceps, fiber-depressing grid, fiber-depressing smooth plate, and velvet-covered plates. The sorter forceps consist of two brass pieces approximately 75 mm long, hinged on one end and slightly curved to present a beaked aspect at the gripping end for gripping the protruding fibers close to the surfaces of the combs.

Usually, one of the gripping edges has a leather or other fibrous padding. The gripping edge is approximately 19 mm wide.

The fiber-depressing grid consists of a series of brass rods spaced 3.2 mm apart so that they may be placed between the combs to press the fibers down between the teeth. The fiber-depressing smooth plate consists of a polished brass plate approximately  $25 \times 50$  mm, with a knob or handle on the upper surface whereby the plate may be smoothed over the fibers as they are laid on the velvet surface of the array plates. The velvet-covered plates, upon which the fibers may be arrayed, are aluminum sheets approximately  $100 \text{ mm} \times 225 \text{ mm} \times 2.4 \text{ mm}$  thick, covered on both sides with high-grade velvet, preferably black.

Selection of Cotton— After unrolling the cotton, prepare a representative laboratory test specimen by taking from a package containing from 8 to 16 ounces, 32 pinches (about 75 mg each) well distributed throughout the bulk of the lap, 16 representative pinches being taken from each longitudinal half of the lap. Avoid the cut ends of the lap, and take particular care to secure portions throughout the thickness of the lap. To avoid biased selection of long or short fibers, remove all fibers of the group pinched and do not allow them to slip from between the fingers.

From packages of not more than 4 ounces in weight, take 8 pinches, and from packages weighing more than 4 ounces and not more than 8 ounces, take 16 pinches, all well distributed.

Mix the pinches in pairs promiscuously, and combine each pair by gently drawing and lapping them in the fingers. Then divide each combined pair by splitting longitudinally into two approximately equal parts and utilize one part in the further mixing. (The other part may be discarded or reserved for any further tests or checks.)

Repeat the process described in the preceding paragraph with the successive halves of the bifurcated series until only 1 pinch, the final composite test portion, results. Gently parallel and straighten the fibers of the final composite test portion by drawing and lapping them in the fingers. Take care to retain all of the fibers, including as far as possible those of the neps (specks of entangled fibers) and naps (matted masses of fibers), discarding only motes (immature seed fragments with fibers) and nonfiber foreign material such as stem, leaf, and fragments of seedcoats.

From the final composite portion described in the preceding paragraph, separate longitudinally a test portion of  $75 \pm 2$  mg, accurately weighed. Retain the residue for any check test necessary.

Procedure— With the fiber-depressing grid carefully insert the weighed test portion into one bank of combs of the cotton sorter, so that it extends across the combs at approximately right angles.

With the sorter forceps, grip by the free ends a small portion of the fibers extending through the teeth of the comb nearest to the operator; gently and smoothly draw them forward out of the combs, and transfer them to the tips of the teeth in the second bank of combs, laying them parallel to themselves, straight, and approximately at right angles to the faces of the combs, releasing the gripped ends as near to the face of the front comb as possible. With the depressor grid carefully press the transferred fibers down into the teeth of the combs. Continue the operation until all of the fibers are transferred to the second bank of combs. During this transfer of the fibers, drop the combs of the first bank in succession when and as all of the protruding fibers have been removed.

Turn the machine through  $180^{\circ}$ , and transfer the cotton fibers back to the first bank of combs in the manner described in the preceding paragraph.

Take great care in evening up the ends of the fibers during both of the above transfers, arranging them as closely as possible to the front surface of the proximal comb. Such evening out of the ends of the protruding fibers may involve drawing out straggling fibers from both the front and rear aspects of the banks of combs, and re-depositing them into and over the main bundle in the combs.

Turn the machine again through  $180^{\circ}$ . Drop successive combs if necessary to expose the ends of the longest fibers. It may be necessary to re-deposit some straggling fibers. With the forceps withdraw the few most protuberant fibers. In this way continue to withdraw successively the remaining protuberant fibers back to the front face of the proximal comb. Drop this comb and repeat the series of operations in the same manner until all of the fibers have been drawn out. In order not to disturb seriously the portion being tested, and thereby vitiate the length fractionation into length groups, make several pulls (as many as 8 to 10) between each pair of combs.

Lay the pulls on the velvet-covered plates alongside each other, as straight as possible, with the ends as clearly defined as possible, and with the distal ends arranged in a straight line, pressing them down gently and smoothly with the fiber-depressing smooth plate before releasing the pull from the forceps. Employ not fewer than 50 and not more than 100 pulls to fractionate the test portion.

Group together all of the fibers measuring 12.5 mm (about 1/2 inch) or more in length, and weigh the group to the nearest 0.3 mg. In the same manner, group together all fibers 6.25 mm (about 1/4 inch) or less in length, and weigh in the same manner. Finally, group the remaining fibers of intermediate lengths together and weigh. The sum of the three weights does not differ from the initial weight of the test portion by more than 3 mg. Divide the weight of each of the first two groups by the weight of the test portion to obtain the percentage by weight of fiber in the two ranges of length.

\* note—The method here described is especially adapted to the Suter-Webb Duplex Cotton Fiber sorting apparatus, but with more or less obvious alteration in procedure, may be carried out with two Baer sorters in tandem arrangement, or with a Johannsen or other similar apparatus.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Radhakrishna S Tirumalai, Ph.D.</a> Senior Scientist 1-301-816-8339	(GTMDB05) General Toxicology and Medical Device Biocompatibility

USP32–NF27 Page 255

### 695 CRYSTALLINITY

This test is provided to determine compliance with the crystallinity requirement where stated in the individual monograph for a drug substance.

Procedure—A detailed test procedure is described under [Optical Microscopy \(776\)](#).

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Kahkashan Zaidi, Ph.D.</a> Senior Scientist 1-301-816-8269	(GC05) General Chapters 05

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### 696 CRYSTALLINITY DETERMINATION BY SOLUTION CALORIMETRY

#### INTRODUCTION—THE CONCEPT OF CRYSTALLINITY

The perfectly ordered crystal lattice with every molecule in its expected lattice position is an ideal that is seldom, if ever, achieved. The other extreme is the amorphous state, in which a crystal contains the maximum possible density of imperfections (defects of various dimensionalities), such that all long-range order is lost while only the short-range order, imposed by its nearest molecular neighbors, remains. Real crystals lie somewhere between these two extremes. A crystal's position on a scale bounded by these two extremes is termed its crystallinity.

All real crystals, even in the pure state, possess some lattice imperfections or defects, which increase both the energy (enthalpy under conditions of constant atmospheric pressure) and the disorder (expressed as the entropy) of the crystal lattice. A crystal with a relatively small density of imperfections is said to be highly crystalline and to possess a high crystallinity. By contrast, a particle with a relatively high density of imperfections is said to be partially amorphous and to possess a low crystallinity. A totally amorphous particle corresponds to zero crystallinity. Even amorphous particles may contain domains of somewhat ordered molecules that can act as nuclei for crystallization; such so-called amorphous particles are said to possess a small but finite crystallinity.

For a powder or a collection of particles, two models of crystallinity have been proposed: the one-state model and the two-state model. According to the one-state model, all particles in the powder possess essentially the same crystallinity. By contrast, the two-state model postulates that each particle in a powder may be either crystalline or amorphous, such that the actual crystallinity is the weighted average of these two extreme crystallinities. In reality, a powder probably contains particles with different degrees of crystallinity, just as it may contain particles with different sizes and shapes. The lower the crystallinity of a particle, the greater its enthalpy and entropy. The increase in enthalpy is never totally compensated by the increase in entropy; however, the Gibbs free energy, which reflects the balance between them, actually increases. Hence, the lower the crystallinity of a particle, and consequently the greater its amorphous character, the greater its apparent intrinsic solubility, dissolution rate, and reactivity, but the lower its stability. Because of the great relevance of these properties, crystallinity is also an important property and requires measurement by a suitable method.

Various methods are available for the measurement of crystallinity. In the present chapter, the crystallinity of a powder is measured by solution calorimetry, although other methods could be used provided they have been validated.

#### DETERMINING CRYSTALLINITY BY SOLUTION CALORIMETRY

Solution calorimetry provides a means of determining enthalpy of solution (i.e., heat of solution under constant atmospheric pressure) of a solid substance. Enthalpy of solution may be defined as the enthalpy of the substance dissolved in the solution to a defined concentration minus the enthalpy of the original solid substance. The solvent for the solution process must be such that the weight of the solid taken (25 to 100 mg) dissolves within a time frame that matches the response time of the calorimeter, as discussed below. The enthalpy of solution is, of course, proportional to the amount of solid being dissolved. This amount may be defined as one mole for molar enthalpy or as one gram for specific enthalpy. If the substance is pure and if its molecular weight is known, the molar enthalpy is preferred, otherwise the specific enthalpy must be used. The enthalpy of solution is weakly dependent on both the temperature, which is usually 25.0°, and the final concentration of the dissolved solute, which is usually on the order of 50 to 200 mg per 100 mL of solvent.

The crystallinity of the solid sample under study is given by the enthalpy of solution of the solid sample  $\Delta H^s_x$ , minus the enthalpy of solution of the chosen reference standard of the same substance,  $\Delta H^s_R$ , when determined under the same conditions. Because the reference standard is usually chosen for its perceived high crystallinity, its enthalpy of solution usually is algebraically greater (more endothermic or less exothermic) than that of the solid sample under study in the same solvent. Consequently, the crystallinity so determined is a negative quantity with the SI units, kJ/mol or J/g (J/kg is avoided because of its unwieldiness and potential for error). The preference for a negative value with respect to a highly crystalline reference standard recognizes the fact that most samples have a lower crystallinity than this reference standard.

A number of substances, including some purified by freeze-drying, may be available in an amorphous form but not in a crystalline form. With such substances, an amorphous form, prepared by a standard procedure, may be used as the reference standard. The enthalpy of solution may then be algebraically smaller than that of the chosen amorphous reference standard, in which case the crystallinity, as defined above, has a positive value.

The use of a single reference standard for each solid substance provides a single crystallinity scale, expressed in terms of energy, for each substance and recognizes that each solid drug or excipient has unique properties. Furthermore, the crystallinity can be recalculated—if the original reference standard is later replaced by a more crystalline (or more amorphous) reference standard—because enthalpies of solution are additive quantities according to Hess's Law of constant heat summation (i.e., the first law of thermodynamics).

In principle, the determination of the crystallinity of polymers may also be carried out using solution calorimetry, but this requires a defined reference standard for the polymer and a solvent in which the polymer is sufficiently soluble as discussed below.

Because the enthalpy of solution depends not only on the crystallinity of the solid but also on the various other solute–solute intermolecular interactions and on the solute–solvent and solvent–solvent intermolecular interactions, a zero value for the enthalpy of solution does not necessarily indicate zero crystallinity of the solid solute.

It is sometimes preferred to express the crystallinity,  $P_c$ , of a substance on a percentage scale, as described by Pikal et al.,<sup>1</sup> who also provide references to relevant earlier literature. This procedure requires two reference standards, namely a highly crystalline sample representing 100% crystallinity and having a measured enthalpy of solution of  $\Delta H^s_c$ , and an essentially amorphous sample representing 0% crystallinity and having a measured enthalpy of solution of  $\Delta H^s_a$ . From these values and from the measured enthalpy,  $\Delta H^s_s$ , of solution of the solid under study, the percentage crystallinity of the solid,  $P_c$ , may be calculated as follows:

$$P_c (\%) = 100(\Delta H^s_s - \Delta H^s_a)/(\Delta H^s_c - \Delta H^s_a)$$

Clearly, crystallinity expressed on a percentage scale depends on three, not two, measured values and the enthalpies of solution may be replaced by other corresponding physical



quantities that depend on crystallinity. The value of the percentage crystallinity of a solid sample, however, depends not only on the nature and method of preparation of the two reference standards, but also on the choice of the physical quantity that is measured.

The enthalpy of solution is measured at  $25.0 \pm 0.1^\circ$  either by an isoperibol (constant perimeter, i.e., jacket) solution calorimeter or by an isothermal (constant temperature) solution calorimeter using a fixed weight from 25 to 100 mg of solid sample, weighed to  $\pm 0.1$  mg, with a fixed weight of solvent from 25 to 100 g, weighed to 0.01 g (usually  $50.00 \pm 0.01$  g). The weight of solid solute and the nature of the solvent should be so chosen that the enthalpy of solution is not less than 200 mJ. At least three measurements are made with each sample, if a sufficient quantity is available, until the measured values of the heat of solution do not differ by more than 5%. The arithmetic mean of these three values is then calculated.

#### Isoperibol Solution Calorimetry

In the isoperibol solution calorimeter, the heat change during the solution process causes a corresponding change in temperature of the solvent–solute system (i.e., solution). This temperature change is measured by a temperature sensor, which is wired to an electrical circuit that records an electrical signal corresponding to the temperature change. Typically, this temperature change in an electronic form is measured at precisely defined time intervals to produce temperature–time data that are collected, analyzed by a computer, and then plotted. A blank run without addition of the solid solute to the solvent should show no discernible change in the slope of the temperature–time plot.

For isoperibol solution calorimeters, response is relatively rapid, but any heat losses to or heat gains from the bath reduce the accuracy and contribute to noise. Therefore, isoperibol solution calorimeters are more advantageous than isothermal solution calorimeters when the solution process is relatively fast. For all measurements of enthalpy of solution using isoperibol solution calorimeters, the choice of solvent and solid is critical. The nature and weight of the solvent and the weight of the solid sample allow the total heat change, corresponding to total dissolution of the solid, to proceed to completion within 10 minutes under vigorous stirring at a constant rotational speed within the range of 400 to 600 revolutions per minute. The rotational speed is checked with a stroboscope.

#### Isothermal Solution Calorimetry

In the isothermal (constant temperature) solution calorimeter, the heat change during the solution process is compensated for by an equal but opposite energy change, such that the temperature of the solvent–solute system (i.e., solution) remains constant. This equal but opposite energy change is measured and, when its sign is reversed, provides the enthalpy of solution. For isothermal calorimeters, response is relatively slow, but the compensation process eliminates the effects of heat losses to or heat gains from the bath. Therefore, isothermal calorimeters are more advantageous than isoperibol calorimetry when the solution process is relatively slow.

#### CALORIMETER CALIBRATION

To ensure the accuracy of the calorimeter, chemical calibrations must be performed daily. For an endothermic solution process, the calibration of the calorimeter is checked by measuring the heat absorbed during the dissolution of potassium chloride in distilled water at  $298.15\text{ K}$  ( $25.0^\circ$ ). The established enthalpy change in this endothermic process is  $235.5\text{ J/g}$  or  $4.196\text{ kcal/mol}$ . For an exothermic solution process, the calorimeter is checked by measuring the heat evolved during the dissolution of  $5\text{ g}$  per  $\text{L}$  of tromethamine [tris(hydroxymethyl)aminomethane, THAM] in a  $0.1\text{ mol/L}$  aqueous hydrochloric acid solution at  $298.15\text{ K}$  ( $25.0^\circ$ ). The established heat for the aforementioned process is  $-29.80\text{ kJ/mol}$  or  $-7.12\text{ kcal/mol}$ .

The effective heat capacity of the calorimeter cell and its contents is determined for every calorimeter run. This determination is accomplished by electrical heating of the contents of the calorimeter cell. The effective heat capacity is determined according to one of two protocols—either by making one determination after ampul breakage or by making one determination before and a second determination after ampul breakage and then averaging the two results. The accuracy and reliability of the electrical heating are established by the accuracy and reliability of the aforementioned chemical calibrations.

#### SAMPLE HANDLING

The thermodynamic stability of solids decreases with decreasing crystallinity. In particular, solids of low crystallinity, especially amorphous solids, tend to sorb water vapor from the atmosphere, leading to crystallization and a corresponding gain in crystallinity. For these reasons, anhydrous solid samples whose crystallinity is to be determined must be stored at zero humidity in sealed chambers containing a desiccant, preferably containing an indicator of effectiveness. If crystallinity–humidity studies are to be carried out, the solid sample should be stored in a sealed chamber containing a saturated salt solution to provide a defined relative humidity at  $25.0 \pm 0.1^\circ$ .

\* See Pikal, M.J.; Lukes, A.L.; Lang, J.E.; Gaines, K. Quantitative crystallinity determinations for  $\beta$ -lactam antibiotics by solution calorimetry: correlations with stability, *J. Pharm. Sci.* 1978, 67, 767–773.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Kahkashan Zaidi, Ph.D.</a> Senior Scientist 1-301-816-8269	(GC05) General Chapters 05

USP32–NF27 Page 256

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#### 698 DELIVERABLE VOLUME

The following tests are designed to provide assurance that oral liquids will, when transferred from the original container, deliver the volume of dosage form that is declared on the label of the article. These tests are applicable to products labeled to contain not more than 250 mL, whether supplied as liquid preparations or liquid preparations that are constituted from solids upon the addition of a designated volume of a specific diluent. They are not required for an article packaged in single-unit containers when the monograph includes the

[Uniformity of Dosage Units](#) [905](#) test.

#### TEST PREPARATIONS

For the determination of deliverable volume, select not fewer than 30 containers, and proceed as follows for the dosage form designated.

Oral Solutions, Oral Suspensions, and Other Oral Liquid Dosage Forms—Shake the contents of 10 containers individually.

Powders that are Labeled to State the Volume of Oral Liquid that Results when the Powder is Constituted with the Volume of Diluent Stated in the Labeling—Constitute 10 containers with the volume of diluent stated in the labeling, accurately measured, and shake individually.

#### PROCEDURE

Being careful to avoid the formation of air bubbles, gently pour the contents of each container into separate dry graduated cylinders of a rated capacity not exceeding two and a half times the volume to be measured, and calibrated “to contain”. Allow each container to drain for a period not to exceed 30 minutes, for multiple-unit containers and 5 seconds for single-unit containers, unless otherwise specified in the monograph. When free from air bubbles, measure the volume of each mixture. Alternatively, in the case of products of low volume packaged in single-unit containers, the volume can be computed as follows: (1) discharge the container contents into a suitable tared container (allowing drainage for not more than 5 seconds); (2) determine the weight of the contents; and (3) compute the volume after determining the density.

## ACCEPTANCE CRITERIA

Use the following criteria to determine compliance with this test.

For Multiple-Unit Containers (see [Figure 1](#))— The average volume of liquid obtained from the 10 containers is not less than 100%, and the volume of no container is less than 95% of the volume declared in the labeling. If A, the average volume is less than 100% of that declared in the labeling, but the volume of no container is less than 95% of the labeled amount, or B, the average volume is not less than 100% and the volume of not more than 1 container is less than 95%, but is not less than 90% of the labeled volume, perform the test on 20 additional containers. The average volume of liquid obtained from the 30 containers is not less than 100% of the volume declared in the labeling; and the volume of liquid obtained from not more than 1 of the 30 containers is less than 95%, but not less than 90% of that declared in the labeling.

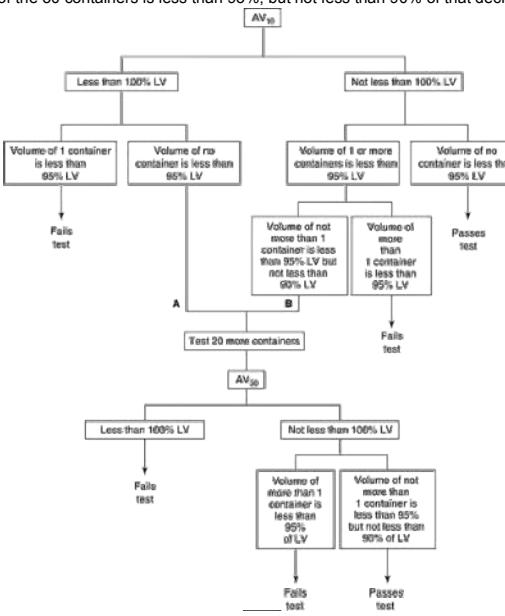


Figure 1. Decision scheme for multiple-unit containers. (AV = Average volume. LV = Labeled volume)

For Single-Unit Containers (see [Figure 2](#))— The average volume of liquid obtained from the 10 containers is not less than 100%, and the volume of each of the 10 containers lies within the range of 95% to 110% of the volume declared in the labeling. If A, the average volume is less than 100% of that declared in the labeling, but the volume of no container is outside the range of 95% to 110%, or if B, the average volume is not less than 100% and the volume of not more than 1 container is outside the range of 95% to 110%, but within the range of 90% to 115%, perform the test on 20 additional containers. The average volume of liquid obtained from the 30 containers is not less than 100% of the volume declared in the labeling; and the volume obtained from not more than 1 of the 30 containers is outside the range of 95% to 110%, but within the range of 90% to 115% of the volume declared on the labeling.

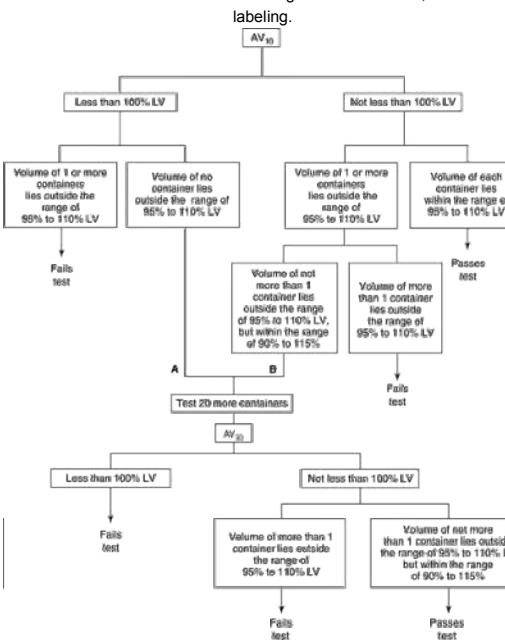


Figure 2. Decision scheme for single-unit containers. (AV = Average volume. LV = Labeled volume)

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">William E. Brown</a> Senior Scientist 1-301-816-8380	(PDF05) Pharmaceutical Dosage Forms 05



Density refers to the average spatial distribution of mass in a material. The density of solids typically is expressed in g per cm<sup>3</sup>, in contrast to fluids, where the density is commonly expressed in g per mL at a stated reference temperature.

The density of a solid particle can assume different values depending on the method used to measure the volume of the particle. It is useful to distinguish among three different possibilities.

The true density of a substance is the average mass per unit volume, exclusive of all voids that are not a fundamental part of the molecular packing arrangement. It is a property of a particular material, and hence should be independent of the method of determination. The true density of a perfect crystal can be determined from the size and composition of the unit cell.

The pycnometric density, as measured by gas pycnometry, is a convenient density measurement for pharmaceutical powders. In a gas pycnometer, the volume occupied by a known mass of powder is determined by measuring the volume of gas displaced by the powder. The quotient of the mass and volume is the pycnometric density. The pycnometric density equals the true density unless the material contains impenetrable voids, or sealed pores, that are inaccessible to the gas used in the pycnometer.

The granular density includes contributions to particle volume from open pores smaller than some limiting size. The size limit depends on the method of measurement. A common measurement technique is mercury porosimetry, where the limiting pore size depends upon the maximum intrusion pressure. Because of the additional contribution from pore volume, the granular density will never be greater than the true density. A related concept is the aerodynamic density, which is the density of the particle with a volume defined by the aerodynamic envelope of the particle in a flowing stream. Both the closed and open pores contribute to this volume, but the open pores fill with the permeating fluid. The aerodynamic density, therefore, depends on the density of the test fluid if the particle is porous.

For brevity, the pycnometric density and the true density are both referred to as density. If needed, these quantities may be distinguished based on the method of measurement.

The density of a material depends on the molecular packing. For gases and liquids, the density will depend only on temperature and pressure. For solids, the density will also vary with the crystal structure and degree of crystallinity. If the solids are amorphous, the density may further depend upon the history of preparation and treatment. Therefore, unlike fluids, the densities of two chemically equivalent solids may be different, and this difference reflects a difference in solid-state structure. The density of constituent particles is an important physical characteristic of pharmaceutical powders.

Beyond these definitions of particle density, the bulk density of a powder includes the contribution of interparticulate void volume. Hence, the bulk density depends on both the density of powder particles and the packing of powder particles.

#### GAS PYCNOMETRY FOR THE MEASUREMENT OF DENSITY

Gas pycnometry is a convenient and suitable method for the measurement of the density of powder particles. A simple schematic of one type of gas pycnometer is shown in [Figure 1](#).

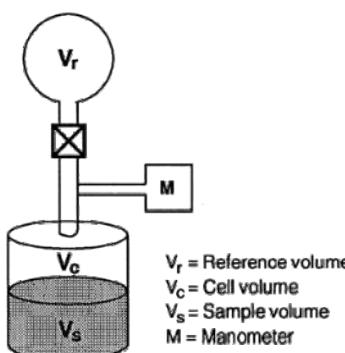


Fig. 1. Schematic of Gas Pycnometer.

The sample, with mass  $w$  and volume  $V_s$ , is placed inside a sealed test cell with an empty cell volume of  $V_c$ . The system reference pressure,  $P_r$ , is determined at the manometer while the valve that connects the reference volume with the test cell is open. The valve is closed to separate the reference volume,  $V_r$ , from the test cell. The test cell is pressurized with the measurement gas to an initial pressure,  $P_i$ . Then the valve is opened to connect the reference volume,  $V_r$ , with the test cell, and the pressure drops to the final pressure,  $P_f$ . If the measurement gas behaves ideally under the conditions of measurement, the sample volume,  $V_s$ , is given by the following expression:

$$V_s = V_c + \frac{V_r}{1 - \left[ \frac{P_i - P_r}{P_f - P_r} \right]} \quad (1)$$

The density,  $\rho$ , is given by the equation:

$$\rho = \frac{w}{V_s} \quad (2)$$

Details of the instrumental design may differ, but all gas pycnometers rely on the measurement of pressure changes as a reference volume is added to, or deleted from, the test cell.

The measured density is a volume-weighted average of the densities of individual powder particles. The density will be in error if the test gas sorbs onto the powder or if volatile contaminants are evolved from the powder during the measurement. Sorption is prevented by an appropriate choice of test gas. Helium is the common choice. Volatile contaminants in the powder are removed by degassing the powder under a constant purge of helium prior to the measurement. Occasionally, powders may have to be degassed under vacuum. Two



Consecutive readings should yield sample volumes that are equal within 0.2% if volatile contaminants are not interfering with the measurements. Because volatiles may be evolved during the measurement, the weight of the sample should be taken after the pycnometric measurement of volume.

#### Method

Ensure that the reference volume and the calibration volume have been determined for the gas pycnometer by an appropriate calibration procedure. The test gas is helium, unless another gas is specified in the individual monograph. The temperature of the gas pycnometer should be between 15° and 30° and should not vary by more than 2° during the course of the measurement. Load the test cell with the substance under examination that has been prepared according to the individual monograph. Where 699D is indicated, dry the substance under examination as directed for Loss on drying in the monograph unless other drying conditions are specified in the monograph Density of solids test. Where 699U is indicated, the substance under examination is used without drying. Use a quantity of powder recommended in the operating manual for the pycnometer. Seal the test cell in the pycnometer, and purge the pycnometer system with the test gas according to the procedure given in the manufacturer's operating instructions. If the sample must be degassed under vacuum, follow the recommendations in the individual monographs and the instructions in the operating manual for the pycnometer.

The measurement sequence above describes the procedure for the gas pycnometer shown in [Figure 1](#). If the pycnometer differs in operation or in construction from the one shown in [Figure 1](#), follow the operating procedure given in the manual for the pycnometer.

Repeat the measurement sequence for the same powder sample until consecutive measurements of the sample volume,  $V_s$ , agree to within 0.2%. Unload the test cell and measure the final powder weight,  $w$ . Calculate the pycnometric density,  $P$ , of the sample according to Equation 2.

Auxiliary Information— Please [check your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Robert H. Lafaver, B.A.</a> Scientist 1-301-816-8335	(EGC05) Excipient General Chapters

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Change to read:

701 DISINTEGRATION

This general chapter is harmonized with the corresponding texts of the European Pharmacopoeia and/or the Japanese Pharmacopoeia. The texts of these pharmacopoeias are therefore interchangeable, and the methods of the European Pharmacopoeia and/or the Japanese Pharmacopoeia may be used for demonstration of compliance instead of the present general chapter. These pharmacopoeias have undertaken not to make any unilateral change to this harmonized chapter.

Portions of the present general chapter text that are national USP text, and therefore not part of the harmonized text, are marked with symbols (♦) to specify this fact.

This test is provided to determine whether tablets or capsules disintegrate within the prescribed time when placed in a liquid medium at the experimental conditions presented below.

♦ Compliance with the limits on Disintegration stated in the individual monographs is required except where the label states that the tablets or capsules are intended for use as troches, or are to be chewed, or are designed as extended-release dosage forms or delayed-release dosage forms. Determine the type of units under test from the labeling and from observation, and apply the appropriate procedure to 6 or more dosage units.♦

For the purposes of this test, disintegration does not imply complete solution of the unit or even of its active constituent. Complete disintegration is defined as that state in which any residue of the unit, except fragments of insoluble coating or capsule shell, remaining on the screen of the test apparatus or adhering to the lower surface of the disk, if used, is a soft mass having no palpably firm core.

#### APPARATUS

The apparatus consists of a basket-rack assembly, a 1000-mL, low-form beaker, 138 to 160 mm in height and having an inside diameter of 97 to 115 mm for the immersion fluid, a thermostatic arrangement for heating the fluid between 35° and 39°, and a device for raising and lowering the basket in the immersion fluid at a constant frequency rate between 29 and 32 cycles per minute through a distance of not less than 53 mm and not more than 57 mm. The volume of the fluid in the vessel is such that at the highest point of the upward stroke the wire mesh remains at least 15 mm below the surface of the fluid and descends to not less than 25 mm from the bottom of the vessel on the downward stroke. At no time should the top of the basket-rack assembly become submerged. The time required for the upward stroke is equal to the time required for the downward stroke, and the change in stroke direction is a smooth transition, rather than an abrupt reversal of motion. The basket-rack assembly moves vertically along its axis. There is no appreciable horizontal motion or movement of the axis from the vertical.

**Basket-Rack Assembly—** The basket-rack assembly consists of six open-ended transparent tubes, each  $77.5 \pm 2.5$  mm long and having an inside diameter of 20.7 to 23 mm and a wall 1.0 to 2.8 mm thick; the tubes are held in a vertical position by two plates, each 88 to 92 mm in diameter and 5 to 8.5 mm in thickness, with six holes, each 22 to 26 mm in diameter, equidistant from the center of the plate and equally spaced from one another. Attached to the under surface of the lower plate is a woven stainless steel wire cloth, which has a plain square weave with 1.8- to 2.2-mm apertures and with a wire diameter of 0.57 to 0.66 mm. The parts of the apparatus are assembled and rigidly held by means of three bolts passing through the two plates. A suitable means is provided to suspend the basket-rack assembly from the raising and lowering device using a point on its axis.

The design of the basket-rack assembly may be varied somewhat, provided the specifications for the glass tubes and the screen mesh size are maintained. The basket-rack assembly conforms to the dimensions found in [Figure 1](#).

**Disk—** The use of disks is permitted only where specified or allowed in the monograph. If specified in the individual monograph, each tube is provided with a cylindrical disk  $9.5 \pm 0.15$  mm thick and  $20.7 \pm 0.15$  mm in diameter. The disk is made of a suitable transparent plastic material having a specific gravity of between 1.18 and 1.20. Five parallel  $2 \pm 0.1$ -mm holes extend between the ends of the cylinder. One of the holes is centered on the cylindrical axis. The other holes are centered  $6 \pm 0.2$  mm from the axis on imaginary lines perpendicular to the axis and parallel to each other. Four identical trapezoidal-shaped planes are cut into the wall of the cylinder, nearly perpendicular to the ends of the cylinder. The trapezoidal shape is symmetrical; its parallel sides coincide with the ends of the cylinder and are parallel to an imaginary line connecting the centers of two adjacent holes 6 mm from the cylindrical axis. The parallel side of the trapezoid on the bottom of the cylinder has a length of  $1.6 \pm 0.1$  mm, and its bottom edges lie at a depth of  $1.5$  to  $1.8$  mm (RB 1-Aug-2008) from the cylinder's circumference. The parallel side of the trapezoid on the top of the cylinder has a length of  $9.4 \pm 0.2$  mm, and its center lies at a depth of  $2.6 \pm 0.1$  mm from the cylinder's circumference. All surfaces of the disk are smooth. If the use of disks is specified in the individual monograph, add a disk to each tube, and operate the apparatus as directed under Procedure. The disks conform to dimensions found in [Figure 1](#).

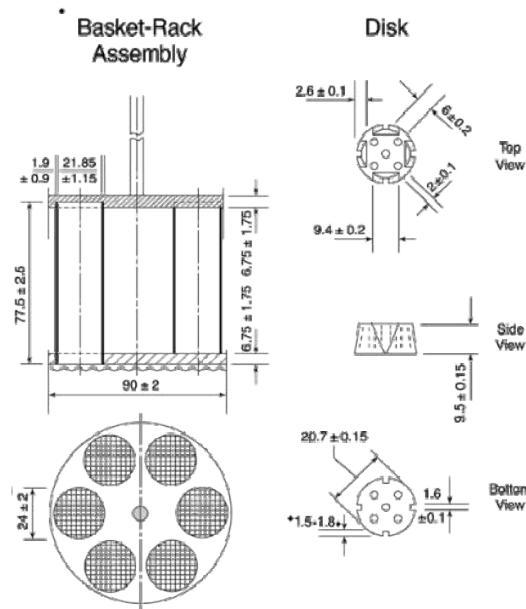


Figure 1. Disintegration apparatus. (All dimensions are expressed in mm.)\* (RB 1-Aug-2008)

## PROCEDURE

Uncoated Tablets— Place 1 dosage unit in each of the six tubes of the basket and, if prescribed, add a disk. Operate the apparatus, using water or the specified medium as the immersion fluid, maintained at  $37 \pm 2^\circ\text{C}$ . At the end of the time limit specified in the monograph, lift the basket from the fluid, and observe the tablets: all of the tablets have disintegrated completely. If 1 or 2 tablets fail to disintegrate completely, repeat the test on 12 additional tablets. The requirement is met if not fewer than 16 of the total of 18 tablets tested are disintegrated.

Plain-Coated Tablets— Apply the test for Uncoated Tablets, operating the apparatus for the time specified in the individual monograph.

Delayed-Release (Enteric-Coated) Tablets— Place 1 tablet in each of the six tubes of the basket and, if the tablet has a soluble external sugar coating, immerse the basket in water at room temperature for 5 minutes. Then operate the apparatus using simulated gastric fluid TS maintained at  $37 \pm 2^\circ\text{C}$  as the immersion fluid. After 1 hour of operation in simulated gastric fluid TS, lift the basket from the fluid, and observe the tablets: the tablets show no evidence of disintegration, cracking, or softening. Operate the apparatus, using simulated intestinal fluid TS maintained at  $37 \pm 2^\circ\text{C}$  as the immersion fluid, for the time specified in the monograph. Lift the basket from the fluid, and observe the tablets: all of the tablets disintegrate completely. If 1 or 2 tablets fail to disintegrate completely, repeat the test on 12 additional tablets: not fewer than 16 of the total of 18 tablets tested disintegrate completely.

Buccal Tablets— Apply the test for Uncoated Tablets. After 4 hours, lift the basket from the fluid, and observe the tablets: all of the tablets have disintegrated. If 1 or 2 tablets fail to disintegrate completely, repeat the test on 12 additional tablets: not fewer than 16 of the total of 18 tablets tested disintegrate completely.

Sublingual Tablets— Apply the test for Uncoated Tablets. At the end of the time limit specified in the individual monograph: all of the tablets have disintegrated. If 1 or 2 tablets fail to disintegrate completely, repeat the test on 12 additional tablets: not fewer than 16 of the total of 18 tablets tested disintegrate completely.

Hard Gelatin Capsules— Apply the test for Uncoated Tablets. Attach a removable wire cloth, which has a plain square weave with 1.8- to 2.2-mm mesh apertures and with a wire diameter of 0.60 to 0.655 mm, as described under Basket-Rack Assembly, to the surface of the upper plate of the basket-rack assembly. Observe the capsules within the time limit specified in the individual monograph: all of the capsules have disintegrated except for fragments from the capsule shell. If 1 or 2 capsules fail to disintegrate completely, repeat the test on 12 additional capsules: not fewer than 16 of the total of 18 capsules tested disintegrate completely.

Soft Gelatin Capsules— Proceed as directed under Hard Gelatin Capsules.♦

1 The use of automatic detection employing modified disks is permitted where the use of disks is specified or allowed. Such disks must comply with the requirements for density and dimension given in this chapter.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">William E. Brown</a> Senior Scientist 1-301-816-8380	(BPC05) Biopharmaceutics05

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711 DISSOLUTION

This general chapter is harmonized with the corresponding texts of the European Pharmacopoeia and/or the Japanese Pharmacopoeia. These pharmacopeias have undertaken not to make any unilateral change to this harmonized chapter.

Portions of the present general chapter text that are national USP text, and therefore not part of the harmonized text, are marked with symbols (♦) to specify this fact.

This test is provided to determine compliance with the dissolution requirements ♦where stated in the individual monograph♦ for dosage forms administered orally. In this general chapter, a dosage unit is defined as 1 tablet or 1 capsule or the amount specified. ♦Of the types of apparatus described herein, use the one specified in the individual monograph. Where the label states that an article is enteric-coated, and where a dissolution or disintegration test that does not specifically state that it is to be applied to delayed-release articles is included in the individual monograph, the procedure and interpretation given for Delayed-Release Dosage Forms is applied unless otherwise specified in the individual monograph. For hard or soft gelatin capsules and gelatin-coated tablets that do not conform to the Dissolution specification, repeat the test as follows. Where water or a medium with a pH of less than 6.8 is specified as the Medium in the individual monograph, the same Medium specified may be used with the addition of purified pepsin that results in an activity of 750,000 Units or

less per 1000 mL. For media with a pH of 6.8 or greater, pancreatin can be added to produce not more than 1750 USP Units of protease activity per 1000 mL.

[USP Reference Standards](#) (11) — USP Chlorpheniramine Maleate Extended-Release Tablets RS. [USP Prednisone Tablets RS](#). [USP Salicylic Acid Tablets RS](#).

## APPARATUS

### Apparatus 1 (Basket Apparatus)

The assembly consists of the following: a vessel, which may be covered, made of glass or other inert, transparent material<sup>1</sup>; a motor; a metallic drive shaft; and a cylindrical basket. The vessel is partially immersed in a suitable water bath of any convenient size or heated by a suitable device such as a heating jacket. The water bath or heating device permits holding the temperature inside the vessel at  $37 \pm 0.5^\circ$  during the test and keeping the bath fluid in constant, smooth motion. No part of the assembly, including the environment in which the assembly is placed, contributes significant motion, agitation, or vibration beyond that due to the smoothly rotating stirring element. An apparatus that permits observation of the specimen and stirring element during the test is preferable. The vessel is cylindrical, with a hemispherical bottom and  $\blacklozenge$  with one of the following dimensions and capacities: for a nominal  $\blacklozenge$  capacity of 1 L, the height is 160 mm to 210 mm and its inside diameter is 98 mm to 106 mm;  $\blacklozenge$  for a nominal capacity of 2 L, the height is 280 mm to 300 mm and its inside diameter is 98 mm to 106 mm; and for a nominal capacity of 4 L, the height is 280 mm to 300 mm and its inside diameter is 145 mm to 155 mm  $\blacklozenge$ . Its sides are flanged at the top. A fitted cover may be used to retard evaporation.<sup>2</sup> The shaft is positioned so that its axis is not more than 2 mm at any point from the vertical axis of the vessel and rotates smoothly and without significant wobble that could affect the results. A speed-regulating device is used that allows the shaft rotation speed to be selected and maintained at the specified rate  $\blacklozenge$  given in the individual monograph,  $\blacklozenge$  within  $\pm 4\%$ .

Shaft and basket components of the stirring element are fabricated of stainless steel, type 316, or other inert material, to the specifications shown in [Figure 1](#). A basket having a gold coating of about 0.0001 inch (2.5  $\mu\text{m}$ ) thick may be used. A dosage unit is placed in a dry basket at the beginning of each test. The distance between the inside bottom of the vessel and the bottom of the basket is maintained at  $25 \pm 2$  mm during the test.

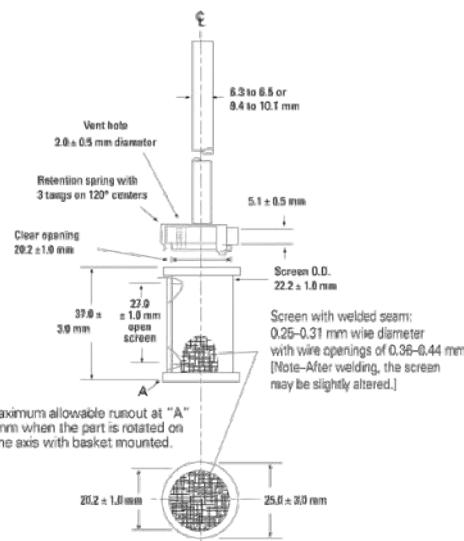


Figure 1. Basket Stirring Element

### Apparatus 2 (Paddle Apparatus)

Use the assembly from Apparatus 1, except that a paddle formed from a blade and a shaft is used as the stirring element. The shaft is positioned so that its axis is not more than 2 mm from the vertical axis of the vessel at any point and rotates smoothly without significant wobble that could affect the results. The vertical center line of the blade passes through the axis of the shaft so that the bottom of the blade is flush with the bottom of the shaft. The paddle conforms to the specifications shown in [Figure 2](#). The distance of  $25 \pm 2$  mm between the bottom of the blade and the inside bottom of the vessel is maintained during the test. The metallic or suitably inert, rigid blade and shaft comprise a single entity. A suitable two-part detachable design may be used provided the assembly remains firmly engaged during the test. The paddle blade and shaft may be coated with a suitable coating so as to make them inert. The dosage unit is allowed to sink to the bottom of the vessel before rotation of the blade is started. A small, loose piece of nonreactive material, such as not more than a few turns of wire helix, may be attached to dosage units that would otherwise float. An alternative sinker device is shown in [Figure 2a](#). Other validated sinker devices may be used.

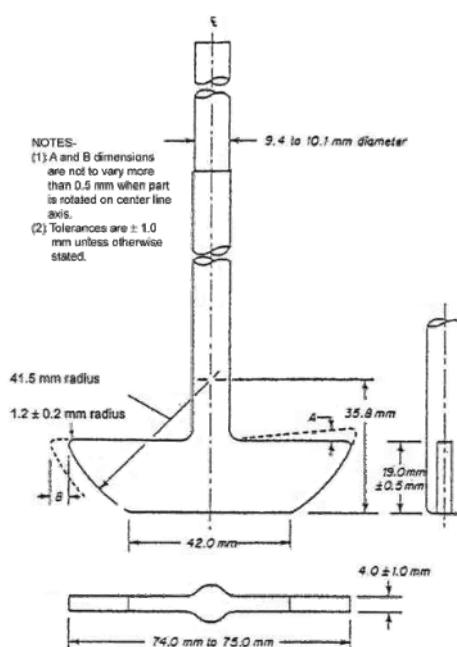


Figure 2. Paddle Stirring Element

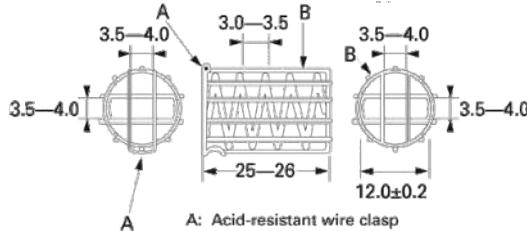


Figure 2a. Alternative sinker. All dimensions are expressed in mm.

Apparatus 3 (Reciprocating Cylinder)

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The assembly consists of a set of cylindrical, flat-bottomed glass vessels; a set of glass reciprocating cylinders; inert fittings (stainless steel type 316 or other suitable material), and screens that are made of suitable nonsorbing and nonreactive material and that are designed to fit the tops and bottoms of the reciprocating cylinders; and a motor and drive assembly to reciprocate the cylinders vertically inside the vessels and, if desired, index the reciprocating cylinders horizontally to a different row of vessels. The vessels are partially immersed in a suitable water bath of any convenient size that permits holding the temperature at  $37 \pm 0.5^\circ$  during the test. No part of the assembly, including the environment in which the assembly is placed, contributes significant motion, agitation, or vibration beyond that due to the smooth, vertically reciprocating cylinder. A device is used that allows the reciprocation rate to be

selected and maintained at the specified dip rate ♦given in the individual monograph♦ within  $\pm 5\%$ . An apparatus that permits observation of the specimens and reciprocating cylinders is preferable. The vessels are provided with an evaporation cap that remains in place for the duration of the test. The components conform to the dimensions shown in [Figure 3](#) unless otherwise specified ♦in the individual monograph♦.

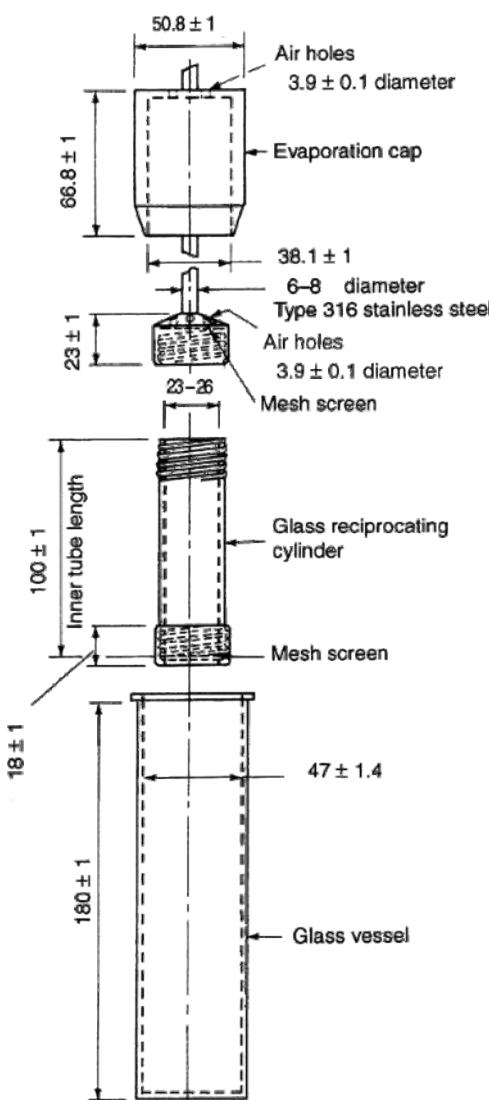


Figure 3. Apparatus 3 (reciprocating cylinder)

#### Apparatus 4 (Flow-Through Cell)

The assembly consists of a reservoir and a pump for the Dissolution Medium; a flow-through cell; and a water bath that maintains the Dissolution Medium at  $37 \pm 0.5^\circ$ . Use the specified cell size ♦ as given in the individual monograph ♦.

The pump forces the Dissolution Medium upwards through the flow-through cell. The pump has a delivery range between 240 and 960 mL per hour, with standard flow rates of 4, 8, and 16 mL per minute. It must deliver a constant flow ( $\pm 5\%$  of the nominal flow rate); the flow profile is sinusoidal with a pulsation of  $120 \pm 10$  pulses per minute.

The flow-through cell (see [Figures 4](#) and [5](#)), of transparent and inert material, is mounted vertically with a filter system (specified in the individual monograph) that prevents escape of undissolved particles from the top of the cell; standard cell diameters are 12 and 22.6 mm; the bottom cone is usually filled with small glass beads of about 1-mm diameter with one bead of about 5 mm positioned at the apex to protect the fluid entry tube; and a tablet holder (see [Figures 4](#) and [5](#)) is available for positioning of special dosage forms, for example, inlay tablets. The cell is immersed in a water bath, and the temperature is maintained at  $37 \pm 0.5^\circ$ .

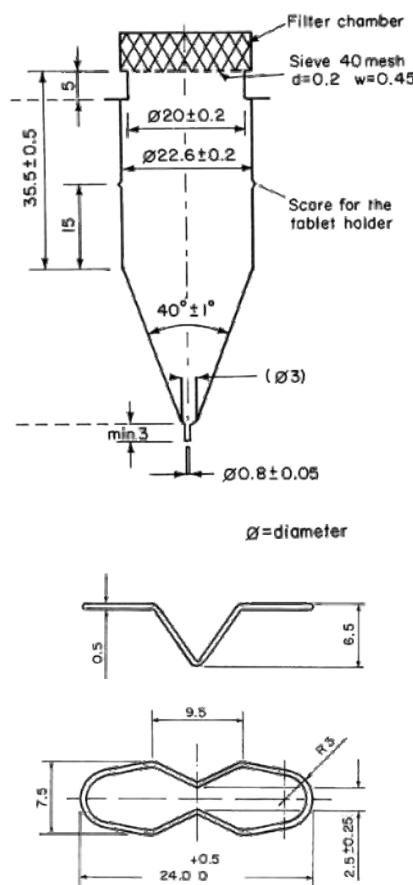


Figure 4. Large cell for tablets and capsules (top) Tablet holder for the large cell (bottom) (All measurements are expressed in mm unless noted otherwise.)

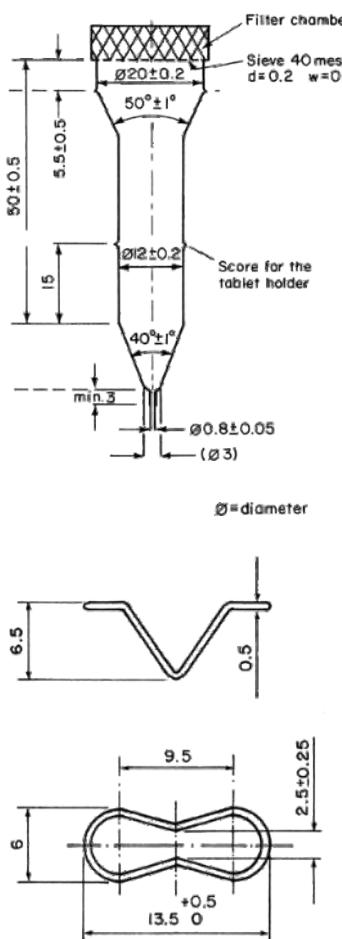


Figure 5. Small cell for tablets and capsules (top) Tablet holder for the small cell (bottom) (All measurements are expressed in mm unless noted otherwise.)

The apparatus uses a clamp mechanism and two O-rings to assemble the cell. The pump is separated from the dissolution unit in order to shield the latter against any vibrations originating from the pump. The position of the pump should not be on a level higher than the reservoir flasks. Tube connections are as short as possible. Use suitably inert tubing, such as polytef, with about 1.6-mm inner diameter and chemically inert flanged-end connections.

#### apparatus suitability

The determination of suitability of a test assembly to perform dissolution testing must include conformance to the dimensions and tolerances of the apparatus as given above. In addition, critical test parameters that have to be monitored periodically during use include volume and temperature of the Dissolution Medium, rotation speed (Apparatus 1 and Apparatus 2), dip rate (Apparatus 3), and flow rate of medium (Apparatus 4).

Determine the acceptable performance of the dissolution test assembly periodically. ♦The suitability for the individual apparatus is demonstrated by the Performance Verification Test.

Performance Verification Test, Apparatus 1 and 2— Individually test 1 tablet of the USP Prednisone Tablets RS and 1 tablet of USP Salicylic Acid Tablets RS, according to the operating conditions specified. The apparatus is suitable if the results obtained are within the acceptable range stated in the certificate for that Reference Standard tablet in the apparatus tested.

Performance Verification Test, Apparatus 3— Individually test 1 tablet of the USP Chlorpheniramine Maleate Extended-Release Tablets RS according to the operating conditions specified. The apparatus is suitable if the results obtained are within the acceptable range stated in the certificate.

Performance Verification Test, Apparatus 4— [To come.]♦

#### PROCEDURE

##### Apparatus 1 and Apparatus 2

##### immediate-release dosage forms

Place the stated volume of the Dissolution Medium ( $\pm 1\%$ ) in the vessel of the specified apparatus ♦given in the individual monograph♦, assemble the apparatus, equilibrate the Dissolution Medium to  $37 \pm 0.5^\circ$ , and remove the thermometer. Place 1 dosage unit in the apparatus, taking care to exclude air bubbles from the surface of the dosage unit, and immediately operate the apparatus at the specified rate ♦given in the individual monograph♦. Within the time interval specified, or at each of the times stated, withdraw a specimen from a zone midway between the surface of the Dissolution Medium and the top of the rotating basket or blade, not less than 1 cm from the vessel wall. [note—Where multiple sampling times are specified, replace the aliquots withdrawn for analysis with equal volumes of fresh Dissolution Medium at  $37^\circ$  or, where it can be shown that replacement of the medium is not necessary, correct for the volume change in the calculation. Keep the vessel covered for the duration of the test, and verify the temperature of the mixture under test at suitable times.] Perform the analysis ♦as directed in the individual monograph♦ using a suitable assay method.<sup>3</sup> Repeat the test with additional dosage form units.

If automated equipment is used for sampling or the apparatus is otherwise modified, verification that the modified apparatus will produce results equivalent to those obtained with the standard apparatus described in this general chapter is necessary.

Dissolution Medium— A suitable dissolution medium is used. Use the solvent specified ♦in the individual monograph♦. The volume specified refers to measurements made between  $20^\circ$  and  $25^\circ$ . If the Dissolution Medium is a buffered solution, adjust the solution so that its pH is within 0.05 unit of the specified pH ♦given in the individual monograph♦. [note—Dissolved gases can cause bubbles to form, which may change the results of the test. If dissolved gases influence the dissolution results, dissolved gases should be removed prior to



testing.<sup>4</sup>

Time— Where a single time specification is given, the test may be concluded in a shorter period if the requirement for minimum amount dissolved is met. Specimens are to be withdrawn only at the stated times within a tolerance of  $\pm 2\%$ .

◆ Procedure for a Pooled Sample for Immediate-Release Dosage Forms —Use this procedure where Procedure for a Pooled Sample is specified in the individual monograph. Proceed as directed in Procedure for Apparatus 1 and Apparatus 2 in Immediate-Release Dosage Forms. Combine equal volumes of the filtered solutions of the six or twelve individual specimens withdrawn, and use the pooled sample as the test specimen. Determine the average amount of the active ingredient dissolved in the pooled sample.◆

extended-release dosage forms

Proceed as directed for Immediate-Release Dosage Forms.

Dissolution Medium— Proceed as directed for Immediate-Release Dosage Forms.

Time— The test-time points, generally three, are expressed in hours.

delayed-release dosage forms

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Use Method A or Method B and the apparatus specified ◆ in the individual monograph◆. All test times stated are to be observed within a tolerance of  $\pm 2\%$ , unless otherwise specified.

Method A—

Procedure ◆(unless otherwise directed in the individual monograph)◆—

acid stage— Place 750 mL of 0.1 N hydrochloric acid in the vessel, and assemble the apparatus. Allow the medium to equilibrate to a temperature of  $37 \pm 0.5^\circ$ . Place 1 dosage unit in the apparatus, cover the vessel, and operate the apparatus at the specified rate ◆ given in the monograph◆.

After 2 hours of operation in 0.1 N hydrochloric acid, withdraw an aliquot of the fluid, and proceed immediately as directed under Buffer Stage.

Perform an analysis of the aliquot using a suitable assay method. ◆ The procedure is specified in the individual monograph.◆

buffer stage— [note—Complete the operations of adding the buffer and adjusting the pH within 5 minutes.]

With the apparatus operating at the rate specified ◆ in the monograph◆, add to the fluid in the vessel 250 mL of 0.20 M tribasic sodium phosphate that has been equilibrated to  $37 \pm 0.5^\circ$ . Adjust, if necessary, with 2 N hydrochloric acid or 2 N sodium hydroxide to a pH of  $6.8 \pm 0.05$ . Continue to operate the apparatus for 45 minutes, or for the specified time ◆ given in the individual monograph◆. At the end of the time period, withdraw an aliquot of the fluid, and perform the analysis using a suitable assay method. ◆ The procedure is specified in the individual monograph. The test may be concluded in a shorter time period than that specified for the Buffer Stage if the requirement for the minimum amount dissolved is met at an

earlier time.◆

Method B—

Procedure ◆(unless otherwise directed in the individual monograph)◆—

acid stage— Place 1000 mL of 0.1 N hydrochloric acid in the vessel, and assemble the apparatus. Allow the medium to equilibrate to a temperature of  $37 \pm 0.5^\circ$ . Place 1 dosage unit in the apparatus, cover the vessel, and operate the apparatus at the rate specified ◆ in the monograph◆. After 2 hours of operation in 0.1 N hydrochloric acid, withdraw an aliquot of the fluid, and proceed immediately as directed under Buffer Stage.

Perform an analysis of the aliquot using a suitable assay method. ◆ The procedure is specified in the individual monograph.◆

buffer stage— [note—For this stage of the procedure, use buffer that previously has been equilibrated to a temperature of  $37 \pm 0.5^\circ$ .] Drain the acid from the vessel, and add to the vessel 1000 mL of pH 6.8 phosphate buffer, prepared by mixing 0.1 N hydrochloric acid with 0.20 M tribasic sodium phosphate (3:1) and adjusting, if necessary, with 2 N hydrochloric acid or 2 N sodium hydroxide to a pH of  $6.8 \pm 0.05$ . [note—This may also be accomplished by removing from the apparatus the vessel containing the acid and replacing it with another vessel containing the buffer and transferring the dosage unit to the vessel containing the buffer.]

Continue to operate the apparatus for 45 minutes, or for the specified time ◆ given in the individual monograph◆. At the end of the time period, withdraw an aliquot of the fluid, and perform the analysis using a suitable assay method. ◆ The procedure is specified in the individual monograph. The test may be concluded in a shorter time period than that specified for the Buffer Stage if the requirement for minimum amount dissolved is met at an earlier time.◆

Apparatus 3 (Reciprocating Cylinder)

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immediate-release dosage forms

Place the stated volume of the Dissolution Medium in each vessel of the apparatus, assemble the apparatus, equilibrate the Dissolution Medium to  $37 \pm 0.5^\circ$ , and remove the thermometer. Place 1 dosage-form unit in each of the six reciprocating cylinders, taking care to exclude air bubbles from the surface of each dosage unit, and immediately operate the apparatus as specified ◆ in the individual monograph◆. During the upward and downward stroke, the reciprocating cylinder moves through a total distance of 9.9 to 10.1 cm. Within the time interval specified, or at each of the times stated, raise the reciprocating cylinders and withdraw a portion of the solution under test from a zone midway between the surface of the Dissolution Medium and the bottom of each vessel. Perform the analysis as directed ◆ in the individual monograph◆. If necessary, repeat the test with additional dosage-form units.

Dissolution Medium —Proceed as directed for Immediate-Release Dosage Forms under Apparatus 1 and Apparatus 2.

Time —Proceed as directed for Immediate-Release Dosage Forms under Apparatus 1 and Apparatus 2.

extended-release dosage forms

Proceed as directed for Immediate-Release Dosage Forms under Apparatus 3.

Dissolution Medium —Proceed as directed for Extended-Release Dosage Forms under Apparatus 1 and Apparatus 2.

Time —Proceed as directed for Extended-Release Dosage Forms under Apparatus 1 and Apparatus 2.

delayed-release dosage forms

Proceed as described for Delayed-Release Dosage Forms, Method B under Apparatus 1 and Apparatus 2 using one row of vessels for the acid stage media and the following row of vessels for the buffer stage media and using the volume of medium specified (usually 300 mL).

Time —Proceed as directed for Immediate-Release Dosage Forms under Apparatus 1 and Apparatus 2.

Apparatus 4 (Flow-Through Cell)

immediate-release dosage forms

Place the glass beads into the cell specified ◆ in the monograph◆. Place 1 dosage unit on top of the beads or, if specified ◆ in the monograph◆, on a wire carrier. Assemble the filter head, and fix the parts together by means of a suitable clamping device. Introduce by the pump the Dissolution Medium warmed to  $37 \pm 0.5^\circ$  through the bottom of the cell to obtain the



ow rate specified ♦ in the individual monograph♦ and measured with an accuracy of 5%. Collect the eluate by fractions at each of the times stated. Perform the analysis as directed in the individual monograph♦. Repeat the test with additional dosage-form units.

Dissolution Medium — Proceed as directed for Immediate-Release Dosage Forms under Apparatus 1 and Apparatus 2.

Time — Proceed as directed for Immediate-Release Dosage Forms under Apparatus 1 and Apparatus 2.  
extended-release dosage forms

Proceed as directed for Immediate-Release Dosage Forms under Apparatus 4.

Dissolution Medium — Proceed as directed for Immediate-Release Dosage Forms under Apparatus 4.

Time — Proceed as directed for Immediate-Release Dosage Forms under Apparatus 4.  
delayed-release dosage forms

Proceed as directed for Delayed-Release Dosage Forms under Apparatus 1 and Apparatus 2, using the specified media.

Time — Proceed as directed for Delayed-Release Dosage Forms under Apparatus 1 and Apparatus 2.

#### INTERPRETATION

##### Immediate-Release Dosage Forms

Unless otherwise specified ♦ in the individual monograph♦, the requirements are met if the quantities of active ingredient dissolved from the dosage units tested conform to Acceptance Table 1. Continue testing through the three stages unless the results conform at either S1 or S2. The quantity, Q, is the amount of dissolved active ingredient ♦ specified in the individual monograph♦, expressed as a percentage of the labeled content of the dosage unit; the 5%, 15%, and 25% values in [Acceptance Table 1](#) are percentages of the labeled content so that these values and Q are in the same terms.

Acceptance Table 1

Stage	Number Tested	Acceptance Criteria
S1	6	Each unit is not less than Q + 5%.
S2	6	Average of 12 units (S1 + S2) is equal to or greater than Q, and no unit is less than Q - 15%.
S3	12	Average of 24 units (S1 + S2 + S3) is equal to or greater than Q, not more than 2 units are less than Q - 15%, and no unit is less than Q - 25%.

♦ Immediate-Release Dosage Forms Pooled Sample — Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient dissolved from the pooled sample conform to the accompanying Acceptance Table for a Pooled Sample. Continue testing through the three stages unless the results conform at either S1 or S2.

The quantity, Q, is the amount of dissolved active ingredient specified in the individual monograph, expressed as a percentage of the labeled content.

Acceptance Table for a Pooled Sample

Stage	Number Tested	Acceptance Criteria
S1	6	Average amount dissolved is not less than Q + 10%.
S2	6	Average amount dissolved (S1 + S2) is equal to or greater than Q + 5%.
S3	12	Average amount dissolved (S1 + S2 + S3) is equal to or greater than Q.

♦

##### Extended-Release Dosage Forms

Unless otherwise specified ♦ in the individual monograph♦, the requirements are met if the quantities of active ingredient dissolved from the dosage units tested conform to [Acceptance Table 2](#). Continue testing through the three levels unless the results conform at either L1 or L2. Limits on the amounts of active ingredient dissolved are expressed in terms of the percentage of labeled content. The limits embrace each value of Qi, the amount dissolved at each specified fractional dosing interval. Where more than one range is specified

♦ in the individual monograph♦, the acceptance criteria apply individually to each range.

Acceptance Table 2

Level	Number Tested	Criteria
L1	6	No individual value lies outside each of the stated ranges and no individual value is less than the stated amount at the final test time.
L2	6	The average value of the 12 units (L1 + L2) lies within each of the stated ranges and is not less than the stated amount at the final test time; none is more than 10% of labeled content outside each of the stated ranges; and none is more than 10% of labeled content below the stated amount at the final test time.
L3	12	The average value of the 24 units (L1 + L2 + L3) lies within each of the stated ranges, and is not less than the stated amount at the final test time; not more than 2 of the 24 units are more than 10% of labeled content outside each of the stated ranges; not more than 2 of the 24 units are more than 10% of labeled content below the stated amount at the final test time; and none of the units is more than 20% of labeled content outside each of the stated ranges or more than 20% of labeled content below the stated amount at the final test time.

##### Delayed-Release Dosage Forms

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Acid Stage — Unless otherwise specified ♦ in the individual monograph♦, the requirements of this portion of the test are met if the quantities, based on the percentage of the labeled content, of active ingredient dissolved from the units tested conform to [Acceptance Table 3](#). Continue testing through all levels unless the results of both acid and buffer stages conform at an earlier level.

Acceptance Table 3

Level	Number Tested	Criteria
A1	6	No individual value exceeds 10% dissolved.
A2	6	Average of the 12 units (A1 + A2) is not more than 10% dissolved, and no individual unit is greater than 25% dissolved.
A3	12	Average of the 24 units (A1 + A2 + A3) is not more than 10% dissolved, and no individual unit is greater than 25% dissolved.

Buffer Stage — Unless otherwise specified ♦ in the individual monograph♦, the requirements are met if the quantities of active ingredient dissolved from the units tested conform to [Acceptance Table 4](#). Continue testing through the three levels unless the results of both stages conform at an earlier level. The value of Q in [Acceptance Table 4](#) is 75% dissolved unless otherwise specified ♦ in the individual monograph♦. The quantity, Q, ♦ specified in the individual monograph♦ is the total amount of active ingredient dissolved in both the



and Buffer Stages, expressed as a percentage of the labeled content. The 5%, 15%, and 25% values in [Acceptance Table 4](#) are percentages of the labeled content so that the values and Q are in the same terms.

Acceptance Table 4

Level	Number Tested	Criteria
B1	6	Each unit is not less than Q + 5%.
B2	6	Average of 12 units (B1 + B2) is equal to or greater than Q, and no unit is less than Q - 15%.
B3	12	Average of 24 units (B1 + B2 + B3) is equal to or greater than Q, not more than 2 units are less than Q - 15%, and no unit is less than Q - 25%.

1 The materials should not sorb, react, or interfere with the specimen being tested.

2 If a cover is used, it provides sufficient openings to allow ready insertion of the thermometer and withdrawal of specimens.

3 Test specimens are filtered immediately upon sampling unless filtration is demonstrated to be unnecessary. Use an inert filter that does not cause adsorption of the active ingredient or contain extractable substances that would interfere with the analysis.

4 One method of deaeration is as follows: Heat the medium, while stirring gently, to about 41°, immediately filter under vacuum using a filter having a porosity of 0.45 µm or less, with vigorous stirring, and continue stirring under vacuum for about 5 minutes. Other validated deaeration techniques for removal of dissolved gases may be used.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	William E. Brown Senior Scientist 1-301-816-8380	(BPC05) Biopharmaceutics05
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## 721 DISTILLING RANGE

To determine the range of temperatures within which an official liquid distills, or the percentage of the material that distills between two specified temperatures, use Method I or Method II as directed in the individual monograph. The lower limit of the range is the temperature indicated by the thermometer when the first drop of condensate leaves the tip of the condenser, and the upper limit is the Dry Point, i.e., the temperature at which the last drop of liquid evaporates from the lowest point in the distillation flask, without regard to any liquid remaining on the side of the flask, or the temperature observed when the proportion specified in the individual monograph has been collected.

note—Cool all liquids that distill below 80° to between 10° and 15° before measuring the sample to be distilled.

### Method I

Apparatus— Use apparatus similar to that specified for Method II, except that the distilling flask is of 50- to 60-mL capacity, and the neck of the flask is 10 to 12 cm long and 14 to 16 mm in internal diameter. The perforation in the upper insulating board, if one is used, should be such that when the flask is set into it, the portion of the flask below the upper surface of the insulating material has a capacity of 3 to 4 mL.

Procedure— Proceed as directed for Method II, but place in the flask only 25 mL of the liquid to be tested.

### Method II

Apparatus— Use an apparatus consisting of the following parts:

Distilling Flask— A round-bottom distilling flask, of heat-resistant glass, of 200-mL capacity, and having a total length of 17 to 19 cm, and an inside neck diameter of 20 to 22 mm. Attached about midway on the neck, approximately 12 cm from the bottom of the flask, is a side-arm 10 to 12 cm long and 5 mm in internal diameter, which forms an angle of 70° to 75° with the lower portion of the neck.

Condenser— A straight glass condenser 55 to 60 cm in length with a water jacket about 40 cm in length, or a condenser of other design having equivalent condensing capacity. The lower end of the condenser may be bent to provide a delivery tube, or it may be connected to a bent adapter that serves as a delivery tube.

Insulating Boards— Two pieces of insulating board, 5 to 7 mm thick and 14 to 16 cm square, suitable for confining the heat to the lower part of the flask. Each board has a hole in its center, and the two boards differ only with respect to the diameter of the hole, i.e., the diameters are 4 cm and 10 cm, respectively. In use, the boards are placed one upon the other, and rest on a tripod or other suitable support, with the board having the larger hole on top.

Receiver— A 100-mL cylinder graduated in 1-mL subdivisions.

Thermometer— In order to avoid the necessity for an emergent stem correction, an accurately standardized, partial-immersion thermometer having the smallest practical subdivisions (not greater than 0.2°) is recommended. Suitable thermometers are available as the ASTM E-1 series 37C through 41C, and 102C through 107C (see [Thermometers](#) 21). When placed in position, the stem is located in the center of the neck, and the top of the contraction chamber (or bulb, if 37C or 38C is used) is level with the bottom of the outlet to the side-arm.

Heat Source— A small Bunsen burner or an electric heater or mantle capable of adjustment comparable to that possible with a Bunsen burner.

Procedure— Assemble the apparatus, and place in the flask 100 mL of the liquid to be tested, taking care not to allow any of the liquid to enter the side-arm. Insert the thermometer, shield the entire burner and flask assembly from external air currents, and apply heat, regulating it so that between 5 and 10 minutes elapse before the first drop of distillate falls from the condenser. Continue the distillation at a rate of 4 to 5 mL of distillate per minute, collecting the distillate in the receiver. Note the temperature when the first drop of distillate falls from the condenser, and again when the last drop of liquid evaporates from the bottom of the flask or when the specified percentage has distilled over. Unless otherwise specified in the individual monograph, apply when necessary the emergent stem correction and report the temperatures adjusting the barometric pressure by the following formula:

$$t = t_0 + [(t_0 - 10 + 0.033)(760 - p)]$$

in which t is the corrected boiling temperature, in Celsius scale;  $t_0$  is the measured boiling temperature, in Celsius scale; and p is the barometric pressure at the time of measurement, in mm Hg.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	Antonio Hernandez-Cardoso, B.S. Scientist, Latin American Specialist	(GC05) General Chapters 05

## 724 DRUG RELEASE

This test is provided to determine compliance with drug-release requirements where specified in individual monographs. Use the apparatus specified in the individual monograph. Replace the aliquots withdrawn for analysis with equal volumes of fresh Dissolution Medium at the temperature specified in the monograph or, where it can be shown that replacement of the medium is not necessary, correct for the volume change in the calculation. Keep the vessel covered for the duration of the test, and verify the temperature of the mixture under test at suitable times.

## TRANSDERMAL DELIVERY SYSTEMS—GENERAL DRUG RELEASE STANDARDS

## Apparatus 5 (Paddle over Disk)

Apparatus— Use the paddle and vessel assembly from Apparatus 2 as described under [Dissolution 711](#), with the addition of a stainless steel disk assembly<sup>1</sup> designed for holding the transdermal system at the bottom of the vessel. Other appropriate devices may be used, provided they do not sorb, react with, or interfere with the specimen being tested<sup>2</sup>. The temperature is maintained at  $32 \pm 0.5^\circ$ . A distance of  $25 \pm 2$  mm between the paddle blade and the surface of the disk assembly is maintained during the test. The vessel may be covered during the test to minimize evaporation. The disk assembly for holding the transdermal system is designed to minimize any “dead” volume between the disk assembly and the bottom of the vessel. The disk assembly holds the system flat and is positioned such that the release surface is parallel with the bottom of the paddle blade (see [Figure 1](#)).

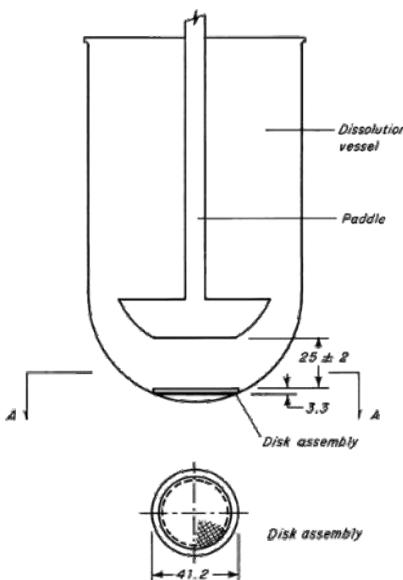


Fig. 1. Paddle Over Disk.(All measurements are expressed in mm unless noted otherwise.)

Apparatus Suitability Test and Dissolution Medium— Proceed as directed for Apparatus 2 under [Dissolution 711](#).

Procedure— Place the stated volume of the Dissolution Medium in the vessel, assemble the apparatus without the disk assembly, and equilibrate the medium to  $32 \pm 0.5^\circ$ . Apply the transdermal system to the disk assembly, assuring that the release surface of the system is as flat as possible. The system may be attached to the disk by applying a suitable adhesive<sup>3</sup> to the disk assembly. Dry for 1 minute. Press the system, release surface side up, onto the adhesive-coated side of the disk assembly. If a membrane<sup>4</sup> is used to support the system, it is applied so that no air bubbles occur between the membrane and the release surface. Place the disk assembly flat at the bottom of the vessel with the release surface facing up and parallel to the edge of the paddle blade and surface of the Dissolution Medium. The bottom edge of the paddle is  $25 \pm 2$  mm from the surface of the disk assembly.

Immediately operate the apparatus at the rate specified in the monograph. At each sampling time interval, withdraw a specimen from a zone midway between the surface of the Dissolution Medium and the top of the blade, not less than 1 cm from the vessel wall. Perform the analysis on each sampled aliquot as directed in the individual monograph, correcting for any volume losses, as necessary. Repeat the test with additional transdermal systems.

Time— The test time points, generally three, are expressed in hours. Specimens are to be withdrawn within a tolerance of  $\pm 15$  minutes or  $\pm 2\%$  of the stated time, the tolerance that results in the narrowest time interval being selected.

Interpretation— Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient released from the system conform to [Acceptance Table 1](#) for transdermal drug delivery systems. Continue testing through the three levels unless the results conform at either L1 or L2.

Acceptance Table 1

Level	Number Tested	Criteria
L1	6	No individual value lies outside the stated range.
L2	6	The average value of the 12 units (L1 + L2) lies within the stated range. No individual value is outside the stated range by more than 10% of the average of the stated range.
L3	12	The average value of the 24 units (L1 + L2 + L3) lies within the stated range. Not more than 2 of the 24 units are outside the stated range by more than 10% of the average of the stated range; and none of the units is outside the stated range by more than 20% of the average of the stated range.

## Apparatus 6 (Cylinder)

Apparatus— Use the vessel assembly from Apparatus 1 as described under [Dissolution 711](#), except to replace the basket and shaft with a stainless steel cylinder stirring element and to maintain the temperature at  $32 \pm 0.5^\circ$  during the test. The shaft and cylinder components of the stirring element are fabricated of stainless steel to the specifications shown in [Figure 2](#). The dosage unit is placed on the cylinder at the beginning of each test. The distance between the inside bottom of the vessel and the cylinder is maintained at  $25 \pm 2$  mm during the test.

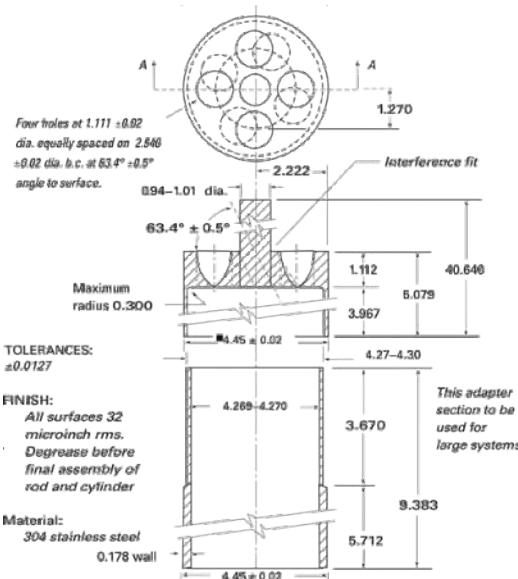


Fig. 2. Cylinder Stirring Element.<sup>5</sup> (All measurements are expressed in cm unless noted otherwise.)

Dissolution Medium— Use the medium specified in the individual monograph (see [Dissolution](#) (711)).

Procedure— Place the stated volume of the Dissolution Medium in the vessel of the apparatus specified in the individual monograph, assemble the apparatus, and equilibrate the Dissolution Medium to  $32 \pm 0.5^{\circ}\text{C}$ . Unless otherwise directed in the individual monograph, prepare the test system prior to test as follows. Remove the protective liner from the system, and place the adhesive side on a piece of Cuprophan<sup>4</sup> that is not less than 1 cm larger on all sides than the system. Place the system, Cuprophan covered side down, on a clean surface, and apply a suitable adhesive<sup>3</sup> to the exposed Cuprophan borders. If necessary, apply additional adhesive to the back of the system. Dry for 1 minute. Carefully apply the adhesive-coated side of the system to the exterior of the cylinder such that the long axis of the system fits around the circumference of the cylinder. Press the Cuprophan covering to remove trapped air bubbles. Place the cylinder in the apparatus, and immediately rotate at the rate specified in the individual monograph. Within the time interval specified, or at each of the times stated, withdraw a quantity of Dissolution Medium for analysis from a zone midway between the surface of the Dissolution Medium and the top of the rotating cylinder, not less than 1 cm from the vessel wall. Perform the analysis as directed in the individual monograph, correcting for any volume losses as necessary. Repeat the test with additional transdermal drug delivery systems.

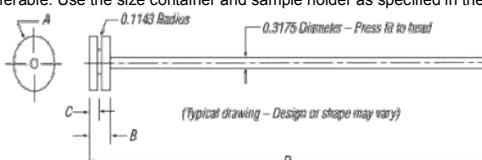
Time— Proceed as directed under Apparatus 5.

Interpretation— Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient released from the system conform to [Acceptance Table 1](#) for transdermal drug delivery systems. Continue testing through the three levels unless the results conform at either L1 or L2.

#### Apparatus 7 (Reciprocating Holder)

note—This apparatus may also be specified for use with a variety of dosage forms.

Apparatus— The assembly consists of a set of volumetrically calibrated or tared solution containers made of glass or other suitable inert material<sup>6</sup>, a motor and drive assembly to reciprocate the system vertically and to index the system horizontally to a different row of vessels automatically if desired, and a set of suitable sample holders (see [Figure 3](#)<sup>7</sup> and Figures 4a–4d). The solution containers are partially immersed in a suitable water bath of any convenient size that permits maintaining the temperature, T, inside the containers at  $32 \pm 0.5^{\circ}\text{C}$  or within the allowable range, as specified in the individual monograph, during the test. No part of the assembly, including the environment in which the assembly is placed, contributes significant motion, agitation, or vibration beyond that due to the smooth, vertically reciprocating sample holder. Apparatus that permits observation of the system and holder during the test is preferable. Use the size container and sample holder as specified in the individual monograph.



Dimensions are in centimeters

System <sup>a</sup>	HEAD			ROD		O-RING (not shown)
	A (Diameter)	B	C	Material <sup>b</sup>	D	
1.6cm <sup>2</sup>	1.428	0.9525	0.4750	SS/VT	30.48	SS/P
2.5cm <sup>2</sup>	1.778	0.9525	0.4750	SS/VT	30.48	SS/P
5cm <sup>2</sup>	2.0824	0.7620	0.3810	SS/VT	8.890	SS/P
7cm <sup>2</sup>	3.1750	0.7620	0.3810	SS/VT	30.48	SS/P
10cm <sup>2</sup>	5.0292	0.6350	0.3505	SS/VT	31.01	SS/P

<sup>a</sup> Typical system sizes.

<sup>b</sup> SS/VT—Either stainless steel or virgin Teflon.

<sup>c</sup> SS/P—Either stainless steel or Plexiglas.

Fig. 3. Reciprocating Disk Sample Holder.7

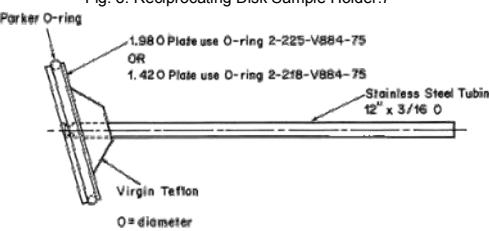


Fig. 4a. Transdermal System Holder—Angled Disk.

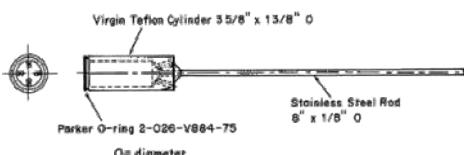


Fig. 4b. Transdermal System Holder—Cylinder.

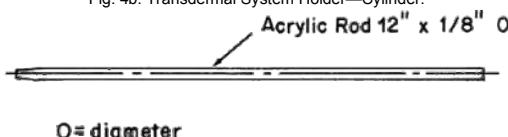


Fig. 4c. Oral Extended-Release Tablet Holder—Rod, Pointed for Gluing.

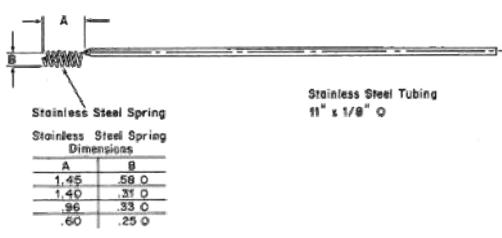


Fig. 4d. Oral Extended-Release Tablet Holder—Spring Holder.

Dissolution Medium— Use the Dissolution Medium specified in the individual monograph (see [Dissolution \(711\)](#)).

Sample Preparation A (Coated tablet drug delivery system)— Attach each system to be tested to a suitable sample holder (e.g., by gluing system edge with 2-cyano acrylate glue onto the end of a plastic rod or by placing the system into a small nylon net bag at the end of a plastic rod or within a metal coil attached to a metal rod).

Sample Preparation B (Transdermal drug delivery system)— Press the system onto a dry, unused piece of Cuprophan<sup>4</sup>, nylon netting, or equivalent with the adhesive side against the selected substrate, taking care to eliminate air bubbles between the substrate and the release surface. Attach the system to a suitable sized sample holder with a suitable O-ring such that the back of the system is adjacent to and centered on the bottom of the disk-shaped sample holder or centered around the circumference of the cylindrical-shaped sample holder. Trim the excess substrate with a sharp blade.

Sample Preparation C (Other drug delivery systems)— Attach each system to be tested to a suitable holder as described in the individual monograph.

Procedure— Suspend each sample holder from a vertically reciprocating shaker such that each system is continuously immersed in an accurately measured volume of Dissolution Medium within a calibrated container pre-equilibrated to temperature, T. Reciprocate at a frequency of about 30 cycles per minute with an amplitude of about 2 cm, or as specified in the individual monograph, for the specified time in the medium specified for each time point. Remove the solution containers from the bath, cool to room temperature, and add sufficient solution (i.e., water in most cases) to correct for evaporative losses. Perform the analysis as directed in the individual monograph. Repeat the test with additional drug delivery systems as required in the individual monograph.

Interpretation— Unless otherwise specified in the individual monograph, the requirements are met if the quantities of the active ingredients released from the system conform to Acceptance Table 2 under [Dissolution \(711\)](#) for coated tablet drug delivery systems, to [Acceptance Table 1](#) for transdermal drug delivery systems, or as specified in the individual monograph. Continue testing through the three levels unless the results conform at either L1 or L2.

1 Disk assembly (stainless support disk) may be obtained from Millipore Corp., Ashley Rd., Bedford, MA 01730.

2 A suitable device is the watchglass-patch-polytef mesh sandwich assembly available as the Transdermal SandwichTM from Hanson Research Corp., 9810 Variel Ave., Chatsworth, CA 91311.

3 Use Dow Corning, MD7-4502 Silicone Adhesive 65% in ethyl acetate, or the equivalent.

4 Use Cuprophan, Type 150 pm,  $11 \pm 0.5\text{-}\mu\text{m}$  thick, an inert, porous cellulosic material, which is available from Medicell International Ltd., 239 Liverpool Road, London NI 1LX, England.

5 The cylinder stirring element is available from Accurate Tool, Inc., 25 Diaz St., Stamford, CT 06907, or from VanKel Technology Group, 13000 Weston Parkway, Cary, NC 27513.

6 The materials should not sorb, react with, or interfere with the specimen being tested.

7 The reciprocating disk sample holder may be purchased from ALZA Corp., 1900 Charleston Road, P.O. Box 7210, Mt. View, CA 94039-7210 or VanKel Technology Group.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">William E. Brown</a> Senior Scientist 1-301-816-8380	(BPC05) Biopharmaceutics05

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### 726 ELECTROPHORESIS

Electrophoresis refers to the migration of electrically charged proteins, colloids, molecules, or other particles when dissolved or suspended in an electrolyte through which an electric current is passed.

Based upon the type of apparatus used, electrophoretic methods may be divided into two categories, one called free solution or moving boundary electrophoresis and the other called zone electrophoresis.

In the free solution method, a buffered solution of proteins in a U-shaped cell is subjected to an electric current which causes the proteins to form a series of layers in order of decreasing mobility, which are separated by boundaries. Only a part of the fastest moving protein is physically separated from the other proteins, but examination of the moving boundaries using a schlieren optical system provides data for calculation of mobilities and information on the qualitative and quantitative composition of the protein mixture.

In zone electrophoresis, the sample is introduced as a narrow zone or spot in a column, slab, or film of buffer. Migration of the components as narrow zones permits their complete separation. Remixing of the separated zones by thermal convection is prevented by stabilizing the electrolyte in a porous matrix such as a powdered solid, or a fibrous material such as paper, or a gel such as starch, agar, or polyacrylamide.

Various methods of zone electrophoresis are widely employed. Gel electrophoresis, particularly the variant called disk electrophoresis, is especially useful for protein separation because of its high resolving power.

Gel electrophoresis, which is employed by the compendium, is discussed in more detail following the presentation of some theoretical principles and methodological practices, which are shared in varying degrees by all electrophoretic methods.

The electrophoretic migration observed for particles of a particular substance depends on characteristics of the particle, primarily its electrical charge, its size or molecular weight, and its shape, as well as characteristics and operating parameters of the system. These latter include the pH, ionic strength, viscosity and temperature of the electrolyte, density or cross-linking of any stabilizing matrix such as gel, and the voltage gradient employed.

**Effect of Charge, Particle Size, Electrolyte Viscosity, and Voltage Gradient**— Electrically charged particles migrate toward the electrode of opposite charge, and molecules with both positive and negative charges move in a direction dependent on the net charge. The rate of migration is directly related to the magnitude of the net charge on the particle and is inversely related to the size of the particle, which in turn is directly related to its molecular weight.

Very large spherical particles, for which Stokes' law is valid, exhibit an electrophoretic mobility,  $u_0$ , which is inversely related to the first power of the radius as depicted in the equation:

$$u_0 = \frac{v}{E} = \frac{Q}{6\pi r \eta}$$

where  $v$  is the velocity of the particle,  $E$  is the voltage gradient imposed on the electrolyte,  $Q$  is the charge on the particle,  $r$  is the particle radius, and  $\eta$  is the viscosity of the electrolyte. This idealized expression is strictly valid only at infinite dilution and in the absence of a stabilizing matrix such as paper or a gel.

Ions, and peptides up to molecular weights of at least 5000, particularly in the presence of stabilizing media, do not obey Stokes' law, and their electrophoretic behavior is best described by an equation of the type:

$$u_0 = \frac{Q}{A\pi r^2 \eta}$$

where  $A$  is a shape factor generally in the range of 4 to 6, which shows an inverse dependence of the mobility on the square of the radius. In terms of molecular weight, this implies an inverse dependence of mobility on the  $2/3$  power of the molecular weight.

**Effect of pH**— The direction and rate of migration of molecules containing a variety of ionizable functional groups, such as amino acids and proteins, depends upon the pH of the electrolyte. For instance, the mobility of a simple amino acid such as glycine varies with pH approximately as shown in [Figure 1](#).

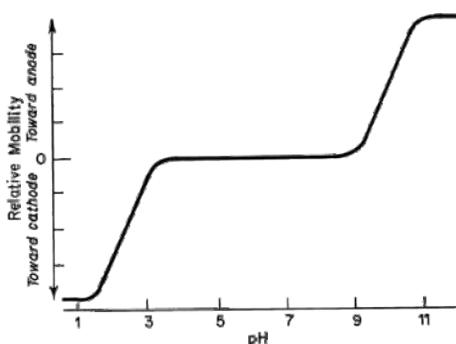


Fig. 1.

The  $pK_a$  values of 2.2 and 9.9 coincide with the inflection points of the sigmoid portions of the plot. Since the respective functional groups are 50% ionized at the pH values where  $pH = pK_a$ , the electrophoretic mobilities at these points are half of the value observed for the fully ionized cation and anion obtained at very low and very high pH, respectively. The zwitterion that exists at the intermediate pH range is electrically neutral and has zero mobility.

**Effect of Ionic Strength and Temperature**— Electrophoretic mobility decreases with increasing ionic strength of the supporting electrolyte. Ionic strength,  $\mu$ , is defined as:

$$\mu = 0.5 \sum C_i Z_i^2$$

where  $C_i$  is the concentration of an ion in moles per L and  $Z_i$  is its valence, and the sum is calculated for all ions in the solution. For buffers in which both the anion and cation are univalent, ionic strength is identical with molarity.

Ionic strengths of electrolytes employed in electrophoresis commonly range from about 0.01 to 0.10. A suitable strength is somewhat dependent on the sample composition, since the buffer capacity must be great enough to maintain a constant pH over the area of the component zones. Zones become sharper or more compact as ionic strength is increased.

Temperature affects mobility indirectly, since the viscosity,  $\eta$ , of the supporting electrolyte is temperature-dependent. The viscosity of water decreases at a rate of about 3% per  $^{\circ}\text{C}$  in the range of  $0^{\circ}$  to  $5^{\circ}$  and at a slightly lower rate in the vicinity of room temperature. Mobility, therefore, increases with increasing electrolyte temperature.

Considerable heat is evolved as a result of current passing through the supporting electrolyte. This heat increases with the applied voltage and with increasing ionic strength. Particularly in larger apparatus, despite the circulation of a coolant, this heat produces a temperature gradient across the bed which may lead to distortion of the separated zones. Therefore, practical considerations and the design of the particular apparatus dictate the choice of ionic strength and operating voltage.

effect of a Stabilizing Medium, Electroosmosis— When an electrical current is passed through an electrolyte contained in a glass tube or contained between plates of glass or plastic, bulk flow of the electrolyte toward one of the electrodes is observed. This flow is called electroosmosis. It results from the surface charge on the walls of the apparatus, which arises either from ionizable functional groups inherent in the structural material or from ions adsorbed on the cell walls from the electrolyte contacting them. The effect is usually increased when the cell is filled with a bed of porous substance, such as a gel, used to stabilize the supporting electrolyte and prevent remixing of separated zones by thermal convection or diffusion. The solution immediately adjacent to the surface builds up an electrical charge, equal but opposite to the surface charge, and the electrical field traversing the cell produces a movement of solution toward the electrode of opposite charge.

The substances commonly used as stabilizing media in zone electrophoresis develop a negative surface charge, and therefore electroosmotic flow of the electrolyte is toward the cathode. As a result, all zones, including neutral substances, are carried toward the cathode during the electrophoretic run.

The degree of electroosmosis observed varies with the stabilizing substance. It is appreciable with agar gel, while it is negligibly small with polyacrylamide gel.

Molecular Sieving— In the absence of a stabilizing medium or in cases where the medium is very porous, electrophoretic separation of molecules results from differences in the ratio of their electrical charge to their size. In the presence of a stabilizing medium, differences in adsorptive or other affinity of molecules for the medium introduces a chromatographic effect that may enhance the separation.

If the stabilizing medium is a highly cross-linked gel such that the size of the resultant pores is of the order of the dimensions of the molecules being separated, a molecular sieving effect is obtained. This effect is analogous to that obtained in separations based on gel permeation or molecular exclusion chromatography, but in gel electrophoresis the effect is superimposed on the electrophoretic separation. Molecular sieving may be visualized to result from a steric barrier to the passage of larger molecules. Small molecules pass through pores of a wide size range, and therefore their electrophoretic passage through the gel will not be impeded. As size increases, fewer pores will permit passage of the molecules, causing a retardation of the migration of substances of large molecular weight.

#### Gel Electrophoresis

Processes employing a gel such as agar, starch, or polyacrylamide as a stabilizing medium are broadly termed gel electrophoresis. The method is particularly advantageous for protein separations. The separation obtained depends upon the electrical charge to size ratio coupled with a molecular sieving effect dependent primarily on the molecular weight.

Polyacrylamide gel has several advantages that account for its extensive use. It has minimal adsorptive properties and produces a negligible electroosmotic effect. Gels of a wide range of pore size can be reproducibly prepared by varying the total gel concentration (based on monomer plus cross-linking agent) and the percentage of cross-linking agent used to form the gel. These quantities are conveniently expressed as

$$T(\%) = \frac{a + b}{V} 100$$
$$C(\%) = \frac{b}{a + b} 100$$

where T is the total gel concentration in %; C is the percentage of cross-linking agent used to prepare the gel; V is the volume, in mL, of buffer used in preparing the gel; and a and b are the weights, in g, of monomer (acrylamide) and cross-linking agent (usually N,N'-methylenebisacrylamide) used to prepare the gel. Satisfactory gels ranging in concentration (T) from about 3% to 30% have been prepared. The amount of cross-linking agent is usually about one-tenth to one-twentieth of the quantity of monomer (C = 10% to 5%), a smaller percentage being used for higher values of T.

In the preparation of the gel, the bed of the electrophoresis apparatus is filled with an aqueous solution of monomer and cross-linking agent, usually buffered to the pH desired in the later run, and polymerized in place by a free radical process. Polymerization may be initiated by a chemical process, frequently using ammonium persulfate plus N,N,N',N'-tetramethylenediamine or photochemically using a mixture of riboflavin and N,N,N',N'-tetramethylenediamine. Polymerization is inhibited by molecular oxygen and by acidic conditions. The gel composition and polymerization conditions chosen must be adhered to rigorously to ensure reproducible qualities of the gel.

Apparatus for Gel Electrophoresis— In general, the bed or medium in which electrophoresis is carried out may be supported horizontally or vertically, depending upon the design of the apparatus. A series of separations to be compared may also be carried out in several individual tubes or by placing different samples in adjacent wells, cast or cut into a single slab of gel. A vertical slab assembly such as that depicted schematically in Figure 2

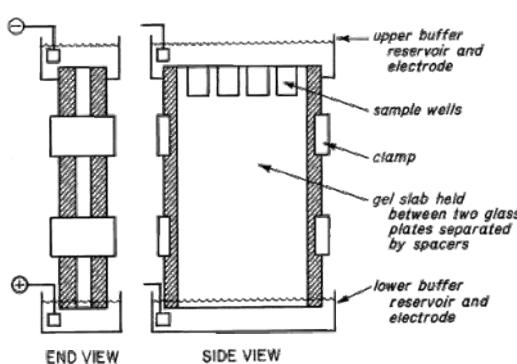


Fig. 2. Vertical Slab Gel Electrophoresis Apparatus.

is convenient for direct comparison of several samples. A particular advantage derives from the comparison of the samples in a single bed of gel which is likely to be more uniform in composition than gels cast in a series of chambers.

A feature of many types of apparatus, not illustrated in the schematic view, seals the lower buffer chamber to the base of the bed and allows the level of the buffer in the lower chamber to be made equal to that in the upper chamber, thereby eliminating hydrostatic pressure on the gel. In addition, some units provide for the circulation of coolant on one or both sides of the gel bed.

In the preparation of the gel, the base of the gel chamber is closed with a suitable device and the unit is filled with the solution of monomer, cross-linking agent, and catalyst. A comb, having teeth of an appropriate size, is inserted in the top, and polymerization is allowed to proceed to completion. Removal of the comb leaves a series of sample wells in the polymerized gel.

In simple gel electrophoresis, an identical buffer is used to fill the upper and lower buffer chambers as well as in the solution used to prepare the gel. After filling the chambers, the samples, dissolved in sucrose or other dense and somewhat viscous solution to prevent diffusion, are introduced with a syringe or micropipet into the bottoms of the sample wells, and

the electrophoresis is begun immediately thereafter.

#### disk electrophoresis

An important variant of polyacrylamide gel electrophoresis, which employs a discontinuous series of buffers and often also a discontinuous series of gel layers, is called disk electrophoresis. The name is derived from the discoid shape of the very narrow zones that result from the technique. As a result of the narrow zones produced, this technique exhibits an extremely high resolving power and is to be recommended for the characterization of protein mixtures and for the detection of contaminants that may have mobilities close to that of the major component.

The basis of disk electrophoresis is outlined in the following paragraphs with reference to an anionic system suitable for separating proteins bearing a net negative charge. To understand disk electrophoresis, it is essential to have a knowledge of the general aspects of electrophoresis and the apparatus already described.

**Basis of Disk Electrophoresis**— The high resolution obtained in disk electrophoresis depends on the use of a buffer system that is discontinuous with respect to both pH and composition. This is usually combined with a discontinuous series of two or three gels that differ in density.

A typical system is illustrated schematically in [Figure 3](#).

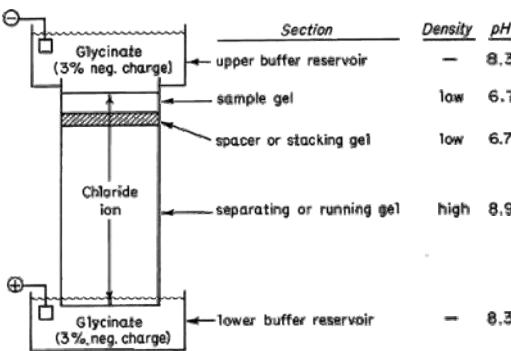


Fig. 3. Terminology, Buffer pH, and Buffer Composition for Acrylamide Gel Disk Electrophoresis.

A high density ( $T = 10\text{ to }30\%$ ) separating gel several centimeters high is polymerized in a tris-chloride buffer in the bed of the apparatus. During polymerization the buffer is overlaid with a thin layer of water to prevent fixation of a meniscus in the top of the gel. The overlayer of water is then removed and a thin layer, 3 mm to 10 mm thick, of low density ( $T = 3\%$ ) gel, called the spacer or stacking gel, is polymerized in a tris-chloride buffer on top of the separating gel. An overlayer of water is again used to ensure a flat surface.

The sample is mixed with a small amount of the spacer gel monomer solution which is applied on top of the spacer gel and allowed to polymerize. The pH of the separating gel is typically 8.9, while that of the spacer and sample gels is 6.7. All three gels are prepared using chloride as the anion.

The upper and lower buffer reservoirs are filled with a pH 8.3 buffer prepared from tris and glycine. At this pH about 3% of the glycine molecules bear a net negative charge.

When a voltage is applied across the system, the glycinate-chloride interface moves downward toward the anode. It was initially positioned at the junction of the buffer in the upper reservoir and the top of the sample gel layer. The chloride anion, by virtue of its small size, migrates faster than any of the proteins present in the sample. The pH of the sample and spacer layers was chosen to be about 3 units below the higher  $pK_a$  of glycine. Therefore, in traversing these layers, only about 0.1% of the glycine molecules bear a net negative charge. Consequently, glycine migrates more slowly than chloride. The tendency for the faster-moving chloride to move away from glycinate lowers the concentration at the interface, producing a greater voltage drop at the interface, which in turn causes the glycinate to catch up to the chloride. Under these conditions, a very sharp interface is maintained, and as it moves through the sample and spacer layers, the proteins in the sample tend to stack themselves at the interface in very thin layers in order of mobility. The process is called stacking and is the source of the disks which are separated.

When the stacked proteins reach the high-density separating gel, they are slowed down by a molecular sieving process. The higher pH encountered in the running gel also causes the glycinate to migrate faster, so that the discontinuous buffer interface overtakes the proteins and eventually reaches the bottom of the separating gel. During this period, the disks of protein continue to separate by electrophoresis and molecular sieving in the separating gel. At the end of the run, the pH of the separating gel will have risen above its original value of 8.9 to a value of about pH 9.5.

**Relative Mobility**— Bromophenol blue is often used as a standard for calculating the relative mobility of separated zones and to judge visually the progress of a run. It may be added to one of the sample wells, or mixed with the sample itself, or simply added to the buffer in the upper sample reservoir.

Relative mobility,  $M_B$ , is calculated as:

$$M_B = \frac{\text{distance from origin to sample zone}}{\text{distance from origin to bromophenol blue zone}}$$

**Visualization of Zones**— Since polyacrylamide is transparent, protein bands may be located by scanning in a densitometer with UV light. The zones may be fixed by immersing in protein precipitants such as phosphotungstic acid or 10% trichloroacetic acid. A variety of staining reagents including naphthalene black (amido black) and Coomassie brilliant blue R250 may be used. The fixed or stained zones may be conveniently viewed and photographed with transmitted light from an X-ray film illuminator.

#### safety precautions

Voltages used in electrophoresis can readily deliver a lethal shock. The hazard is increased by the use of aqueous buffer solutions and the possibility of working in damp environments.

The equipment, with the possible exception of the power supply, should be enclosed in either a grounded metal case or a case made of insulating material. The case should have an interlock that deenergizes the power supply when the case is opened, after which reactivation should be prevented until activation of a reset switch is carried out.

High-voltage cables from the power supply to the apparatus should preferably be a type in which a braided metal shield completely encloses the insulated central conductor, and the shield should be grounded. The base of the apparatus should be grounded metal or contain a grounded metal rim which is constructed in such a way that any leakage of electrolyte will produce a short which will deenergize the power supply before the electrolyte can flow beyond the protective enclosure.

If the power supply contains capacitors as part of a filter circuit, it should also contain a bleeder resistor to ensure discharge of the capacitors before the protective case is opened. A shorting bar that is activated by opening the case may be considered as an added precaution.

Because of the potential hazard associated with electrophoresis, laboratory personnel should be completely familiar with electrophoresis equipment before using it.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Kahkashan Zaidi, Ph.D.</a> Senior Scientist	(GC05) General Chapters 05

## 72 CAPILLARY ELECTROPHORESIS

Electrophoresis refers to the migration of charged electrical species when dissolved or suspended in an electrolyte through which an electric current is passed. Cations migrate toward the negatively charged electrode (cathode), while anions are attracted toward the positively charged electrode (anode). Neutral particles are not attracted toward either electrode.

The use of capillaries as a migration channel in electrophoresis has enabled analysts to perform electrophoretic separations on an instrumental level comparable to that of high-performance liquid chromatography (HPLC), albeit with some distinct operational differences, advantages, and disadvantages relative to HPLC. This method of analysis is commonly known as capillary electrophoresis (CE). During typical CE operation with an uncoated capillary filled with a buffer, referred to as the "operating buffer," silanol groups present on the inner wall of the glass capillary release hydrogen ions to the buffer and the wall surface becomes negatively charged, even at a fairly low pH. Cations, or solutes having partial positive charges in the medium, are electrostatically attracted to the negatively charged wall, forming an electrical double layer. The initiation of electrophoresis by applying voltage across the length of the capillary causes the solution portion of the electrical double layer to move toward the cathode end of the capillary, drawing the bulk solution. This movement of the bulk solution under the force of the electrical field is called the electroosmotic flow (EOF). The degree of ionization of the inner-wall capillary silanol groups depends mainly on the pH of the operating buffer and on the modifiers that may have been added to the electrolyte. At low pH, the silanol groups generally have a low ionization and the EOF is low. At higher pH, silanol groups become more ionized and the EOF increases. In some cases organic solvents, such as methanol or acetonitrile, are added to the aqueous buffer to increase the solubility of the solute and other additives or to affect the degree of ionization of the sample. The addition of such organic modifiers generally causes a decrease in the EOF. The detector is located toward the cathode end of the capillary. The EOF is usually greater than the electrophoretic mobility; thus, even anions are swept toward the cathode and the detector. When an uncoated capillary containing pH 7.0 phosphate buffer is used, the usual order of appearance of solutes in an electropherogram is cationic species, neutral solutes, and anionic species.

Currently, there are five major modes of operation of CE: capillary zone electrophoresis (CZE), also referred to as free solution or free flow capillary electrophoresis; micellar electrokinetic chromatography (MEKC); capillary gel electrophoresis (CGE); capillary isoelectric focusing (CIEF); and capillary isotachophoresis (CITP).

In CZE, separations are controlled by differences in the relative electrophoretic mobilities of the individual components in the sample or test solution. The mobility differences are functions of analyte charge and size under specific method conditions. They are optimized by appropriate control of the composition of the buffer, its pH, and its ionic strength.

In MEKC, ionic surfactants are added to the operating buffer at a concentration above their critical micelle concentration. The micelles provide a pseudostationary phase with which analytes can partition. This technique is useful for the separation of neutral and ionic species.

CGE, which is analogous to gel filtration, uses gel-filled capillaries to separate molecules on the basis of relative differences in their respective molecular weight or molecular size. It was first used for the separation of proteins, peptides, and oligomers. Gels may have the advantage of decreasing the EOF and also significantly reducing protein adsorption onto the inner wall of the capillary, which can significantly reduce analyte peak tailing effects.

In CIEF, substances are separated on the basis of their relative differences in isoelectric points. This is accomplished by achieving steady-state sample zones within a buffer pH gradient, where the pH is low at the anode and high at the cathode. The gradient is established by applying a voltage across a capillary filled with a mixture of carrier components consisting of amphoteric substances having different pI values.

CITP employs two buffers that enclose the analyte zones between them. Either anions or cations can be analyzed in sharply separated zones. In addition, the analyte concentrations are the same in each zone; thus, the length of each zone is proportional to the amount of the particular analyte.

The most commonly utilized capillary electrophoresis techniques are CZE and MEKC. These are briefly discussed in the following sections. Pertinent general principles and theory, instrumental considerations, analysis, and operational considerations and parameters are discussed as well.

### PRINCIPLES OF CAPILLARY ZONE ELECTROPHORESIS

CZE makes use of the principles of electrophoresis and electroosmosis to achieve separation of charged species.

(1) The electrophoretic mobility of an ion,  $\mu_{EP}$ , is described by the equation:

$$\mu_{EP} = q / (6\pi\eta r)$$

in which  $q$  is the charge of the ion,  $\eta$  is the solution viscosity, and  $r$  is the radius of the hydrated ion. This relationship infers that small, highly charged analytes have high mobilities and large, slightly charged analytes have low mobilities.

(2) The velocity of migration,  $V_{EP}$ , in cm per second, is represented by the equation:

$$V_{EP} = \mu_{EP}(V / L)$$

in which  $\mu_{EP}$  is the electrophoretic mobility;  $V$  is the applied voltage; and  $L$ , in cm, is the total capillary length.

(3) The velocity of the EOF,  $V_{EO}$ , in cm per second, is described by the equation:

$$V_{EO} = \mu_{EO}(V / L)$$

in which  $\mu_{EO}$  is the EOF mobility (the coefficient of electroosmotic flow), and the other terms are as defined above.

(4) The time,  $t$ , in seconds, necessary for a solute to migrate the entire effective length of the capillary (from the inlet to the detector),  $l$ , is represented by the relationship:

$$t = l / E(\mu_{EP} + \mu_{EO}) = l / V(\mu_{EP} + \mu_{EO})$$

in which  $E$  is the strength of the applied electrical field, and the other terms are as defined above.

(5) Efficiency of an electrophoretic system can be related to mobility and EOF and expressed in terms of the number of theoretical plates,  $N$ , by the equation:

$$N = (\mu_{EP} + \mu_{EO})V / 2D$$

in which  $D$  is the diffusion coefficient of the solute, and the other terms are as defined above.

(6) The resolution,  $R$ , of two consecutively eluting solutes can be defined by the equation:

$$R = 0.18(\mu_{EP1} - \mu_{EP2})[V/D(\mu_{EP} + \mu_{EO})]^{1/2}$$

where  $\mu_{EP1}$  and  $\mu_{EP2}$  are the mobilities of the two solutes,

# $\mu_{EP}$

is their average, and the other terms are as defined above.

## PRINCIPLES OF MICELLAR ELECTROKINETIC CHROMATOGRAPHY

In MEKC, the supporting electrolyte medium contains a surfactant at a concentration above its critical micelle concentration (CMC). In this aqueous medium, the surfactant self-aggregates and forms micelles whose hydrophilic head groups form an outer shell and whose hydrophobic tail groups form a nonpolar core into which the solutes can partition. Generally, the micelles are anionic on their surface, and, under the applied voltage, they migrate in the opposite direction to the EOF. This type of partitioning is analogous to that in solvent extraction or reverse-phase HPLC. The differential partitioning of neutral molecules between the buffered aqueous mobile phase and the micellar pseudostationary phase is the sole basis for separation. The buffer and micelles form a two-phase system, and the analyte can partition between these two phases.

A micellar system suitable for MEKC meets the following criteria: the surfactant is highly soluble in the buffer, and the micellar solution is homogeneous and transparent when UV detection is employed. The most common surfactant for MEKC is sodium dodecyl sulfate (anionic surfactant). Others include cetyltrimethylammonium bromide (cationic surfactant) and bile salts (chiral surfactant). The selectivity of an MEKC system is mainly dependent on the nature of the surfactant. Organic solvents are often added to the MEKC buffer to adjust the capacity factors, just as in reverse-phase HPLC separations. MEKC may be used for the separation of enantiomers. For such separations, a chiral additive is added to the buffer or a chiral surfactant, such as a bile salt, is used.

A general knowledge of conventional column chromatographic principles aids in understanding MEKC principles. However, in MEKC the micelles are not truly stationary; therefore, the column chromatographic theory needs to be modified. The major modification introduced to MEKC principles is the finite nature of the separation window for neutral molecules.

(7) The migration time,  $t_R$ , for a neutral species is expressed with the following equation:

$$t_R = (1 + k')t_0 / [1 + (t_0 / t_{MC})]$$

in which  $t_0$  is the time required for an unretained substance to travel the effective length of the capillary;  $t_{MC}$  is the time required for a micelle to traverse the capillary;  $k'$  is the capacity factor; and  $t_R$  is always between  $t_0$  and  $t_{MC}$ .

(8) The capacity factor,  $k'$ , for a neutral species, is calculated by the equation:

$$k' = (t_R / t_0 - 1) / (1 - t_R / t_{MC})$$

in which the terms are as defined above.

(9) For practical purposes,  $k'$  is calculated by the equation:

$$k' = t_R / t_0 - 1$$

in which  $t_R$  is the time measured from the point of voltage application (or injection) to the peak maximum; and  $t_0$  is measured from the point of voltage application (or injection) to the leading edge of the solvent front or of an unretained substance. In contrast with CZE,  $k'$  in MEKC is significant and is a characteristic of a given solute in a given MEKC system. Further discussion of  $k'$  appears later in the System Suitability section under Operational Parameters.

(10) The resolution,  $R_S$ , for neutral species is calculated by the equation:

$$R_S = [(\sqrt{N})/4][(\alpha - 1)/\alpha][k'_2/(1 + k'_2)][(1 - (t_0/t_{MC}))/\{1 + (t_0/t_{MC})k'_1\}]$$

in which  $\alpha$  is the selectivity, defined as the ratio of  $k'_2$  to  $k'_1$ , of the operating conditions for separating two solutes. If the two solutes elute close together ( $\alpha \leq 1.1$ ), either  $k'$  may be used. The equation shows that, just as with conventional chromatography, resolution in MEKC can be improved through controlling efficiency, selectivity, retention, and the chemical nature of the resolving surfactant-medium system. The last term of the equation is due to the limited elution range. Although MEKC is particularly useful in the separation of neutral species, this technique may also be used for the separation of charged solutes. The latter procedure involves a combination of chromatographic and electrophoretic separation mechanisms. The additional interaction between charged solutes and micelle can be used to optimize a separation. Ion-pairs may form if the charges borne on the surfactant and solute are opposite; otherwise, surfactant and solute repel each other. These differences can significantly influence the separation of charged molecules.

## INSTRUMENTAL CONSIDERATIONS

A typical CE system (see [Figure 1](#)) contains a fused-silica capillary having an internal diameter of 50 to 100  $\mu\text{m}$  and a length of 20 to 100 cm. The ends of the capillary are placed in separate electrolyte reservoirs. The direct-current power supply is capable of furnishing high voltages, typically ranging from 0 to 30 kV. A detector and autosampler with some form of data-recording device complete the system. An automatic buffer replenishment system and a computer-based control and data acquisition system may also be found on the standard commercial systems. Temperature controls for both the capillary and the autosampler are also available on commercial instruments.

The primary considerations of instrumentation include capillary type and configuration, modes of sampling, power supply and detector modes.

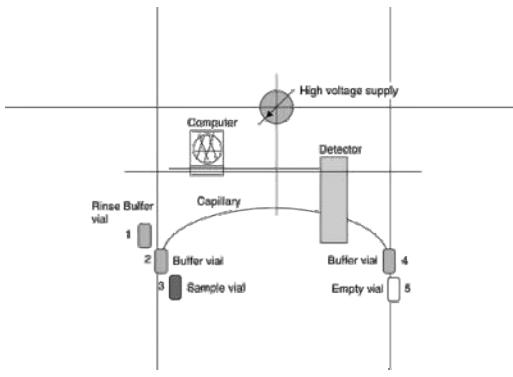


Fig. 1. Typical CE Instrument Configuration.  
Capillary Type and Configuration

Capillaries used in CZE are usually made of fused silica and with no internal coating. Some instruments are configured with a "free-swinging" style of capillary; that is, the capillary is not encased within an enclosure. In most commercial instruments, the capillary is housed in a cartridge. Both configurations offer specific advantages and disadvantages. The ability of the instrument to accommodate different types of capillaries and capillaries of various diameters and lengths is an important consideration. Capillaries with a variety of internal coatings are also available; therefore, the ability of the instrument to accommodate different capillaries is important. Internal capillary coatings may be employed to alter the magnitude or direction of EOF or to reduce sample absorption. If an internally coated capillary is to be used, then sufficient details and the indication of the supplier must be included in the method. Capillaries from an alternate supplier can be used if it is demonstrated that they are suitable.

#### Sample Introduction and Injector Technology

Modes of sample introduction onto the capillary include electromigration (electrokinetic mode) and negative- and positive-pressure injection (hydrostatic mode).

For injection via electromigration, the sample solution is electrophoresed into the capillary by inserting the capillary and electrode into the sample vials and applying a brief, high voltage. The sample enters the capillary by a combination of electrophoresis and EOF. Therefore, analytes with different mobilities are loaded into the capillary to different extents. The conductivities of the sample and standard solutes also affect the EOF and the volume injected.

Negative-pressure injectors place negative pressure at the detector end of the capillary and draw the sample solution into the injection end of the capillary. Positive-pressure injectors pressurize the sample vial, forcing the sample into the capillary. Pressure injection loads all sample components into the capillary to the same extent, and it is generally the most reproducible and the most frequently applied injection mode. The sample volume injected depends on the capillary length and internal diameter and the voltage or pressure applied. The typical sample volumes injected into the capillary are between 1 and 20 nL.

Each injection method offers specific advantages and disadvantages, depending on the sample composition, the separation mode, and the application of the method. None of the above injection modes is as reproducible as commercially available HPLC injectors. Based on the circumstances, it may be necessary to use internal standards for specific methods where high injection precision is required.

#### Power Supply

Most commercially available CE units have direct-current power supplies that are capable of furnishing power on a ramp-up or step-function mode to achieve and maintain the desired operational voltage in a smooth manner. This will help to ensure a relatively smooth baseline.

Another essential feature of the power supply is its utility in introducing a sample at the cathodic or the anodic end of the capillary. Because it is impractical to relocate the on-line detector from one end of the instrument to the other, it is beneficial to be able to specify whether the sample injection end is at the cathode or the anode.

#### Detector Modes

CE systems generally offer UV-visible absorbance and laser-induced fluorescence (LIF) detectors. Scanning UV detectors or photodiode-array detectors are also available for many commercial CE instruments.

The coupling of CE to a mass spectrometer offers the possibility of obtaining structural information in conjunction with electrophoretic migration data.

Fluorescence detection offers an enhanced sensitivity for samples containing only very small amounts of UV-active analytes. Application of fluorescent tags to non-UV-absorbing compounds can be useful. Alternately, non-UV-absorbing or nonfluorescent analytes can be detected indirectly by adding a chromophore or a fluorophore, respectively, to the buffer: the non-absorbing species are detected through the absence of expected signal from the absorbing species. Conductivity and pulsed amperometric detectors can also be used but are not generally available on commercial CE instruments.

#### ANALYTICAL CONSIDERATIONS

Several parameters, namely, capillary dimensions, voltage, ionic strength, and pH, are optimized to give adequate resolution and separation. Care should be taken to avoid changes in temperature that will affect the viscosity of the buffer and, in turn, influence both the EOF and the solute mobilities.

**Capillary Dimensions**— Variation of the capillary diameter and length can affect the electrophoretic resolution. Increasing the capillary length results in longer migration times, usually increasing resolution and generating a lower current. Increasing the capillary diameter will usually increase current and associated internal temperature gradients that decrease resolution. Conversely, a reduction in capillary diameter will result in lower heat and better resolution. However, larger capillary diameters have advantages of better mass loading and improved signal-to-noise ratio.

**Voltage Effects**— When higher voltages are applied, additional internal heating of the operating buffer occurs because of the current flow through the buffer. This heating effect, known as Joule heating, must be controlled because resistance, dielectric constant, and viscosity are temperature-dependent and alter the velocity of the EOF and solute mobilities.

In general, increasing the voltage will result in increased efficiency and resolution (up to the point where Joule heat cannot be adequately dissipated). Maximum resolution is obtained by maintaining the voltage below the level at which Joule heating and diffusion become limiting factors.

**Ionic Strength Effects**— Control of ionic strength and its manipulation allow adjustment of resolution, efficiency, and sensitivity. Increasing ionic strength will generally improve resolution, peak efficiency, and peak shape. Sensitivity may be improved because better focusing is achieved. However, because the current generated is directly proportional to the buffer concentration, more heat is produced when ionic strength of the buffer is increased, hence limiting the ionic strengths that can be utilized.

**pH Effects**— Resolution, selectivity, and peak shape can be dramatically altered by changes in pH as this parameter affects the extent of solute ionization and the level of EOF. The EOF is high at high pH and low at low pH in uncoated fused-silica capillaries.

#### OPERATIONAL PARAMETERS

The major steps in operating a CE system are system setup, capillary rinsing procedure, running a sample, system suitability testing, sample analysis, data handling, and system shutdown.



System Setup— An appropriate capillary of specific length, inner diameter, and coating is selected, with considerations made for separation and resolution, ionic strength of buffer, and pH effects. A buffer of appropriate composition, ionic strength, and pH is prepared, degassed, if necessary, and passed through an appropriate filter. All solvents, including water, are HPLC or CE grade.

Capillary Rinsing Procedure— Improved consistency of migration times and resolution may generally be obtained if a defined rinsing procedure is followed. Capillary conditioning and rinsing procedures are very specific to the analyte, matrix, and method. Therefore, these procedures are developed as part of the method and are specified in the individual monograph. Rinsing may involve the use of solutions such as 0.1 M phosphoric acid, water, and 0.1 M sodium hydroxide. Before beginning analysis of the test specimen, the capillary may be rinsed with five column volumes of the operating buffer that is to be used for the test. When changing buffer composition, it is advisable to rinse the capillary with five column volumes of each new buffer to allow the capillary to be cleansed of the previous buffer. Use of a new uncoated fused-silica capillary usually requires a regeneration procedure to activate the surface silanol groups. This procedure may include an extended rinse with a sodium hydroxide solution. Coated capillaries are rinsed according to the manufacturer's guidelines because inappropriate rinsing can remove or damage the coating. Columns may be dedicated to particular methods or buffer types to prevent cross-contamination.

Running a Sample— An appropriate capillary, electrolyte, and injection procedure are selected to achieve adequate resolution, sensitivity, and separation, with well-shaped and well-defined peaks. The required injection precision for a specific method may require use of an internal standard. The internal standard is selected with consideration of its ability to adequately separate from the analyte. The performance of the system may be improved by rinsing the capillary between injections and supplying fresh buffer to the source and destination vials used during voltage application, namely, vials 2 and 4 in [Figure 1](#). Replicate injections from the same sample vial may be performed provided that no cross-contamination occurs. If cross-contamination occurs, the capillary tip may be rinsed by briefly inserting it into a vial containing the buffer prior to inserting the capillary into the electrolyte or sample vial.

The operational parameters are specified in each individual monograph so as to minimize voltage effects, ionic strength effects, and pH effects. The instrument is set up to run with the appropriate capillary configuration and injection conditions, within the established linear dynamic range of the detector; and acceptable migration precision is ensured by appropriate choice of sample diluent, separation electrolyte, electrolyte additives, and capillary pretreatment conditions. Exercise caution to avoid overloading the capillary with sample, as this decreases efficiency and reproducibility.

System Suitability— Parameters measured may include injector reproducibility, system selectivity, system efficiency, and tailing. Resolution between the analytes and other compounds may be determined by using test mixture standards.

Parameters typically used to determine system suitability include relative standard deviation (RSD), capacity factor ( $k'$ ), the number of theoretical plates (N), sensitivity (limit of detection or quantitation), number of theoretical plates per meter (TPM), tailing factor (T), and resolution (R).

The peak shape is closely examined; ideally, the peak is symmetrical, with no shoulders and no excessive tailing. If these conditions are not met, corrective actions are taken before proceeding with the analysis. Peak integration is also closely examined to ensure that the peak response is correctly quantitated.

Replicate injections of a Standard preparation of known concentration can be used to determine the reproducibility of the CE system. Data from five or more replicate injections are used to calculate RSD. Unless otherwise specified in the individual monograph, the relative standard deviation for replicate injections is not more than 3.0%. Minimum injection precision values may be specified in specific CE methods, especially when determining trace-level components. Calculation of electrophoretic parameters in MEKC, as in other forms of CE, may involve a combination of chromatographic and electrophoretic relationships. Hence, capacity factor,  $k'$ , for neutral analyte migration in MEKC can be calculated by the equation:

$$k' = tR - t0(1 - tR / tMC)$$

in which  $tR$ ,  $t0$ , and  $tMC$  are the migration times of the analyte, the bulk solution (EOF), and the micelle, respectively.

The number of theoretical plates, N, is a measure of the efficiency of the system and is calculated by the equation:

$$N = 16(tR / W)^2 \text{ or } N = 5.54(tR / W1/2)^2$$

in which W is the analyte peak width at baseline,  $W1/2$  is the analyte peak width at half-height, and  $tR$  is the analyte migration time.

The number of theoretical plates per meter, TPM, is a measure of the efficiency of the capillary as a function of peak width at baseline and can be calculated by the equation:

$$TPM = 1600(tR / W)^2 / L$$

in which L, in cm, is the total capillary length; and the other terms are as defined above. The tailing factor, T, of the analyte peak is a measure of peak symmetry, and it represents the degree of deviation of the symmetry of the peak from an ideally symmetrical Gaussian peak. This factor can be calculated by the equation:

$$T = W0.05 / 2f$$

in which  $W0.05$  is the length of a line constructed parallel to the peak base from the leading edge to the tailing edge of the peak at 5% of peak height; and  $f$  is the distance along the same line from the leading edge of the peak, appearing to the left of the peak maximum in the electropherogram, to the intercept of a perpendicular line dropped from the peak maximum to the base. A ratio of 1.0 indicates a perfectly symmetrical peak. If electrodispersive effects occur, they can generate highly asymmetrical peaks. This can occur when high sample concentrations are used, such as those for testing of impurities. Use of highly asymmetrical peaks is acceptable provided that they are reproducible and that they do not compromise separation selectivity.

The resolution factor, R, is a measure of the ability of the capillary system to separate consecutively migrating analytes. Resolution is determined for all sample analytes of interest, with the pH of the buffer adjusted as necessary to meet system suitability requirements. It can be calculated by the equation:

$$R = 2(t2 - t1) / (W1 + W2)$$

in which  $t2$  and  $t1$  are the migration times, measured at peak maxima, for the slower migrating peak and the faster migrating peak, respectively; and  $W2$  and  $W1$  are the corresponding widths of these two peaks measured at their bases.

Sample Analysis— Once the suitability of the CE system has been established, aliquots of both the Standard preparation and the test preparation are injected. Standards are injected before or after the samples and intermittently throughout the run.

Data Handling— Time-normalized peak areas are often used in quantitative calculations. These are determined by dividing the observed integrated peak area by the migration time of the analyte. This compensates for the fact that in CE, unlike HPLC, each analyte travels through the detector at a different velocity. Unless this normalization is performed, slowly moving (later-migrating) analytes will have disproportionately large peak areas compared with those for early migrating components.

System Shutdown— After analysis, the capillary is rinsed according to the directions specified in each monograph or as recommended by the manufacturer. For example, the capillary might be rinsed with distilled water to remove buffer components and then filled with air or nitrogen by performing a rinse from an empty vial. Naturally, the destination and source vials, namely, vials 4 and 2 in [Figure 1](#), are emptied of buffer and rinsed thoroughly with deionized water.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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## INTRODUCTION

Lipid injectable emulsions used in total parenteral nutrition (TPN) therapy are sterile oil-in-water emulsions of soybean oil, used to provide an ample supply of essential fatty acids, linoleic and linolenic, dispersed with the aid of an emulsifying agent in [Water for Injection](#). Alternatively, soybean oil can be mixed with other suitable oils (neutral triglycerides), such as safflower oil, medium-chain triglycerides (MCT) derived from coconut or palm kernel oils, olive oil, or a marine oil, such as menhaden oil. The size of the lipid droplets is critical: because of mechanical filtration, larger-size fat globules ( $>5 \mu\text{m}$ ) can be trapped in the lungs. The essential size characteristics of a lipid injectable emulsion for intravenous use includes the mean diameter of the lipid droplets and the range of the various droplet diameters distributed around the mean diameter, expressed as the standard deviation. In particular, the amounts of fat globules comprising the large-diameter tail of the globule size distribution are especially important with respect to infusion safety. These two regions of the globule size distribution (mean droplet size and large-diameter tail) must be controlled within specified limits.

The two methods described below are used for determination of the mean lipid droplet diameter and the distribution of large-diameter globule sizes in lipid injectable emulsions. Method I and Method II must be validated. The methods described below to assess the quality of lipid injectable emulsions are to be performed in two stages.

### METHOD I—LIGHT-SCATTERING METHOD

For the determination of the mean droplet size of lipid injectable emulsions, either of two common light-scattering techniques may be employed: (1) dynamic light scattering (DLS), also known as photon correlation spectroscopy (PCS), or (2) classical light scattering, based on Mie scattering theory. The DLS, or PCS, technique is based on analyzing the rapid temporal fluctuations in the scattered light intensity that occur due to the random Brownian motion, or diffusion, of any particles, including lipid droplets, suspended in liquid. The intensity is measured at a given angle (usually  $90^\circ$ ) by a suitable detector (e.g., photomultiplier tube) able to measure the rapidly fluctuating scattered light intensity produced by the suspended, diffusing droplets. These scattered intensity data are typically used to calculate the intensity autocorrelation function, which is a simple decaying exponential function in time for droplets of uniform size. A distribution of droplet sizes expresses itself by exponential functions of different decay times. The autocorrelation function generated by the scattered intensity data obtained from a given emulsion can be “inverted” by means of an appropriate deconvolution algorithm in order to obtain the approximate distribution of intensity-weighted diffusion coefficients. From the latter, the distribution of small-diameter droplets is calculated, using the Stokes-Einstein equation and the rules of classical (Mie) light scattering.

By contrast, classical light scattering based on Mie theory analyzes the spatial, rather than temporal, variation of the scattered light intensity by measuring the latter as a function of the scattering angle, typically over a large range of detected angles. The temporal fluctuations in the scattering intensity due to Brownian motion are averaged out in time for each angular measurement. This angular variation occurs as a consequence of the mutual interference of individual scattered waves arriving at the detector with different phases from different points within a given lipid droplet, as well as from different particles. The extent of the angular variation is significant whenever the droplet diameter is not small compared with the wavelength of the laser light (typically 635 nm). Droplets of a given size and refractive index yield a unique curve of scattering intensity vs. angle. A distribution of droplet sizes gives rise to a final angular dependence that represents the superposition, or summation, of individual (different) intensity vs. angle curves. The measured angular dependence of the scattering intensity obtained from a given emulsion sample can be inverted by means of an appropriate deconvolution algorithm and Mie scattering theory in order to obtain the approximate droplet size distribution.

Thus, light scattering, using either dynamic light scattering (i.e., temporal fluctuations due to droplet diffusion) or classical light scattering/Mie theory (i.e., average intensity vs. angle), can provide acceptable results for both the mean diameter and standard deviation of the droplet size distribution. For purposes of illustrating the method used in Method I, a dynamic light-scattering technique is described. For guidance regarding instruments employing classical Mie-theory light scattering, see [Light Diffraction Measurement of Particle Size](#) (429).

**Apparatus**—A suitable DLS/PCS instrument with or without the capability of automatic sample dilution is controlled by validated software and is used to perform the measurement, with the scattering angle typically set at  $90^\circ$ . The intensity-weighted results (mean diameter and standard deviation) are reported, provided it is clearly stated which values are given and that the necessary parameter values required for all requisite calculations are also given.

**Water**—Pass distilled water through a filter having a  $0.2\text{-}\mu\text{m}$  porosity, and degas by sonication, or use [Sterile Water for Injection](#) stored in a glass container.

**Standard Preparation**—To a pre-established volume of Water add an appropriate amount of concentrated suspension, containing NIST-traceable polystyrene latex standard particles or other suitable nanospheres. Gently mix the fluids to achieve a homogeneous suspension. The diluted suspension will be slightly turbid in appearance. If the DLS/PCS instrument is equipped with an automatic dilution system, the starting concentrated sample can be analyzed by injection directly into the instrument via a syringe, with further dilution occurring automatically to optimize the droplet concentration for analysis. Alternatively, the sample would require greater manual dilution with Water (typically by at least a factor of 10 over the first dilution), and then this sample would be instilled into a “drop-in” cuvette. The optimum dilution scheme that achieves the proper scattering intensity for the cuvette-based analysis will be determined by the instrument specifications. Thus, the concentration of latex in the final sample must be optimized for the DLS/PCS instrument used. This should be performed separately for three different size standards of approximately 100, 250, and 400 nm (triplicate analyses per size), and the corresponding results of intensity-weighted mean diameter and standard deviation should coincide with the expected values within acceptable errors.

**Test Preparation**—To a pre-established volume of Water add an appropriate volume of sample from the lipid injectable emulsion. Gently mix the fluids to achieve a homogeneous suspension. The diluted suspension will be slightly turbid in appearance. Gently mix the fluids. If the DLS/PCS instrument is equipped with an automatic dilution system, the starting concentrated sample can be analyzed by injection directly into the instrument via a syringe. Further dilution of the sample then occurs automatically to optimize the droplet concentration for analysis, ensuring that it is not so high as to cause artifacts due to multiple scattering or interdroplet interactions. Alternatively, the sample would require greater manual dilution with Water (typically by at least a factor of 10 over the first dilution), and then this sample would be instilled into a “drop-in” cuvette. The optimum dilution scheme that achieves the proper scattering intensity for the cuvette-based analysis will be determined by the instrument specifications. Thus, the concentration of lipid injectable emulsion in the final sample must be optimized for the DLS/PCS instrument used.

**System Suitability**—Using the Standard Preparation, measure the intensity-weighted mean particle diameter and the corresponding standard deviation. The system is suitable once the sample temperature has reached equilibration and the results have stabilized and triplicate mean droplet diameter measurements are obtained. The coefficient of variation (CV) should not exceed 10% of the NIST-traceable mean droplet diameter. A larger CV value indicates that the latex microspheres are not suitable as a standard because they either inherently lack uniformity or have become aggregated to an unacceptable extent. In this case, another standard latex suspension must be selected and tested.

**Procedure and Interpretation**—If the DLS/PCS instrument is equipped with an automatic dilution system, use a disposable syringe to load the Standard Preparation or Test Preparation. If no automatic dilution system is used, transfer the appropriately diluted preparation to a cuvette and place the cuvette in the spectrometer. Allow the sample to equilibrate to a preset controlled temperature close to ambient (between  $20^\circ$  and  $25^\circ$ , as in the USP definition found in the General Notices under Preservation, Packaging, Storage, and Labeling). Set the instrument scattering angle to  $90^\circ$ , and carry out the measurements. As long as the chi-square ( $\chi^2$ ) goodness-of-fit parameter remains acceptably low (per instrument specifications), the results for the Test Preparation are acceptable. Excessive values of the  $\chi^2$  parameter suggest that the droplet distribution is not normal and may indicate an unstable emulsion. The intensity-weighted mean droplet diameter (MDD) for lipid injectable emulsions must be less than 500 nm or  $0.5\text{ }\mu\text{m}$ , irrespective of the concentration of the dispersed lipid phase.

### METHOD II—MEASUREMENT OF LARGE GLOBULE CONTENT BY LIGHT OBSCURATION OR EXTINCTION METHOD

For determination of the extent of the large-diameter droplet tail ( $>5\text{ }\mu\text{m}$ ) of lipid injectable emulsions, a light obscuration (LO) or light extinction (LE) method that employs a single-particle (globule) optical sizing (SPOS) technique is used. During application of the LE/SPOS technique, passage of a droplet through a thin optical sensing zone results in blockage of a portion of the incident light beam, causing a momentary decrease in the light intensity reaching the “extinction” detector. The magnitude of this decrease in the signal is ideally proportional to the cross-sectional area of the droplet (assumed smaller than the sensing zone thickness), i.e., to the square of the droplet diameter. During optimization of the LE/SPOS instrument for a given emulsion sample, a series of dilutions should be tested to achieve an acceptably low coefficient of variation (i.e.,  $<10\%$ ) between samples. The goal is to identify a standard range of dilutions that yield consistent data and are most applicable to the formulation tested. Ideally, when comparing different emulsions, the same approximate number of globules are sized each time, and once a standard is achieved, it should be incorporated into the routine sampling plan for validation testing. As long as the fat globule concentration is below the “coincidence limit” of the sensor (determined by the flow cell and optical design), only one globule at most will pass through the sensing zone at any given time, allowing it to be counted and accurately sized (with less than 1% coincidence events). Both the coincidence limit and the optimal flow rate must be known for the LE/SPOS sensor



used. Furthermore, it is prudent to perform the large-diameter measurements at a reduced emulsion concentration such that the measurable droplet concentration (i.e., >1.8  $\mu$ m) is only approximately one-third of the nominal coincidence limit for the sensor used. The resulting single pulse heights are converted to droplet diameters using a standard calibration curve previously constructed from NIST-traceable monosized polystyrene microspheres of known diameters. For additional guidance in the use of the light obscuration methodology, see the general chapter [Particulate Matter in Injections](#) (788).

**Apparatus**— A suitable light obscuration instrument with or without the capability of automatic sample dilution and controlled by a personal computer (PC) is used for the measurement. The number- and volume-weighted particle size distribution data are reported, provided it is clearly stated which values are given and that the necessary parameter values required for all necessary calculations are also given.

Water— Pass distilled water through a filter having a 0.2- $\mu$ m porosity, and degas by sonication, or use [Sterile Water for Injection](#) stored in a glass container.

**Standard Preparation**— To a pre-established volume of Water add an appropriate amount of concentrated suspension, containing NIST-traceable polystyrene latex standard particles or other suitable microspheres. Gently mix the fluids to achieve a homogeneous suspension. If the light obscuration instrument is equipped with an automatic dilution system, the starting concentrated sample can be analyzed by injection directly into the instrument via a syringe or Teflon sample line. Further dilution of the sample then occurs automatically to optimize the particle concentration for analysis. Alternatively, the sample would require greater manual dilution with water (typically by at least a factor of 10 over the first dilution). The resulting diluted sample is then instilled in an appropriate, clean container, such as a sterile Type I glass container, before being passed through the sensor. In either case the final particle concentration is caused to lie below the coincidence limit of the sensor. The sizing and counting accuracy of the light obscuration instrument should be obtained using three different size standards of approximately 5  $\mu$ m, 10  $\mu$ m, and 25  $\mu$ m (triplicate analyses per size). The corresponding results for the mean diameter should coincide with the expected values, within a 10% acceptable error. In addition, the number of particle counts obtained per unit volume of diluted sample suspension should also agree, within a 10% acceptable error, with the concentration values certified in the documentation provided with each NIST-traceable size standard.

**Test Preparation**— To a pre-established volume of Water add an appropriate volume of sample from the lipid injectable emulsion. Gently mix the fluids to achieve a homogeneous suspension. The diluted emulsion will be slightly turbid in appearance. If the light obscuration instrument is equipped with an automatic dilution system, the starting concentrated sample can be analyzed by injection directly into the instrument via a syringe or nonreactive Teflon sample line. Further dilution then occurs automatically to optimize the droplet/globule concentration for analysis. Alternatively, the sample would require greater manual dilution with water (typically by at least a factor of 10 over the first dilution). The resulting diluted sample is then instilled in an appropriate, clean container such as a sterile Type I glass container. In either case the final droplet/globule concentration is caused to lie below the coincidence limit of the sensor.

**System Suitability**— Using the Standard Preparation, measure the number-weighted particle diameter and the corresponding standard deviation. The system is suitable once the sample has equilibrated and the results have stabilized and triplicate mean number-weighted particle diameter measurements are obtained within 10% of each other. The measured coefficient of variation (CV) for the number-weighted particle size distribution should not deviate by more than 25% from the CV value stipulated for the NIST-traceable standard. The latter value is usually very small, assuming nearly uniform-size standard particles. Therefore, in practice the measured CV value is usually considerably larger than this ideal value, being dictated instead by the resolution of the LE/SPOS sensor. The resolution of the sensor should be sufficiently good that the measured CV value does not exceed 15% of the mean diameter of the NIST-traceable standard. A larger CV value indicates that the latex microspheres are not suitable as a standard because they either inherently lack uniformity or have become aggregated to an unacceptable extent. In this case, another standard latex suspension must be selected and tested.

**Procedure and Interpretation**— If the light obscuration instrument is equipped with an automatic dilution system, use a disposable syringe or Teflon sample line to load the Standard Preparation or Test Preparation. If no automatic dilution system is used, transfer the sample to an appropriate large-volume, clean container such as a sterile Type I glass vessel containing an appropriate volume of Water. Allow the sample and Water to mix thoroughly to achieve a homogeneous suspension. Set the instrument threshold of detection at 1.8  $\mu$ m, extended to an upper limit of 50  $\mu$ m, and employ measurement times of 120, 180, and 240 seconds for each run of each replicate of the sample (n = 3 runs per sample). As long as the three measurements of the volume-weighted percentage of fat greater than 5  $\mu$ m (PFAT5) for each sample fall within 10% of each other (irrespective of run time), the results for the Test Preparation are acceptable. Values exceeding this reproducibility tolerance suggest either that the sample is unstable or that the dilution has not been optimized. The volume-weighted, large-diameter fat globule limits of the dispersed phase, expressed as the percentage of fat residing in globules larger than 5  $\mu$ m (PFAT5) for a given lipid injectable emulsion, must be less than 0.05%.

\* Polyvinyl chloride (PVC) with diethylhexylphthalate (DEHP) has been shown to induce breakdown of lipid injectable emulsions (Drug Product Problem Reporting System. USP File Access No. 11173, May 15, 1991).

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Desmond G. Hunt, Ph.D.</a> Scientist 1-301-816-8341	(PPI05) Parenteral Products-Industrial 05

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### 730 PLASMA SPECTROCHEMISTRY

Plasma-based instrumental techniques that are useful for pharmaceutical analyses fall into two major categories: those based on the inductively coupled plasma, and those where a plasma is generated at or near the surface of the sample. An inductively coupled plasma (ICP) is a high-temperature excitation source that desolvates, vaporizes, and atomizes aerosol samples and ionizes the resulting atoms. The excited analyte ions and atoms can then subsequently be detected by observing their emission lines, a method termed inductively coupled plasma-atomic emission spectroscopy (ICP-AES), also known as inductively coupled plasma-optical emission spectroscopy (ICP-OES); or the excited or ground state ions can be determined by a technique known as inductively coupled plasma-mass spectrometry (ICP-MS). ICP-AES and ICP-MS may be used for either single- or multi-element analysis, and they provide good general-purpose procedures for either sequential or simultaneous analyses over an extended linear range with good sensitivity.

An emerging technique in plasma spectrochemistry is laser-induced breakdown spectroscopy (LIBS). In LIBS, a solid, liquid, or gaseous sample is heated directly by a pulsed laser, or indirectly by a plasma generated by the laser. As a result, the sample is volatilized at the laser beam contact point, and the volatilized constituents are reduced to atoms, molecular fragments, and larger clusters in the plasma that forms at or just above the surface of the sample. Emission from the atoms and ions in the sample is collected, typically using fiber optics or a remote viewing system, and measured using an array detector such as a charge-coupled device (CCD). LIBS can be used for qualitative analysis or against a working standard curve for quantitative analysis. Although LIBS is not currently in wide use by the pharmaceutical industry, it might be suited for at-line or on-line measurements in a production setting as well as in the laboratory. Because of its potential, it should be considered a viable technique for plasma spectrochemistry in the pharmaceutical laboratory. However,

because LIBS is still an emerging technique, details will not be further discussed in this general chapter.<sup>1</sup>

### SAMPLE PREPARATION

Sample preparation is critical to the success of plasma-based analysis and is the first step in performing any analysis via ICP-AES or ICP-MS. Plasma-based techniques are heavily dependent on sample transport into the plasma, and because ICP-AES and ICP-MS share the same sample introduction system, the means by which samples are prepared may be applicable to either technique. The most conventional means by which samples are introduced into the plasma is via solution nebulization. If solution nebulization is employed, solid samples must be dissolved in order to be presented into the plasma for analysis. Samples may be dissolved in any appropriate solvent. There is a strong preference for the use of aqueous or dilute nitric acid solutions, because there are minimal interferences with these solvents compared to other solvent choices. Hydrogen peroxide, hydrochloric acid, sulfuric acid, perchloric acid, combinations of acids, or various concentrations of acids can all be used to dissolve the sample for analysis. Dilute hydrofluoric acid may also be used, but great care must be taken to ensure the safety of the analyst, as well as to protect the quartz sample introduction equipment when using this acid; specifically, the nebulizer, spray chamber, and inner torch tube should be manufactured from hydrofluoric acid-tolerant materials. Additionally, alternative means of dissolving the sample can be employed. These include, but are not limited to, the use of dilute bases, straight or diluted organic solvents, combinations of acids or bases, and combinations of organic solvents.



When samples are introduced into the plasma via solution nebulization, it is important to consider the potential matrix effects and interferences that might arise from the solvent. The use of an appropriate internal standard and/or matching the standard matrix with samples should be applied for ICP-AES and ICP-MS analyses in cases where accuracy and precision are not adequate. In either event, the selection of an appropriate internal standard should consider the analyte in question, ionization energy, wavelengths or masses, and the nature of the sample matrix.

Where a sample is found not to be soluble in any acceptable solvent, a variety of digestion techniques can be employed. These include hot-plate digestion and microwave-assisted digestions, including open-vessel and closed-vessel approaches. The decision regarding the type of digestion technique to use depends on the nature of the sample being digested, as well as on the analytes of interest.

Open-vessel digestion is generally not recommended for the analysis of volatile metals, e.g., selenium and mercury. The suitability of a digestion technique, whether open-vessel or closed-vessel, should be supported by spike recovery experiments in order to verify that, within an acceptable tolerance, volatile metals have not been lost during sample preparation.

Use acids, bases, and hydrogen peroxide of ultra-high purity, especially when ICP-MS is employed. Deionized water must be at least 18 megaohm. Check diluents for interferences before they are used in an analysis. Because it is not always possible to obtain organic solvents that are free of metals, use organic solvents of the highest quality possible with regard to metal contaminants.

It is important to consider the selection of the type, material of construction, pretreatment, and cleaning of analytical labware used in ICP-AES and ICP-MS analyses. The material must be inert and, depending on the specific application, resistant to caustics, acids, and/or organic solvents. For some analyses, diligence must be exercised to prevent the adsorption of analytes onto the surface of a vessel, particularly in ultra-trace analyses. Contamination of the sample solutions from metal and ions present in the container can also lead to inaccurate results.

The use of labware that is not certified to meet Class A tolerances for volumetric flasks is acceptable if the linearity, accuracy, and precision of the method have been experimentally demonstrated to be suitable for the purpose at hand.

#### SAMPLE INTRODUCTION

There are two ways to introduce the sample into the nebulizer: by a peristaltic pump and by self-aspiration. The peristaltic pump is preferred and serves to ensure that the flow rate of sample and standard solution to the nebulizer is the same irrespective of sample viscosity. In some cases, where a peristaltic pump is not required, self-aspiration can be used.

A wide variety of nebulizer types is available, including pneumatic (concentric and cross-flow), grid, and ultrasonic nebulizers. Micronebulizers, high-efficiency nebulizers, direct-injection high-efficiency nebulizers, and flow-injection nebulizers are also available. The selection of the nebulizer for a given analysis should consider the sample matrix, analyte, and desired sensitivity. Some nebulizers are better suited for use with viscous solutions or those containing a high concentration of dissolved solids, whereas others are better suited for use with organic solutions.

Note that the self-aspiration of a fluid is due to the Bernoulli, or Venturi, effect. Not all types of nebulizers will support self-aspiration. The use of a concentric nebulizer, for example, is required for self-aspiration of a solution.

Once a sample leaves the nebulizer as an aerosol, it enters the spray chamber, which is designed to permit only the smallest droplets of sample solution into the plasma; as a result, typically only 1% to 2% of the sample aerosol reaches the ICP, although some special-purpose nebulizers have been designed that permit virtually all of the sample aerosol to enter the ICP. As with nebulizers, there is more than one type of spray chamber available for use with ICP-AES or ICP-MS. Examples include the Scott double-pass spray chamber, as well as cyclonic spray chambers of various configurations. The spray chamber must be compatible with the sample and solvent and must equilibrate and wash out in as short a time as possible. When a spray chamber is selected, the nature of the sample matrix, the nebulizer, the desired sensitivity, and the analyte should all be considered.

Gas and liquid chromatography systems can be interfaced with ICP-AES and ICP-MS for molecular speciation, ionic speciation, or other modes of separation chemistry, based on elemental emission or mass spectrometry.

Ultimately, the selection of sample introduction hardware should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision of the analysis at hand.

In addition to solution nebulization, it is possible to analyze solid samples directly via laser ablation (LA). In such instances, the sample enters the torch as a solid aerosol. LA-ICP-AES and LA-ICP-MS are better suited for qualitative analyses of pharmaceutical compounds because of the difficulty in obtaining appropriate standards. Nonetheless, quantitative analyses can be performed if it can be demonstrated through appropriate method validation that the available standards are adequate.<sup>2</sup>

#### STANDARD PREPARATION

Single- or multi-element standard solutions, whose concentrations are traceable to primary reference standards, such as those of the National Institute of Standards and Technology (NIST), can be purchased for use in the preparation of working standard solutions. Alternatively, standard solutions of elements can be accurately prepared from standard materials and their concentrations, determined independently, as appropriate. Working standard solutions, especially those used for ultra-trace analyses, may have limited shelf life. As a general rule, working standard solutions should be retained for no more than 24 hours unless stability is demonstrated experimentally. The selection of the standard matrix is of fundamental importance in the preparation of element standard solutions. Spike recovery experiments should be conducted with specific sample matrices in order to determine the accuracy of the method. If sample matrix effects cause excessive inaccuracies, standards, blanks, and sample solutions should be matrix matched, if possible, in order to minimize matrix interferences.

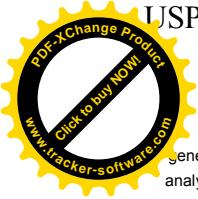
In cases where matrix matching is not possible, an appropriate internal standard or the method of standard additions should be used for ICP-AES or ICP-MS. Internal standards can also be introduced through a T connector into the sample uptake tubing. In any event, the selection of an appropriate internal standard should consider the analytes in question, their ionization and excitation energies, their chemical behavior, their wavelengths or masses, and the nature of the sample matrix. Ultimately, the selection of an internal standard should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision of the analysis at hand.

The method of standard additions involves adding a known concentration of the analyte element to the sample at no fewer than two concentration levels plus an unspiked sample preparation. The instrument response is plotted against the concentration of the added analyte element, and a linear regression line is drawn through the data points. The absolute value of the x-intercept multiplied by any dilution factor is the concentration of the analyte in the sample.

The presence of dissolved carbon at concentrations of a small percentage in aqueous solutions enhances ionization of selenium and arsenic in an inductively coupled argon plasma. This phenomenon frequently results in a positive bias for ICP-AES and ICP-MS selenium and arsenic quantification measurements, which can be remedied by using the method of standard additions or by adding a small percentage of carbon, such as analytically pure glacial acetic acid, to the linearity standards.

#### ICP

The components that make up the ICP excitation source include the argon gas supply, torch, radio frequency (RF) induction coil, impedance-matching unit, and RF generator. Argon gas is almost universally used in an ICP. The plasma torch consists of three concentric tubes designated as the inner, the intermediate, and the outer tube. The intermediate and outer tubes are almost universally made of quartz. The inner tube can be made of quartz or alumina if the analysis is conducted with solutions containing hydrofluoric acid. The nebulizer gas flow carries the aerosol of the sample solution into and through the inner tube of the torch and into the plasma. The intermediate tube carries the intermediate (sometimes referred to as the auxiliary) gas. The intermediate gas flow helps to lift the plasma off the inner and intermediate tubes to prevent their melting and the deposition of carbon and salts on the inner tube. The outer tube carries the outer (sometimes referred to as the plasma or coolant) gas, which is used to form and sustain the toroidal plasma. The tangential flow of the coolant gas through the torch constricts the plasma and prevents the ICP from expanding to fill the outer tube, keeping the torch from melting. An RF induction coil, also called the load coil, surrounds the torch and produces an oscillating magnetic field, which in turn sets up an oscillating current in the ions and electrons produced from the argon. The impedance-matching unit serves to efficiently couple the RF energy from the generator to the load coil. The unit can be of either the active or the passive type. An active matching unit adjusts the impedance of the RF power by means of a capacitive network, whereas the passive type adjusts the impedance directly through the generator circuitry. Within the load coil of the RF



generator, the energy transfer between the coil and the argon creates a self-sustaining plasma. Collisions of the ions and electrons liberated from the argon ionize and excite the analyte atoms in the high-temperature plasma. The plasma operates at temperatures of 6,000 to 10,000 K, so most covalent bonds and analyte-to-analyte interactions have been eliminated.

#### ICP-AES

An inductively coupled plasma can use either an optical or a mass spectral detection system. In the former case, ICP-AES, analyte detection is achieved at an emission wavelength of the analyte in question. Because of differences in technology, a wide variety of ICP-AES systems are available, each with different capabilities, as well as different advantages and disadvantages. Simultaneous-detection systems are capable of analyzing multiple elements at the same time, thereby shortening analysis time and improving background detection and correction. Sequential systems move from one wavelength to the next to perform analyses, and often provide a larger number of analytical lines from which to choose. Array detectors, including charge-coupled devices and charge-injection devices, with detectors on a chip, make it possible to combine the advantages of both simultaneous and sequential systems. These types of detection devices are used in the most powerful spectrometers, providing rapid analysis and a wide selection of analytical lines.

The ICP can be viewed in either axial or radial (also called lateral) mode. The torch is usually positioned horizontally in axially viewed plasmas and is viewed end on, whereas it is positioned vertically in radially viewed plasmas and is viewed from the side. Axial viewing of the plasma can provide higher signal-to-noise ratios (better detection limits and precision); however, it also incurs greater matrix and spectral interferences. Methods validated on an instrument with a radial configuration will probably not be completely transferable to an instrument with an axial configuration, and vice versa.

Additionally, dual-view instrument systems are available, making it possible for the analyst to take advantage of either torch configuration. The selection of the optimal torch configuration will depend on the sample matrix, analyte in question, analytical wavelength(s) used, cost of instrumentation, required sensitivity, and type of instrumentation available in a given laboratory.

Regardless of torch configuration or detector technology, ICP-AES is a technique that provides a qualitative and/or quantitative measurement of the optical emission from excited atoms or ions at specific wavelengths. These measurements are then used to determine the analyte concentration in a given sample. Upon excitation, an atom or atomic ion emits an array of different frequencies of light that are characteristic of the distinct energy transition allowed for that element. The intensity of the light is generally proportional to the analyte concentration. It is necessary to correct for the background emission from the plasma. Sample concentration measurements are usually determined from a working curve of known standards over the concentration range of interest. It is, however, also possible to perform a single-point calibration under certain circumstances, such as with limit tests, if the methodology has been validated for sufficient specificity, sensitivity, linearity, accuracy, precision, ruggedness, and robustness.

Because there are distinct transitions between atomic energy levels, and because the atoms in an ICP are rather dilute, emission lines have narrow bandwidths. However, because the emission spectra from the ICP contain many lines, and because "wings" of these lines overlap to produce a nearly continuous background on top of the continuum that arises from the recombination of argon ions with electrons, a high-resolution spectrometer is required in ICP-AES. The decision regarding which spectral line to measure should include an evaluation of potential spectral interferences. All atoms in a sample are excited simultaneously; however, the presence of multiple elements in some samples can lead to spectral overlap.

Spectral interference can also be caused by background emission from the sample or plasma. Modern ICPs usually have background correction available, and a number of background correction techniques can be applied. Simple background correction typically involves measuring the background emission intensity at some point away from the main peak and subtracting this value from the total signal being measured. Mathematical modeling to subtract the interfering signal as a background correction can also be performed with certain types of ICP-AES spectrometers.

The selection of the analytical spectral line is critical to the success of an ICP-AES analysis, regardless of torch configuration or detector type. Though some wavelengths are preferred, the final choice must be made in the context of the sample matrix, the type of instrument being used, and the sensitivity required. Analysts might choose to start with the wavelengths recommended by the manufacturer of their particular instrument and select alternative wavelengths based on manufacturer recommendations or published wavelength tables.<sup>3,4,5,6,7</sup> Ultimately, the selection of analytical wavelengths should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision of the analysis at hand.

Forward power, gas flow rates, viewing height, and torch position can all be optimized to provide the best signal. However, it must also be kept in mind that these same variables can influence matrix and spectral interferences.

In general, it is desirable to operate the ICP under robust conditions, which can be gauged on the basis of the MgII / MgI line pair at (280.270 nm / 285.213 nm). If that ratio of intensities is above 6.0 in an aqueous solution, the ICP is said to be robust, and is less susceptible to matrix interferences. A ratio of about 10.0 is generally what is sought. Note that the term robust conditions is unrelated to robustness as applied to analytical method validation. Operation of an instrument with an MgII / MgI ratio greater than 6.0 is not mandated, but is being suggested as a means of optimizing instrument parameters in many circumstances.

The analysis of the Group I elements can be an exception to this strategy. When atomic ions are formed from elements in this group, they assume a noble gas electron configuration, with correspondingly high excitation energy. Because the first excited state of these ions is extremely high, few are excited, so emission intensity is correspondingly low. This situation can be improved by reducing the fractional ionization, which can in turn be achieved by using lower forward power settings in combination with adjusted viewing height or nebulizer gas flow, or by adding an ionization suppression agent to the samples and standards.

When organic solvents are used, it is often necessary to use a higher forward power setting, higher intermediate and outer gas flows, and a lower nebulizer gas flow than would be employed for aqueous solutions, as well as a reduction in the nebulizer gas flow. When using organic solvents, it may also be necessary to bleed small amounts of oxygen into the torch to prevent carbon buildup in the torch.

#### Calibration

The wavelength accuracy for ICP-AES detection must comply with the manufacturer's applicable operating procedures. Because of the inherent differences among the types of instruments available, there is no general system suitability procedure that can be employed. Calibration routines recommended by the instrument manufacturer for a given ICP-AES instrument should be followed. These might include, but are not limited to, use of a multi-element wavelength calibration with a reference solution, internal mercury (Hg) wavelength calibration, and peak search. The analyst should perform system checks in accordance with the manufacturer's recommendations.

#### Standardization

The instrument must be standardized for quantification at time of use. However, because ICP-AES is a technique generally considered to be linear over a range of 6 to 8 orders of magnitude, it is not always necessary to continually demonstrate linearity by the use of a standard curve composed of multiple standards. Once a method has been developed and is in routine use, it is possible to calibrate with a blank and a single standard. One-point standardizations are suitable for conducting limit tests on production materials and final products if the methodology has been rigorously validated for sufficient specificity, sensitivity, linearity, accuracy, precision, ruggedness, and robustness. The use of a single-point standardization is also acceptable for qualitative ICP-AES analyses, where the purpose of the experiment is to confirm the presence or absence of elements without the requirement of an accurate quantification.

An appropriate blank solution and standards that bracket the expected range of the sample concentrations should be assayed and the detector response plotted as a function of analyte concentration, as in the case where the concentration of a known component is being determined within a specified tolerance. However, it is not always possible to employ a bracketing standard when an analysis is performed at or near the detection limit. This lack of use of a bracketing standard is acceptable for analyses conducted to demonstrate the absence or removal of elements below a specified limit. The number and concentrations of standard solutions used should be based on the purpose of the quantification, the analyte in question, the desired sensitivity, and the sample matrix. Regression analysis of the standard plot should be employed to evaluate the linearity of detector response, and individual monographs may set criteria for the residual error of the regression line. Optimally, a correlation coefficient of not less than 0.99, or as indicated in the individual monograph, should be demonstrated for the working curve. Here, too, however, the nature of the sample matrix, the analyte(s), the desired sensitivity, and the type of instrumentation available may dictate a correlation coefficient lower than 0.99. The analyst should use caution when proceeding with such an analysis, and should employ additional working standards.

To demonstrate the stability of the system's initial standardization, a solution used in the initial standard curve must be reassayed as a check standard at appropriate intervals throughout the analysis of the sample set. The reassayed standard should agree with its expected value to within  $\pm 10\%$ , or as specified in an individual monograph, for single-element analyses when analytical wavelengths are between 200 and 500 nm, or concentrations are  $>1 \mu\text{g per mL}$ . The reassayed standard should agree with its theoretical value to within



–20%, or as specified in an individual monograph, for multi-element analyses, when analytical wavelengths are <200 nm or >500 nm, or at concentrations of <1 µg per mL. In cases where an individual monograph provides different guidance regarding the reassayed check standard, the requirements of the monograph take precedence.

#### Procedure

Follow the procedure as directed in the individual monograph for the instrumental parameters. Because of differences in manufacturers' equipment configurations, the manufacturer's suggested default conditions may be used and modified as needed. The specification of definitive parameters in a monograph does not preclude the use of other suitable operating conditions, and adjustments of operating conditions may be necessary. Alternative conditions must be supported by suitable validation data, and the conditions in the monograph will take precedence for official purposes. Data collected from a single sample introduction are treated as a single result. This result might be the average of data collected from replicate sequential readings from a single solution introduction of the appropriate standard or sample solution. Sample concentrations are calculated versus the working curve generated by plotting the detector response versus the concentration of the analyte in the standard solutions. This calculation is often performed directly by the instrument.

#### ICP-MS

When an inductively coupled plasma uses a mass spectral detection system, the technique is referred to as inductively coupled plasma–mass spectrometry (ICP–MS). In this technique, analytes are detected directly at their atomic masses. Because these masses must be charged to be detected in ICP–MS, the method relies on the ability of the plasma source to both atomize and ionize sample constituents. As is the case with ICP–AES, a wide variety of ICP–MS instrumentation systems are available.

The systems most commonly in use are quadrupole-based systems. Gaining in interest is time-of-flight ICP–MS. Although still not in widespread use, this approach may see greater use in the future. Additionally, high-resolution sector field instruments are available.

Regardless of instrument design or configuration, ICP–MS provides both a qualitative and a quantitative measurement of the components of the sample. Ions are generated from the analyte atoms by the plasma. The analyte ions are then extracted from the atmospheric-pressure plasma through a sampling cone into a lower-pressure zone, ordinarily held at a pressure near 1 Torr. In this extraction process, the sampled plasma gases, including the analyte species, form a supersonic beam, which dictates many of the properties of the resulting analyte ions. A skimmer cone, located behind the sampling cone, "skims" the supersonic beam of ions as they emerge from the sampling cone. Behind the skimmer cone is a lower-pressure zone, often held near a milliTorr. Lastly, the skinned ions pass a third-stage orifice to enter a zone held near a microTorr, where they encounter ion optics and are passed into the mass spectrometer. The mass spectrometer separates the ions according to their mass-to-charge (m/z) ratios. The ICP–MS has a mass range up to 240 atomic mass units (amu). Depending on the equipment configuration, analyte adducts can form with diluents, with argon, or with their decomposition products. Also formed are oxides and multiply-charged analyte ions, which can increase the complexity of the resulting mass spectra. Interferences can be minimized by appropriate optimization of operational parameters, including gas flows (central, intermediate, and outer gas flow rates), sample-solution flow, RF power, extraction-lens voltage, etc., or by the use of collision or reaction cells, or cool plasma operation, if available on a given instrument. Unless a laboratory is generating or examining isotopes that do not naturally occur, a list of naturally occurring isotopes will provide the analyst with acceptable isotopes for analytical purposes. Isotopic patterns also serve as an aid to element identification and confirmation. Additionally, tables of commonly found interferences and polyatomic isobaric interferences and correction factors can be used.

ICP–MS generally offers considerably lower (better) detection limits than ICP–AES, largely because of the extremely low background that it generates. This ability is a major advantage of ICP–MS for determination of very low analyte concentrations or when elimination of matrix interferences is required. In the latter case, some interferences can be avoided simply by additional dilution of the sample solution. In some applications, analytes can be detected below the parts per trillion (ppt) level using ICP–MS. As a general rule, ICP–MS as a technique requires that samples contain significantly less total dissolved solids than does ICP–AES.

The selection of the analytical mass to use is critical to the success of an ICP–MS analysis, regardless of instrument design. Though some masses are often considered to be the primary ones, because of their high natural abundance, an alternative mass for a given element is often used to avoid spectral overlaps (isobaric interferences). Selection of an analytical mass must always be considered in the context of the sample matrix, the type of instrument being used, and the concentrations to be measured. Analysts might choose to start with masses recommended by the manufacturer of their particular instrument and select alternate masses based on manufacturer's recommendations or published tables of naturally occurring isotopes.<sup>2</sup>

Optimization of an ICP–MS method is also highly dependent on the plasma parameters and means of sample introduction. Forward power, gas flow rates, and torch position may all be optimized to provide the best signal. When organic solvents are used, it is often necessary to use a higher forward power setting and a lower nebulizer flow rate than would be used for aqueous solutions. Additionally, when organic solvents are used, it might be necessary to introduce small amounts of oxygen into the central or intermediate gas to prevent carbon buildup in the torch or on the sampler cone orifice. The use of a platinum-tipped sampling or skimmer cone may also be required in order to reduce cone degradation with some organic solvents.

#### Calibration

The mass spectral accuracy for ICP–MS detection must be in accordance with the applicable operating procedures. Because of the inherent differences between the types of instruments available, there is no general system suitability procedure that can be employed. Analysts should refer to the tests recommended by the instrument manufacturer for a given ICP–MS instrument. These may include, but are not limited to, tuning on a reference mass or masses, peak search, and mass calibration. The analyst should perform system checks recommended by the instrument manufacturer.

#### Standardization

The instrument must be standardized for quantification at the time of use. Because the response (signal vs. concentration) of ICP–MS is generally considered to be linear over a range of 6 to 8 orders of magnitude, it is not always necessary to continually demonstrate linearity by the use of a working curve. Once a method has been developed and is in routine use, it is common practice to calibrate with a blank and a single standard. One-point standardizations are suitable for conducting limit tests on production materials and final products, provided that the methodology has been rigorously validated for sufficient specificity, sensitivity, linearity, accuracy, precision, ruggedness, and robustness. An appropriate blank solution and standards that bracket the expected range of the sample concentrations should be assayed and the detector response plotted as a function of analyte concentration. The number and concentration of standard solutions used should be based on the analyte in question, the expected concentrations, and the sample matrix, and should be left to the discretion of the analyst. Optimally, a correlation coefficient of not less than 0.99, or as indicated in the individual monograph, should be demonstrated for the working standard curve. Here, too, however, the nature of the sample matrix, the analyte, the desired sensitivity, and the type of instrumentation available might dictate a correlation coefficient lower than 0.99.

The analyst should use caution when proceeding with such an analysis and should employ additional working standards.

To demonstrate the stability of the system since initial standardization, a solution used in the initial standard curve must be reassayed as a check standard at appropriate intervals throughout the analysis of the sample set. Appropriate intervals may be established as occurring after every fifth or tenth sample, or as deemed adequate by the analyst, on the basis of the analysis being performed. The reassayed standard should agree with its expected value to within  $\pm 10\%$  for single-element analyses when analytical masses are free of interferences and when concentrations are >1 ng per mL. The reassayed standard should agree with its expected value to within  $\pm 20\%$  for multi-element analyses, or when concentrations are <1 ng per mL. In cases where an individual monograph provides different guidance regarding the reassayed check standard, the requirements of the monograph take precedence.

The method of standard additions should be employed in situations where matrix interferences are expected or suspected. This method involves adding a known concentration of the analyte element to the sample solution at no fewer than two concentration levels. The instrument response is plotted against the concentration of the added analyte element, and a linear regression line is drawn through the data points. The absolute value of the x-intercept multiplied by any dilution factor is the concentration of the analyte in the sample.

#### Procedure

Follow the procedure as directed in the individual monograph for the detection mode and instrument parameters. The specification of definitive parameters in a monograph does not preclude the use of other suitable operating conditions, and adjustments of operating conditions may be necessary. Alternative conditions must be supported by suitable validation data, and the conditions in the monograph will take precedence for official purposes. Because of differences in manufacturers' equipment configurations, the analyst may wish to begin with the manufacturer's suggested default conditions and modify them as needed. Data collected from a single sample introduction are treated as a single result. Data collected from replicate sequential readings from a single introduction of the appropriate standard or sample solutions are averaged as a single result. Sample concentrations are calculated versus the working curve generated by plotting the detector response versus the concentration of the analyte in the standard solutions. With modern instruments, this calculation is often



performed by the instrument.

## GLOSSARY

AUXILIARY GAS: See Intermediate (or Auxiliary) Gas.

AXIAL VIEWING: A configuration of the plasma for AES in which the plasma is directed toward the spectrometer optical path, also called "end-on viewing."

CENTRAL (OR NEBULIZER) GAS: One of three argon gas flows in an ICP torch. The central gas is used to help create a fine mist of the sample solution when solution nebulization is employed. This fine mist is then directed through the central tube of the torch and into the plasma.

COLLISION CELL: A design feature of some ICP-MS instruments. Collision cells are used to reduce interferences from argon species or polyatomic ions and facilitate the analysis of elements that might be affected by those interferences.

COOL PLASMA: Plasma conditions used for ICP-MS that result in a plasma that is cooler than that normally used for an analysis. This condition is achieved by using a lower forward power setting and higher central-gas flow rate, and is used to help reduce isotopic interferences caused by argon and some polyatomic ions.

COOLANT GAS: See Outer (or Coolant or Plasma) Gas.

FORWARD POWER: The number of watts used to ignite and sustain the plasma during an analysis. Forward power requirements may vary, depending on sample matrix and analyte.

INTERMEDIATE (OR AUXILIARY) GAS: Gas used to "lift" the plasma off the surface of the torch, thereby preventing melting of the intermediate tube and the formation of carbon and salt deposits on the inner tube.

INTERNAL STANDARD: An element added to or present in the same concentration in blanks, standards, and samples to act as an intensity reference for the analysis. An internal standard should be used for ICP-AES work and must always be used for quantitative ICP-MS analyses.

LATERAL VIEWING: See Radial Viewing.

m: The ion mass of interest.

MULTIPLY-CHARGED IONS: Atoms that, when subjected to the high-ionization temperature of the ICP, can form doubly or triply charged ions ( $X^{++}$ ,  $X^{+++}$ , etc.). When detected by MS, the apparent mass of these ions will be  $\frac{1}{2}$  or  $\frac{1}{3}$  that of the atomic mass.

NEBULIZER: Used to form a consistent sample aerosol that mixes with the argon gas, which is subsequently sent into the ICP.

OUTER (OR COOLANT OR PLASMA) GAS: The main gas supply for the plasma.

PLASMA GAS: See Outer (or Coolant or Plasma) Gas.

RADIAL VIEWING: A configuration of the plasma for AES in which the plasma is viewed orthogonal to the spectrometer optic path. Also called "side-on viewing." See also Lateral Viewing.

REACTION CELL: Similar to Collision Cell, but operating on a different principle. Designed to reduce or eliminate spectral interferences.

SAMPLING CONE: A metal cone (usually nickel-, aluminum-, or platinum-tipped) with a small opening, through which ionized sample material flows after leaving the plasma.

SEQUENTIAL: A type of detector configuration for AES or MS in which discrete emission lines or isotopic peaks are observed by scanning or hopping across the spectral range by means of a monochromator or scanning mass spectrometer.

SIMULTANEOUS: A type of detector configuration for AES or MS in which all selected emission lines or isotopic peaks are observed at the same time by using a polychromator or simultaneous mass spectrometer, offering increased analysis speed for analyses of multi-element samples.

SKIMMER CONE: A metal cone through which ionized sample flows after leaving the sampling cone and before entering the high-vacuum region of an ICP-MS.

STANDARD ADDITIONS: A method used to determine the actual analyte concentration in a sample when viscosity or matrix effects might cause erroneous results.

TORCH: A series of three concentric tubes, usually manufactured from quartz, in which the ICP is formed.

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Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Kahkashan Zaidi, Ph.D.</a> Senior Scientist 1-301-816-8269	(GC05) General Chapters 05

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### 731 LOSS ON DRYING

The procedure set forth in this chapter determines the amount of volatile matter of any kind that is driven off under the conditions specified. For substances appearing to contain water as the only volatile constituent, the procedure given in the chapter, [Water Determination](#) (921), is appropriate, and is specified in the individual monograph.

Mix and accurately weigh the substance to be tested, and, unless otherwise directed in the individual monograph, conduct the determination on 1 to 2 g. If the test specimen is in the form of large crystals, reduce the particle size to about 2 mm by quickly crushing. Tare a glass-stoppered, shallow weighing bottle that has been dried for 30 minutes under the same conditions to be employed in the determination. Put the test specimen in the bottle, replace the cover, and accurately weigh the bottle and the contents. By gentle, sidewise shaking, distribute the test specimen as evenly as practicable to a depth of about 5 mm generally, and not more than 10 mm in the case of bulky materials. Place the loaded bottle in the drying chamber, removing the stopper and leaving it also in the chamber. Dry the test specimen at the temperature and for the time specified in the monograph. [note—The temperature specified in the monograph is to be regarded as being within the range of  $\pm 2^{\circ}$  of the stated figure.] Upon opening the chamber, close the bottle promptly, and allow it to come to room temperature in a desiccator before weighing.

If the substance melts at a lower temperature than that specified for the determination of Loss on drying, maintain the bottle with its contents for 1 to 2 hours at a temperature  $5^{\circ}$  to  $10^{\circ}$

below the melting temperature, then dry at the specified temperature.

Where the specimen under test is Capsules, use a portion of the mixed contents of not fewer than 4 capsules.

Where the specimen under test is Tablets, use powder from not fewer than 4 tablets ground to a fine powder.

Where the individual monograph directs that loss on drying be determined by thermogravimetric analysis, a sensitive electrobalance is to be used.

Where drying in vacuum over a desiccant is directed in the individual monograph, a vacuum desiccator or a vacuum drying pistol, or other suitable vacuum drying apparatus, is to be used.

Where drying in a desiccator is specified, exercise particular care to ensure that the desiccant is kept fully effective by frequent replacement.

Where drying in a capillary-stoppered bottle\* in vacuum is directed in the individual monograph, use a bottle or tube fitted with a stopper having a  $225 \pm 25 \mu\text{m}$  diameter capillary, and maintain the heating chamber at a pressure of 5 mm or less of mercury. At the end of the heating period, admit dry air to the heating chamber, remove the bottle, and with the capillary stopper still in place allow it to cool in a desiccator before weighing.

\* Available as an "antibiotic moisture content flask" from Kontes, 1022 Spruce St., Vineland, NJ 08360-2841.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Horacio N. Pappa, Ph.D.</a> Senior Scientist and Latin American Liaison 1-301-816-8319	(GC05) General Chapters 05

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### 733 LOSS ON IGNITION

This procedure is provided for the purpose of determining the percentage of test material that is volatilized and driven off under the conditions specified. The procedure, as generally applied, is nondestructive to the substance under test; however, the substance may be converted to another form such as an anhydride.

Perform the test on finely powdered material, and break up lumps, if necessary, with the aid of a mortar and pestle before weighing the specimen. Weigh the specimen to be tested without further treatment, unless a preliminary drying at a lower temperature, or other special pretreatment, is specified in the individual monograph. Unless other equipment is designated in the individual monograph, conduct the ignition in a suitable muffle furnace or oven that is capable of maintaining a temperature within  $25^{\circ}\text{C}$  of that required for the test, and use a suitable crucible, complete with cover, previously ignited for 1 hour at the temperature specified for the test, cooled in a desiccator, and accurately weighed.

Unless otherwise directed in the individual monograph, transfer to the tared crucible an accurately weighed quantity, in g, of the substance to be tested, about equal to that calculated by the formula:

$$10 / L$$

in which L is the limit (or the mean value of the limits) for Loss on ignition, in percentage. Ignite the loaded uncovered crucible, and cover at the temperature ( $\pm 25^{\circ}\text{C}$ ) and for the period of time designated in the individual monograph. Ignite for successive 1-hour periods where ignition to constant weight is indicated. Upon completion of each ignition, cover the crucible, and allow it to cool in a desiccator to room temperature before weighing.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Horacio N. Pappa, Ph.D.</a> Senior Scientist and Latin American Liaison 1-301-816-8319	(GC05) General Chapters 05

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### 736 MASS SPECTROMETRY

A mass spectrometer produces ions from the substance under investigation, separates them according to their mass-to-charge ratio ( $m/z$ ), and records the relative abundance of each ionic species present. The instrument consists of three major components (see [Figure 1](#)):

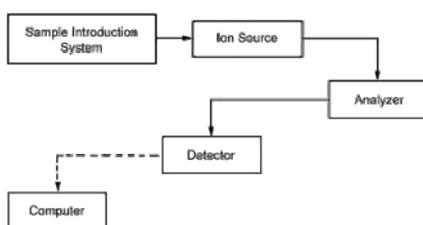


Fig. 1. Major components of a mass spectrometer.

an ion source for producing gaseous ions from the substance being studied, an analyzer for resolving the ions into their characteristic mass components according to their mass-to-charge ratios, and a detector system for detecting the ions and recording the relative abundance of each of the resolved ionic species. In addition, a sample introduction system is necessary to admit the samples to be studied to the ion source while maintaining the high vacuum requirements ( $\sim 10^{-6}$  to  $10^{-8}$  mm of mercury) of the technique; and a computer is required to control the instrument, acquire and manipulate data, and compare spectra to reference libraries.

This chapter gives an overview of the theory, construction, and use of mass spectrometers. The discussion is limited to those instruments and measurements with actual or potential application to compendial and other pharmaceutical requirements: generally, the identification and quantitation of specific compounds.

#### SAMPLE INTRODUCTION

Samples are introduced either as a gas to be ionized in the ion source, or by ejection of charged molecular species from a solid surface or solution. In some cases sample introduction and ionization take place in a single process, making a distinction between them somewhat artificial.

Substances that are gases or liquids at room temperature and atmospheric pressure can be admitted to the source as a neutral beam via a controllable leak system. Volatilizable



Compounds dissolved or adsorbed in solids or liquids can be removed and concentrated with a headspace analyzer. Vapors are flushed from the solid or liquid matrix with a stream of carrier gas and trapped on an adsorbing column. The trapped vapors are subsequently desorbed by programmed heating of the trap and introduced into the mass spectrometer by a capillary connection.

For volatilizable solids, the most frequently used method of sample introduction is the direct insertion probe. Here, the sample is placed in a small crucible at the tip of the probe, which is heated under high vacuum in close proximity to the ion source. A variation of this technique involves desorption of samples inside the ionization chamber from a rapidly heated wire or with the aid of a laser beam. Such desorption techniques, in combination with electron, chemical, or field ionization, are preferred for the analysis of heat sensitive or poorly volatile samples.

Sample introduction techniques that involve the ejection of charged molecules from the surface of solid samples include the field desorption method and various sputtering techniques, where the samples are bombarded by high energy photons, by a primary ion beam, or by a neutral particle beam. Similarly, ions can be ejected from solutions either by bombardment with a primary beam, or by one of the various spray techniques described below.

Gas and liquid chromatographs are widely used as sample inlet devices for mass spectrometers. These chromatographs provide for an initial sample purification, since only that portion of the chromatographic effluent containing the compound of interest need be admitted to the mass spectrometer. Gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS) combinations are valuable tools for the identification of unknown impurities in drugs. These combination methods have the capacity to separate complex mixtures with the opportunity to obtain structural information on the individual components.

#### Gas Chromatography/Mass Spectrometry

Gas chromatographic effluents are already in the vapor state and can be admitted directly into the mass spectrometer. Bridging the several orders of magnitude difference in the operating pressures of the two systems was initially accomplished with the use of various carrier gas separators. However, with the advent of capillary gas chromatographic columns and high capacity vacuum pumps for mass spectrometers, the gas chromatographic effluents are now fed directly into the ion source.

#### Liquid Chromatography/Mass Spectrometry

This technique is particularly useful for analyzing materials that cannot be analyzed by GC/MS, either because of thermal instability, high polarity, or high molecular weight. Compounds of biological interest such as drugs and their metabolites, polar endogenous substances, and macromolecules—including peptides, proteins, nucleic acids, and oligosaccharides—often fall into one of these categories.

Currently available LC/MS interfaces encompass a number of approaches to separating the compound of interest from the liquid chromatographic mobile phase and transforming it into an ionized species suitable for mass spectrometry. These include transport devices such as the particle beam; various spray techniques including thermospray, electrospray, and ionspray; and particle-induced desorption such as continuous-flow fast atom bombardment (CF-FAB).

#### particle beam interface

The solvent is removed from an aerosol of the liquid chromatographic effluent, and the resulting neutral analyte molecules are introduced into the ion source of the mass spectrometer where they are ionized by electron ionization (EI) or chemical ionization (CI). The resulting spectra are thus classical EI or CI spectra, the former with a wealth of structural information. There are limitations with respect to polarity, thermal lability, and molecular weight, so this technique is best suited for small organic molecules with molecular weights of less than 1000 daltons.

#### thermospray

The compound of interest in a volatile buffer mobile phase, such as ammonium acetate, is passed through heated, narrow bore tubing directly into the ion source of a mass spectrometer. The solution is vaporized in the tubing, and analyte ions desorb into the gas phase and pass into the mass analyzer. Neutral analyte molecules in the gas phase may undergo chemical ionization by reaction with gas phase buffer ions such as NH4+. Thermospray is compatible with relatively high flow rates of 1 to 2 mL per minute, solvents containing a high percentage of water, and many types of polar analytes. Thermal degradation may occur, since the analytes are exposed to relatively high temperatures during the volatilization process.

#### electrospray

The mobile phase is sprayed through a small opening (needle tip) held at a potential of several kilovolts. The charged droplets so produced are desolvated by passing through a drying gas, and the resulting ions are injected directly into the high vacuum of the analyzer through an orifice or glass capillary. Classical electrospray is limited to flow rates of 1 to 5  $\mu$ L per minute, and is therefore compatible with either microbore HPLC or post-column stream splitting techniques.

The ions may carry multiple charges, so that the  $m/z$  value of high molecular weight substances will fall into the usable range for most quadrupole or magnetic sector mass analyzers ( $m/z < 4000$ ). Analytes of up to 150,000 daltons can thus be successfully analyzed.

#### ionspray

A variant of electrospray in which nebulization with a gas flow is used to assist the formation of microdroplets of mobile phase. The technique can extend the upper limit of usable flow rates to 0.1 mL per minute. Volatile buffers must be used with both techniques.

#### desorption techniques

Microflow liquid chromatography can also be interfaced to particle induced desorption techniques such as fast atom bombardment (FAB) and liquid secondary ion mass spectroscopy (LSIMS), described in the following section on ionization techniques. Typically, column effluent flowing at a rate of 1 to 10  $\mu$ L per minute is mixed with a small percentage of nonvolatile liquid such as glycerol. The mixture is introduced via a capillary inlet onto a target within the ion source where it is bombarded with high energy (5 to 20 keV) atoms or ions. The resulting spectra are similar to FAB or LSIMS spectra but with the background from the sample matrix greatly reduced. Frit-FAB is a variant of continuous flow FAB where the sample is introduced through a porous frit target.

### IONIZATION TECHNIQUES

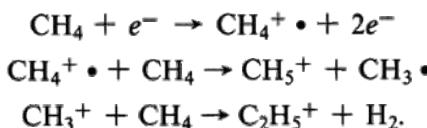
#### Electron Impact

Molecules of the sample under analysis enter the ionization chamber in the vapor state. Positive ions are produced by passing a beam of electrons, obtained from tungsten or rhenium filaments, through the vapor, which is maintained at a pressure of  $10^{-4}$  to  $10^{-6}$  mm of mercury. Provided the energy of the electron beam is greater than the ionization potential of the sample, the sample is ionized and/or fragmented, as represented by the following equation:



#### Chemical Ionization (CI)

In this process, a reagent gas at a pressure between 0.1 to 10 mm of mercury is admitted to the source and ionized by a high energy electron beam or discharge. At these pressures, ion-molecule reactions occur and the primary reagent gas ions react further. The most commonly used reagent gases are methane, isobutane, and ammonia. Typical reactions for methane are shown in the following equations:



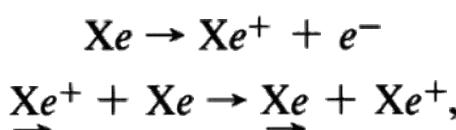
The  $\text{CH}_5^+$  species is a strong Bronsted acid and readily transfers a proton to most organic compounds



In the case of methane, the protonated ion ( $MH^+$ ) initially formed may be sufficiently energetic to dissociate further.

#### Fast Atom Bombardment (FAB)

The sample is ionized by bombardment with a beam of high speed xenon atoms, produced by exchange with highly accelerated xenon ions in a collision cell. The process is summarized as follows:



where the subscript arrows indicate the fast-moving particles.

FAB is a surface analysis technique, and care must be taken during sample preparation to optimize the condition of the surface. When the sample is coated on a probe by evaporation of a solution, the sample ion beam obtained is often transitory. Molecular adducts with alkali metals, such as ( $M + Na$ ) and ( $M + K$ ), favor ion formation. This phenomenon is used to assist in the ionization of biological molecules. Thus, treatment of the sample surface with sodium chloride solution may enhance the yield of adduct ions. Heating the sample during analysis may also increase the ion yield.

The declining yield of sample ions during analysis is probably due to destruction of the sample surface. The surface can, in effect, be continuously replaced by dissolving the sample in a suitable nonvolatile liquid and by coating the mixture onto the top of the probe. Using this approach, the lifetime of samples in the source has been extended to more than 1 hour and the range of compounds amenable to FAB analysis expanded dramatically. The long sample lifetimes and higher sensitivities so achieved make FAB an important mass spectral technique for biochemical analysis, providing the elemental formula of the sample through accurate mass determination. A further advantage of FAB, unlike CI, is the presence of fragment ions within the spectra, which aid in structural elucidation.

Recently, neutral atom bombardment has been replaced by cesium ion bombardment. Although this technique is still referred to as FAB, it is more correctly described as liquid secondary ion mass spectrometry (LSIMS).

Negative and positive ions are formed in the various ionization processes described above, and both are readily analyzed by modern mass spectrometers. Samples with a high electron capture cross section, notably those containing halide atoms, yield an abundance of negative ions. For this reason, halide derivatives of compounds to be studied are often prepared. Negative ion mass spectrometry has been successfully applied to the analysis of pesticide residues, since the structures of these compounds are well suited to the technique.

#### ANALYZERS

Mass analyzers separate the charged species in the ionized sample according to their  $m/z$  ratios, thus permitting the mass and abundance of each species to be determined. Four commonly used analyzers are the magnetic sector, the quadrupole, the time-of-flight, and the Fourier transform analyzers.

##### Magnetic Sector Analyzers

Ions generated in the ion source are collimated into a beam through the focusing action of a magnetic field and a slit assembly. After exiting the source, ions are subjected to a magnetic field perpendicular to the direction of the beam. Each ion experiences a force at right angles to both its direction of travel and the direction of the magnetic field, thereby deflecting the beam. The motion of each ion is given by

$$m/z = H^2 r^2 / 2V$$

where  $m$  is the mass in atomic mass units,  $z$  is the number of electronic charges,  $H$  is the magnetic field strength in gauss,  $r$  is the radius of the ion trajectory in centimeters, and  $V$  is the accelerating voltage. The mass spectrum is scanned by varying the strength of the magnetic field and detecting those ions passing through an exit slit as they come into "focus."

The magnetic sector mass spectrometer affords spatial resolution of ions, giving a unique trajectory at a given field strength for each value of  $m/z$ .

##### Quadrupole Analyzers

The instrument is based on four parallel rods in a square array. The ion beam is focused down the axis of the array and an electrical potential of fixed (DC) and radio frequency (RF) components is applied to diagonally opposed rods. For a given combination of DC and RF components, ions of one specific  $m/z$  ratio have a stable path down the axis. All others are deflected to the sides and lost. Mass scanning is achieved by changing the DC and RF components of the voltage, while maintaining a fixed ratio. The quadrupole analyzer is a mass filter because it separates ions on the basis of their  $m/z$  ratio.

##### Ion-trap Analyzer

This quadrupole-type device is composed of a ring electrode placed between two end cap electrodes. Depending upon the commercial version employed, the end caps are either held at ground potential or have an RF voltage applied to them, while an RF voltage is placed on the ring electrode. As a result of these potentials, the hyperbolic surfaces of the three elements form a three-dimensional quadrupole analyzer.

Both ionization and mass analysis take place within the three-dimensional quadrupole field. In the ionization step, the RF voltage on the ring electrode is set low enough so that the ions within the mass range of interest are trapped within the device. Following ionization, mass analysis is accomplished through use of the "mass selective instability" mode of operation. That is, by raising the RF voltage on the ring electrode, ions of successively higher mass are ejected from the ion trap into an electron multiplier detector. In its most common application, the ion-trap analyzer is used in conjunction with a gas chromatograph and covers the mass range of 10 to 560 daltons. However, recent advances in ion-trap technology have extended the workable mass range to many thousands of daltons.

##### Time-of-flight Analyzers

In separation is based on the principle that ions of different masses, possessing equal kinetic energy, have different velocities. If there is a fixed distance for the ions to travel, the time of travel will vary with their mass, the lighter ions traveling faster and reaching the detector in a shorter period of time. The time-of-flight is given by

$$t_f = k \sqrt{m/z}$$

where  $t_f$  is the time-of-flight in seconds. Thus, the time-of-flight of the various ions is simply proportional to the square root of the mass-to-charge ratio of the ions. To measure the time-of-flight, ions are introduced into the mass spectrometer in discrete packets so that a starting point for the timing process can be established. Ion packets are generated either through a pulsed ionization process or through a gating system in which ions are produced continuously, but are introduced only at given times into the flight tube.

#### Fourier Transform Analyzers

In a magnetic field of flux density  $B$ , ions move in circular orbits. The angular frequency,  $\omega$ , of the orbital motion is given by

$$\omega = (z/m)B$$

In this type of mass spectrometer, the orbits are varied by subjecting the ions to a resonant alternating electric field. When the frequency of the alternating field matches the orbital frequency, the ions are steadily accelerated to larger and larger orbits in coherent motion, developing a high level of kinetic energy. After the alternating electric field is turned off, the orbiting ions give rise to an alternating image current on the electrodes. A frequency analysis of this signal yields the mass of the ions involved. Thus, the Fourier transform of the time domain transient signal yields the corresponding frequency spectrum from which the mass spectrum is computed. This is a high resolution technique, yielding  $m/z$  ratios accurate to about one thousandth of a dalton.

#### TANDEM MASS SPECTROMETRY

Two mass spectrometers connected in series (MS/MS), tandem mass spectrometry, refers to the use of two or more sequential mass analysis steps. In its simplest form MS/MS (Figure 2)

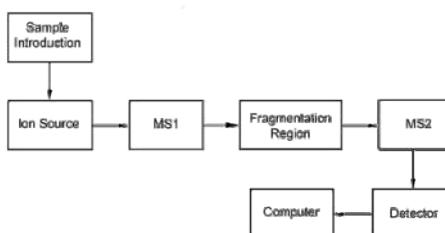


Fig. 2. Tandem Mass Spectrometry.

consists of two mass spectrometers linked in such a way that ions preselected by the first mass analyzer (MS1) are chemically or energetically modified and the results analyzed by the second mass analyzer (MS2).

The basic concept of MS/MS involves the ability to determine the mass relationship between a precursor ion in MS1 and a product ion in MS2. Different mass relationships can be probed depending on how MS1 and MS2 are scanned. These include fragmentation of a precursor and measurement of all its fragments (a product scan), selection of multiple precursors and testing for a common fragment (a precursor scan), or scanning to see if a number of precursors all lose the same neutral species (a constant neutral loss scan).

Fragmentation of the precursor ion can be induced by momentum transfer through collision with gas molecules and/or solid surfaces or by electronic excitation using lasers. These techniques are known as collision-induced dissociation, surface-induced dissociation, or laser-induced dissociation, respectively. Allowing the ion to fragment without additional activation is known as metastable decomposition.

There are many applications of MS/MS to pharmaceutical problems. Product scans can be used to obtain qualitative information from precursor ions of drug substances, impurities, and contaminants. This can aid in the identification of unknowns. The method can also be used to determine the amino acid sequence of peptides and protein fragments.

MS/MS has advantages for mixture analysis. Even when the mass spectrometer is coupled to a separation device such as a liquid or gas chromatograph, the resulting signals may be a result of overlapping or unresolved components. MS/MS can be employed to select the precursor ion from one component and obtain structural information without interference from the others.

Selected reaction monitoring is used to reduce the interference encountered during quantitative analysis for low levels of drugs in biological matrices, as in pharmacokinetic studies. If analysis is for a drug specific ion, interfering signals from other compounds in the matrix can mask the desired signal. Interference is reduced if a drug-specific fragment is selected with MS1 and a structure-specific fragment with MS2. The odds of another molecule producing the same mass relationship are diminishingly small.

MS/MS can also be used in metabolism studies to search for molecules with common structural features such as metabolites related to the drug substance. All of the metabolites might contain the same functional group that is lost as a neutral fragment. In this case a constant-neutral-loss scan will show all of these species. For instance, carboxylic acids will all lose neutral carbon dioxide. If the common functionality is lost as an ionic fragment, then a precursor scan will show all of the molecules that produce that fragment ion.

#### DATA ANALYSIS AND INTERPRETATION

The mass spectral experiment yields information on the molecular weight of ions derived from the sample and the relative abundance of each of these ions. Spectra are often complex, and not all of the ions may be separated by the mass spectrometer. The ability of the instrument to separate ions is called the resolving power, commonly described by the "10% valley" definition. This states that the resolving power is the highest mass number at which two peaks differing by one molecular weight unit and of equal height have a valley between them that is equal to 10% of the peak height. For low, medium, and high resolution mass spectrometers, this value is between 100 and 2000, 2000 and 10,000, and greater than 10,000, respectively.

If one electron is removed or added to a neutral molecule, a molecular ion of essentially the same molecular weight as the parent molecule results. It is often possible to determine the



mass of this ion with sufficient precision to enable the empirical formula of the compound to be calculated. Molecular masses may be determined accurately by using high resolution instruments or by peak-matching measurements using reference compounds.

Fragment ions are those produced from the molecular ion by various bond cleavage processes. Numerous papers in the literature relate bond cleavage patterns (fragmentation patterns) to molecular structure.

In addition to measurement of the mass of a molecular ion and its associated fragment ions, mass spectrometers are also used to quantitate compounds with a high degree of selectivity, precision, and accuracy. Compounds are introduced into the mass spectrometer either via direct insertion probe, gas inlet, or, as is more common, via gas or liquid chromatographic interfaces, which provide sample purification. Ionization may be by EI, CI, FAB, thermospray, or electrospray and mass separation by magnetic sector, quadrupole, or quadrupole ion-trap mass spectrometers. Quantitative mass spectrometry involves measuring the abundance of a specific ion, or set of ions, and relating the response to a known standard. External or internal standards may be used, but the latter are preferred for greater accuracy.

For mass spectrometry, internal standards may be either structural or stable isotope analogs. The former have the advantage of lower cost and availability while precision and accuracy are typically achieved by use of a stable isotope (2H, 13C, 15N) labeled analog of the analyte. The only requirements for labeling the analyte are that the ion monitored for the internal standard must retain an isotopic label after ionization and the label must not be exchangeable under the sampling, separation, or ionization conditions. Stable isotope internal standards are often required for acceptable quantitation, particularly with FAB and LC/MS techniques such as thermospray and electrospray.

Relative abundances of the analyte and internal standard ions are typically determined by selected ion monitoring, by which only specific ions due to the analyte and the internal standard are monitored. This technique has the advantage over scanning the full mass range in that more time is spent integrating the ion current at a selected mass-to-charge ratio, thereby increasing sensitivity. Chromatographic peak area or amount of analyte in a sample is calculated from the ratio of analyte to internal standard peak area (or height) and the regression parameters as determined by a calibration curve, using standard techniques.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Kahkashan Zaidi, Ph.D.</a> Senior Scientist 1-301-816-8269	(GC05) General Chapters 05

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### 741 MELTING RANGE OR TEMPERATURE

For Pharmacopeial purposes, the melting range, melting temperature, or melting point is defined as those points of temperature within which, or the point at which, the first detectable liquid phase is detected to the temperature at which no solid phase is apparent, except as defined otherwise for Classes II and III below. A melting transition may be instantaneous for a highly pure material, but usually a range is observed from the beginning to the end of the process. Factors influencing this transition include the sample size, the particle size, the efficiency of heat diffusion, and the heating rate, among other variables, that are controlled by procedure instructions. In some articles, the melting process is accompanied by simultaneous decomposition, which is visually evidenced as a side event like darkening of the material, charring, bubbling, or other incident. The visual impact of this side reaction frequently obscures the end of the melting process, which it may be impossible to accurately determine. In those circumstances, only the beginning of the melting can be accurately established; and it is to be reported as the melting temperature. The accuracy of the apparatus to be used as described below should be checked at suitable intervals by the use of one or more of the six USP Melting Point Reference Standards, preferably those that melt nearest the melting temperatures of the compounds being tested (see [USP Reference Standards](#) (11)).

Five procedures for the determination of melting range or temperature are given herein, varying in accordance with the nature of the substance. When no class is designated in the monograph, use the procedure for Class Ia for crystalline or amorphous substances and the procedure for Class II for waxy substances.

The procedure known as the mixed-melting point determination, whereby the melting range or temperature of a solid under test is compared with that of an intimate mixture of equal parts of the solid and an authentic specimen of it, e.g., the corresponding USP Reference Standard, if available, may be used as a confirmatory identification test. Agreement of the observations on the original and the mixture constitutes reliable evidence of chemical identity.

Apparatus I—An example of a suitable melting range Apparatus I consists of a glass container for a bath of transparent fluid, a suitable stirring device, an accurate thermometer (see [Thermometers](#) (21)); and a controlled source of heat. The bath fluid is selected with a view to the temperature required, but light paraffin is used generally and certain liquid silicones are well adapted to the higher temperature ranges. The fluid is deep enough to permit immersion of the thermometer to its specified immersion depth so that the bulb is still about 2 cm above the bottom of the bath. The heat may be supplied by an open flame or electrically. The capillary tube is about 10 cm long and 0.8 to 1.2 mm in internal diameter with walls 0.2 to 0.3 mm in thickness.

Apparatus II—An instrument may be used in the procedures for Classes I, Ia, and Ib. An example of a suitable melting range Apparatus II consists of a block of metal that may be heated at a controlled rate, its temperature being monitored by a sensor. The block accommodates the capillary tube containing the test substance and permits monitoring of the melting process, typically by means of a beam of light and a detector. The detector signal may be processed by a microcomputer to determine and display the melting point or range, or the detector signal may be plotted to allow visual estimation of the melting point or range.

Procedure for Class I, Apparatus I—Reduce the substance under test to a very fine powder, and, unless otherwise directed, render it anhydrous when it contains water of hydration by drying it at the temperature specified in the monograph, or, when the substance contains no water of hydration, dry it over a suitable desiccant for not less than 16 hours.

Charge a capillary glass tube, one end of which is sealed, with a sufficient amount of the dry powder to form a column in the bottom of the tube 2.5 to 3.5 mm high when packed down as closely as possible by moderate tapping on a solid surface.

Heat the bath until the temperature is about 30° below the expected melting point. Remove the thermometer, and quickly attach the capillary tube to the thermometer by wetting both with a drop of the liquid of the bath or otherwise, and adjust its height so that the material in the capillary is level with the thermometer bulb. Replace the thermometer, and continue the heating, with constant stirring, sufficiently to cause the temperature to rise at a rate of about 3° per minute. When the temperature is about 3° below the lower limit of the expected melting range, reduce the heating so that the temperature rises at a rate of about 1° to 2° per minute. Continue heating until melting is complete.

The temperature at which the column of the substance under test is observed to collapse definitely against the side of the tube at any point indicates the beginning of melting, and the temperature at which the test substance becomes liquid throughout corresponds to the end of melting or the melting point. The two temperatures fall within the limits of the melting range. If melting occurs with decomposition, the melting temperature corresponding to the beginning of the melting is within the range specified.

Procedure for Class Ia, Apparatus I—Prepare the test substance and charge the capillary as directed for Class I, Apparatus I. Heat the bath until the temperature is about 10° below the expected melting point and is rising at a rate of  $1 \pm 0.5$ ° per minute. Insert the capillary as directed under Class I, Apparatus I when the temperature is about 5° below the lower limit of the expected melting range, and continue heating until melting is complete. Record the melting range as directed for Class I, Apparatus I.

Procedure for Class Ib, Apparatus I—Place the test substance in a closed container and cool to 10°, or lower, for at least 2 hours. Without previous powdering, charge the cooled material into the capillary tube as directed for Class I, Apparatus I, then immediately place the charged tube in a vacuum desiccator and dry at a pressure not exceeding 20 mm of mercury for 3 hours. Immediately upon removal from the desiccator, fire-seal the open end of the tube, and as soon as practicable proceed with the determination of the melting range



as follows: Heat the bath until a temperature  $10 \pm 1^\circ$  below the expected melting range is reached, then introduce the charged tube, and heat at a rate of rise of  $3 \pm 0.5^\circ$  per minute until melting is complete. Record the melting range as directed for Class I, Apparatus I.

If the particle size of the material is too large for the capillary, pre-cool the test substance as above directed, then with as little pressure as possible gently crush the particles to fit the capillary, and immediately charge the tube.

Procedure for Class I, Apparatus II— Prepare the substance under test and charge the capillary tube as directed for Class I, Apparatus I. Operate the apparatus according to the manufacturer's instructions. Heat the block until the temperature is about  $30^\circ$  below the expected melting point. Insert the capillary tube into the heating block, and continue heating at a rate of temperature increase of about  $1^\circ$  to  $2^\circ$  per minute until melting is complete.

The temperature at which the detector signal first leaves its initial value indicates the beginning of melting, and the temperature at which the detector signal reaches its final value corresponds to the end of melting, or the melting point. The two temperatures fall within the limits of the melting range. In the event of dispute, only the melting range or temperature obtained as directed for Class I, Apparatus I, is definitive. If melting occurs with decomposition, the melting temperature corresponding to the beginning of the melting is within the range specified. In the event of dispute, only the melting range or temperature obtained as directed for Class I, Apparatus I, is definitive.

Procedure for Class II— Carefully melt the material to be tested at as low a temperature as possible, and draw it into a capillary tube, which is left open at both ends, to a depth of about 10 mm. Cool the charged tube at  $10^\circ$ , or lower, for 24 hours, or in contact with ice for at least 2 hours. Then attach the tube to the thermometer by suitable means, adjust it in a water bath so that the upper edge of the material is 10 mm below the water level, and heat as directed for Class I, Apparatus I except, within  $5^\circ$  of the expected melting temperature, to regulate the rate of rise of temperature to  $0.5^\circ$  to  $1.0^\circ$  per minute. The temperature at which the material is observed to rise in the capillary tube is the melting temperature.

Procedure for Class III— Melt a quantity of the test substance slowly, while stirring, until it reaches a temperature of  $90^\circ$  to  $92^\circ$ . Remove the source of the heat and allow the molten substance to cool to a temperature of  $8^\circ$  to  $10^\circ$  above the expected melting point. Chill the bulb of a suitable thermometer (see [Thermometers](#) 21) to  $5^\circ$ , wipe it dry, and while it is still cold dip it into the molten substance so that approximately the lower half of the bulb is submerged. Withdraw it immediately, and hold it vertically away from the heat until the wax surface dulls, then dip it for 5 minutes into a water bath having a temperature not higher than  $16^\circ$ .

Fix the thermometer securely in a test tube so that the lower point is 15 mm above the bottom of the test tube. Suspend the test tube in a water bath adjusted to about  $16^\circ$ , and raise the temperature of the bath at the rate of  $2^\circ$  per minute to  $30^\circ$ , then change to a rate of  $1^\circ$  per minute, and note the temperature at which the first drop of melted substance leaves the thermometer. Repeat the determination twice on a freshly melted portion of the test substance. If the variation of three determinations is less than  $1^\circ$ , take the average of the three as the melting point. If the variation of three determinations is  $1^\circ$  or greater than  $1^\circ$ , make two additional determinations and take the average of the five.

\* ASTM Method E77 deals with "Verification and Calibration of Liquid-in-glass Thermometers."

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Antonio Hernandez-Cardoso, B.S.</a> Scientist, Latin American Specialist 1-301-816-8308	(GC05) General Chapters 05

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#### 751 METAL PARTICLES IN OPHTHALMIC OINTMENTS

The following test is designed to limit to a level considered to be unobjectionable the number and size of discrete metal particles that may occur in ophthalmic ointments.

Procedure— Extrude, as completely as practicable, the contents of 10 tubes individually into separate, clear, flat-bottom, 60-mm Petri dishes that are free from scratches. Cover the dishes, and heat at  $85^\circ$  for 2 hours, increasing the temperature slightly if necessary to ensure that a fully fluid state is obtained. Taking precautions against disturbing the melted sample, allow each to cool to room temperature and to solidify.

Remove the covers, and invert each Petri dish on the stage of a suitable microscope adjusted to furnish 30 times magnification and equipped with an eye-piece micrometer disk that has been calibrated at the magnification being used. In addition to the usual source of light, direct an illuminator from above the ointment at a  $45^\circ$  angle. Examine the entire bottom of the Petri dish for metal particles. Varying the intensity of the illuminator from above allows such metal particles to be recognized by their characteristic reflection of light.

Count the number of metal particles that are  $50 \mu\text{m}$  or larger in any dimension: the requirements are met if the total number of such particles in all 10 tubes does not exceed 50, and if not more than 1 tube is found to contain more than 8 such particles. If these results are not obtained, repeat the test on 20 additional tubes: the requirements are met if the total number of metal particles that are  $50 \mu\text{m}$  or larger in any dimension does not exceed 150 in all 30 tubes tested, and if not more than 3 of the tubes are found to contain more than 8 such particles each.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Robert H. Lafaver, B.A.</a> Scientist 1-301-816-8335	(EGC05) Excipient General Chapters

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#### 755 MINIMUM FILL

The following tests and specifications apply to articles such as creams, gels, jellies, lotions, ointments, pastes, powders, and aerosols, including pressurized and nonpressurized topical sprays that are packaged in containers in which the labeled content is not more than 150 g or 150 mL.

PROCEDURE FOR DOSAGE FORMS OTHER THAN AEROSOLS— For containers labeled by weight, select a sample of 10 filled containers, and remove any labeling that might be altered in weight during the removal of the container contents. Thoroughly cleanse and dry the outside of the containers by a suitable means, and weigh individually. Quantitatively remove the contents from each container, cutting the latter open and washing with a suitable solvent, if necessary, taking care to retain the closure and other parts of each container. Dry, and again weigh each empty container together with its corresponding parts. The difference between the two weights is the net weight of the contents of the container. For containers labeled by volume, pour the contents of 10 containers into 10 suitable graduated cylinders, and allow to drain completely. Record the volume of the contents of each of the 10 containers. The average net content of the 10 containers is not less than the labeled amount, and the net content of any single container is not less than 90% of the labeled amount where the labeled amount is 60 g or 60 mL or less, or not less than 95% of the labeled amount where the labeled amount is more than 60 g or 60 mL but not more than 150 g or 150 mL. If this requirement is not met, determine the content of 20 additional containers. The average content of the 30 containers is not less than the labeled amount, and the net content of not more than 1 of the 30 containers is less than 90% of the labeled amount where the labeled amount is 60 g or 60 mL or less, or less than 95% of the labeled amount where the



labeled amount is more than 60 g or 60 mL but not more than 150 g or 150 mL.

PROCEDURE FOR AEROSOLS— Select a sample of 10 filled containers, and remove any labeling that might be altered in weight during the removal of the container contents. Thoroughly cleanse and dry the outsides of the containers by suitable means, and weigh individually. Remove the contents from each container by employing any safe technique (e.g., chill to reduce the internal pressure, remove the valve, and pour). Remove any residual contents with suitable solvents, then rinse with a few portions of methanol. Retain as a unit the container, the valve, and all associated parts, and heat them at 100° for 5 minutes. Cool, and again weigh each of the containers together with its corresponding parts. The difference between the original weight and the weight of the empty aerosol container is the net fill weight. Determine the net fill weight for each container tested. The requirements are met if the net weight of the contents of each of the 10 containers is not less than the labeled amount.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Desmond G. Hunt, Ph.D.</a> Scientist 1-301-816-8341	(PS05) Packaging and Storage 05

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### 761 NUCLEAR MAGNETIC RESONANCE

Nuclear magnetic resonance (NMR) spectroscopy is an analytical procedure based on the magnetic properties of certain atomic nuclei. It is similar to other types of spectroscopy in that absorption or emission of electromagnetic energy at characteristic frequencies provides analytical information. NMR differs in that the discrete energy levels between which the transitions take place are created artificially by placing the nuclei in a magnetic field.

Atomic nuclei are charged and behave as if they were spinning on the nuclear axis, thus creating a magnetic dipole of moment  $\mu$  along this axis. The angular momentum of the spinning nucleus is characterized by a spin quantum number ( $I$ ). If the mass number is odd,  $I$  is  $1/2$  or an integer plus  $1/2$ ; otherwise, it has a value of 0 or a whole number.

Nuclei having a spin quantum number,  $I \neq 0$ , when placed in an external uniform static magnetic field of strength,  $H_0$ , align with respect to the field in  $(2I + 1)$  possible orientations. Thus, for nuclei with  $I = 1/2$ , which include most isotopes of analytical significance ([Table 1](#)), there are two possible orientations, corresponding to two different energy states. A nuclear resonance is the transition between these states, by absorption or emission of the corresponding amount of energy. In a static magnetic field the nuclear magnetic axis precesses (Larmor precession) about the external field axis. The precessional angular velocity,  $\omega_0$ , is related to the external magnetic field strength through the equation:

$$\omega_0 = \gamma H_0$$

in which  $\gamma$  is the magnetogyric ratio and is a constant for all nuclei of a given isotope. If energy from an oscillating radio-frequency field is introduced, the absorption of radiation takes place according to the relationship:

$$\Delta E = h\nu = \mu H_0 / I$$

where  $h$  is Planck's constant, and

$$\nu = \omega_0 / 2\pi = \gamma H_0 / 2\pi$$

Thus, when the frequency ( $\nu$ ) of the external energy field ( $E = h\nu$ ) is the same as the precessional angular velocity, resonance is achieved.

Table 1. Properties of Some Nuclei Amenable to NMR Study

Nucleus	I	Natural Abundance, %	Sensitivity	Resonance Frequency (MHz) at		
				1.4093 T*	2.3488 T	4.6975 T
1H	1/2	99.98	1.00	60.000	100.000	200.000
13C	1/2	1.108	0.0159	15.087	25.144	50.288
19F	1/2	100	0.83	56.446	94.077	188.154
31P	1/2	100	0.0663	24.289	40.481	80.961
11B	(3/2)	80.42	0.17	19.250	32.084	64.167

\* T = tesla: 1.4093 T = 14.093 kilogauss.

The energy difference between the two levels corresponds to electromagnetic radiation in the radio-frequency range. It is a function of  $\gamma$ , which is a property of the nucleus, and  $H_0$ , the external field strength. As shown in [Table 1](#), the resonance frequency of a nucleus increases with the increase of the magnetic field strength.

NMR is a technique of high specificity but relatively low sensitivity. The basic reason for the low sensitivity is the comparatively small difference in energy between the excited and the ground states (0.02 calories at 15 to 20 kilogauss field strength), which results in a population difference between the two levels of only a few parts per million. Another important aspect of the NMR phenomenon, with negative effects on the sensitivity, is the long lifetime of most nuclei in the excited state, which affects the design of the NMR analytical test, especially in pulsed repetitive experiments. Simultaneous acquisition of the entire spectrum instead of frequency-swept spectra can give sensitivity enhancement.

#### Apparatus

The distinctive components of an NMR spectrometer are a magnet and a source of radio frequency. The instruments are described by the approximate resonance frequency of the analytical nucleus, e.g., 1H NMR. More recently, instruments are being referred to by their field strengths. Some spectrometers are dedicated to the analysis of one type of nucleus; others are designed to obtain spectra of different nuclei.

There are two types of commercial NMR spectrometers: the classical continuous wave (CW) instruments and the more modern pulse Fourier-transform (FT) instruments. The CW spectrometers use a technique similar to that of classical optical spectrometers: a slow scan of radio frequency (at fixed magnetic field) or of the magnetic field (at fixed radio frequency) over a domain corresponding to the resonance of the nuclei being studied. The signal generated by the absorption of energy is detected, amplified, and recorded.

Various instrument configurations are possible. The arrangement of a typical double-coil spectrometer, as one might see in the lower resolution 60-MHz and 100-MHz CW instruments, is illustrated in [Figure 1](#).

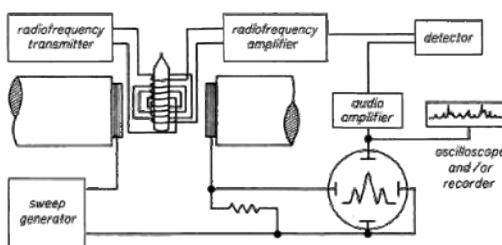


Fig. 1. Block diagram of a typical NMR spectrometer.

The limitations of the CW spectrometers are low sensitivity and long analysis time. In pulsed NMR spectrometers, a single pulse of radio frequency energy is used to simultaneously activate all nuclei. The excited nuclei returning to the lower energy level generate a free induction decay (FID) signal that contains in a time domain all the information obtained in a frequency domain with a CW spectrometer. The time domain and the frequency domain responses form a pair of FTs; the mathematical operation is performed by a computer after analog-to-digital conversion. After a delay allowing for relaxation of the excited nuclei, the pulse experiment (transient) may be repeated and the response coherently added in the computer memory, with random noise being averaged out. (A similar signal-to-noise increase can be obtained by combining CW spectrometers with computers that average transients.)

The block diagram of a typical high-resolution pulsed spectrometer is shown in [Figure 2](#).

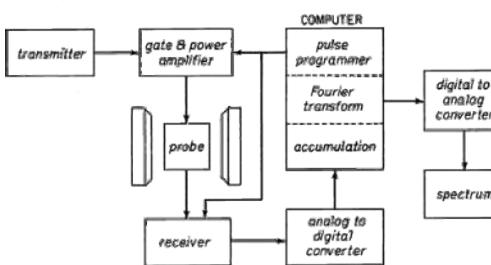


Fig. 2. Block diagram of a typical pulsed FT-NMR spectrometer.

It is a typical configuration of the high-resolution spectrometer that uses a superconducting (cryogenic) solenoid as the source of the magnetic field. Introduction of the pulsed NMR spectrometer has made the acquisition of spectra of many nuclei, other than protons, routine. It has also allowed proton spectra to be obtained in much less time, and with smaller amounts of specimen, as compared to CW techniques.

NMR spectrometers have strict stability and homogeneity requirements. Stability is often achieved by a field-frequency locking system that "locks" the magnetic field to the resonance frequency of a reference signal. The lock signal can be homonuclear or heteronuclear. In the latter case, the reference resonance is usually a deuterium signal from a deuterated solvent. On older spectrometers, using deuterium as a locking nucleus permits noise decoupling of protons to be carried out while studying nuclei like  $^{13}\text{C}$ . While internal homonuclear locks are still used in CW proton spectrometers (where tetramethylsilane at about 0.5% provides a convenient lock), they are hardly ever used in pulsed FT spectrometers.

No type of magnet is capable of producing a homogeneous field over the space occupied by the specimen. Two techniques are usually employed to compensate for this lack of homogeneity: specimen spinning and the use of additional (shim) coils. Because of design, particularly probe design, the spinning in the case of the electromagnet or permanent magnet is perpendicular to the basic field. In the superconducting magnet, the axis of rotation can only be parallel to the basic magnetic field. The spin rate should be sufficient to produce averaging of the field, but not fast enough to produce an extended vortex in the specimen tube. A vortex extended near the region exposed to the radio-frequency coils decreases resolution. The shim coils are adjusted by the operator until instrumental contributions to the observed line width are minimized.

An electronic integrator is a feature of most NMR spectrometers. On a CW instrument (1H and 19F) the integrator, connected to the spectrometer output stage, determines the relative areas of the resonance peaks and presents these areas as a series of stepped horizontal lines when a sweep is made in the integration mode. On FT-NMR spectrometers, an integration algorithm is included in the spectrometer software, and the resonance peak areas may be presented graphically as stepped lines or tabulated as numeric values. The use of computer-generated tabulated/numeric integration data should not be accepted without a specific demonstration of precision and accuracy on the spectrometer in question.

#### The Spectrum

The signals (peaks) in an NMR spectrum are characterized by four attributes: resonance frequency, multiplicity, line width, and relative intensity. The analytical usefulness of the NMR technique resides in the fact that the same types of nuclei, when located in different molecular environments, exhibit different resonance frequencies. The reason for this difference is that the effective field experienced by a particular nucleus is a composite of the external field provided by the instrument and the field generated by the circulation of the surrounding electrons. (The latter is generally opposed to the external field and the phenomenon is called "shielding.") In contrast with other spectroscopic methods, it is not possible to measure accurately the absolute values of transition frequencies. However, it is possible to measure accurately the difference in frequencies between two resonance signals. The position of a signal in an NMR spectrum is described by its separation from another resonance signal arbitrarily taken as standard. This separation is called chemical shift.

The chemical shift, being the difference between two resonance frequencies, is directly proportional to the magnetic field strength (or to the frequency of the oscillator). However, the ratio between the chemical shift, in frequency units, and the instrument frequency is constant. This allows definition of a dimensionless chemical shift parameter ( $\delta$ ) that is independent of the instrument frequency:

$$\delta = \frac{(\nu_s - \nu_r)}{\nu_o} + \delta_r$$

in which  $\nu_s$  is the test substance line frequency,  $\nu_r$  is the reference line frequency,  $\nu_o$  is the instrument frequency, in mHz, and  $\delta_r$  is the chemical shift of the reference.

By employing the above equation, it is possible to use (with appropriate caution) the chemical shift of any known species (such as the residual  $^1\text{H}$ -containing species in deuterated solvent) as a chemical shift reference. The above equation, now in common use, is applicable to nearly all methods except in the relatively rare cases where extremely precise chemical shift values must be determined, and is readily adaptable to nuclei where non-zero reference standards are the only practical method of chemical shift determinations.



For CW instruments, tetramethylsilane (TMS) is the most widely used chemical shift reference for proton and carbon spectra. It is chemically inert, exhibits only one line, which is at a higher field than most signals, and is volatile, thus allowing for ready specimen recovery. Sodium 3-(trimethylsilyl)propionate (TSP) or sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) are used as NMR references for aqueous solutions. The resonance frequency of the TSP or DSS methyl groups closely approximate that of the TMS signal; however, DSS has the disadvantage of showing a number of methylene multiplets that may interfere with signals from the test substance. Where the use of an internal NMR reference material is not desirable, an external reference may be used.

Conventional NMR spectra are shown with the magnetic field strength increasing from left to right. Nuclei that resonate at high magnetic field strengths (to the right) are said to be more shielded (greater electron density) than those that resonate at lower magnetic field strengths: these are said to be de-shielded (lower electron density).

[Figure 3](#) shows the proton NMR spectrum of 2,3-dimethyl-2-butene methyl ether. This compound contains protons in a methylene group (marked d in the graphic formula) and in four methyl groups (a, a, b, and c). Methyl groups b and c are situated in distinctly different molecular environments than the two a methyl groups. Three different methyl proton resonances are observed as spectral peaks in addition to the peak corresponding to methylene proton resonance. The two a methyl groups, being in very similar environments, have the same chemical shift. Interaction between magnetically active nuclei situated within a few bond lengths of each other leads to coupling, which results in a mutual splitting of the respective signals into sets of peaks or multiplets.

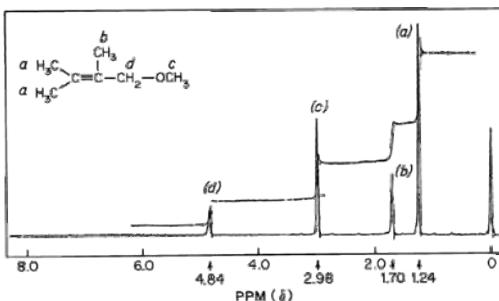


Fig. 3. NMR spectrum of 2,3-dimethyl-2-butene methyl ether (15% in  $\text{CCl}_4$ ) showing four nonequivalent, apparently uncoupled protons with a normal integral trace (peak area ratio from low H0 to high H0 of 2:3:3:6). (Tetramethylsilane, the NMR Reference, appears at 0 ppm.) The system of units represented by  $\delta$  is defined under The Spectrum, in this chapter.

The coupling between two nuclei may be described in terms of the spin-spin coupling constant,  $J$ , which is the separation (in hertz) between the individual peaks of the multiplet. Where two nuclei interact and cause reciprocal splitting, the measured coupling constants in the two resulting multiplets are equal. Furthermore,  $J$  is independent of magnetic field strength.

In a first-order, comparatively noncomplex spin system, the number of individual peaks that are expected to be present in a multiplet and the relative peak intensities are predictable. The number of peaks is determined by  $2n + 1$ , where  $n$  is the number of nuclei in adjacent groups that are active in splitting. For protons this becomes  $(n + 1)$  peaks. In general, the relative intensity of each peak in the multiplet follows the coefficient of the binomial expansion  $(a + b)^n$ . These coefficients may conveniently be found by use of Pascal's triangle, which produces the following relative areas for the specified multiplets: doublet, 1:1; triplet, 1:2:1; quartet, 1:3:3:1; quintet, 1:4:6:4:1; sextet, 1:5:10:10:5:1; and septet, 1:6:15:20:15:6:1. This orderly arrangement, generally referred to as first-order behavior, may be expected when the ratio of  $\Delta\delta$  to  $J$  is greater than about 10;  $\Delta\delta$  is the chemical shift difference between two nuclei or two groups of equivalent nuclei. Two examples of idealized spectra arising from first-order coupling are shown in [Figure 4](#).

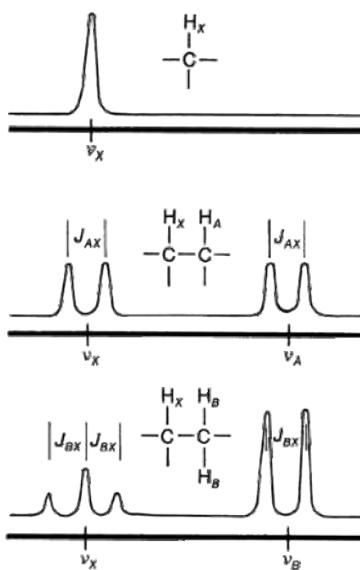


Fig. 4. Diagrammatic representation of simple first-order coupling of adjacent protons.

[Figure 5](#) shows a spectrum displaying triplet signals resulting from the mutual splitting of two adjacent methylene groups.

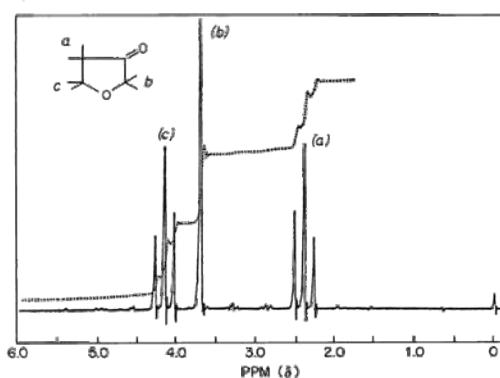


Fig. 5. NMR spectrum of 3-keto-tetrahydrofuran (10% in  $\text{CCl}_4$ ) showing three nonequivalent protons, with a normal integral trace (peak area ratio from low  $\text{H}_0$  to high  $\text{H}_0$  of 1:1:1). Note two sets of methylene groups coupled to each other at 4.2 and 2.4 ppm. (Tetramethylsilane, the NMR Reference, appears at 0 ppm.)

Coupling may occur between  $1\text{H}$  and other nuclei, such as  $19\text{F}$ ,  $13\text{C}$ , and  $31\text{P}$ . In some cases, e.g., in the CW mode, the coupling constants may be large enough so that part of the multiplet is off scale at either the upfield or downfield end. This type of coupling may occur over the normal “three-bond distance,” as for  $1\text{H}$ - $1\text{H}$  coupling.

Magnetically active nuclei with  $I \geq 1$ , such as  $14\text{N}$ , possess an electrical quadrupole moment, which produces line-broadening of the signal due to neighboring nuclei.

Another characteristic of the signal, its relative intensity, has wide analytical applications. In carefully designed experiments (see the section General Method), the area or intensity of a signal is directly proportional to the number of protons giving rise to the signal. As a result, it is possible to determine the relative ratio of the different kinds of protons or other nuclei in a specimen or to perform NMR assays with the aid of an internal standard.

The NMR spectra may contain extraneous signals due to the inhomogeneity of the magnetic field throughout the specimen. These artifacts, called spinning side bands, appear as minor lines symmetrically located around each signal. The presence of large spinning side bands indicates that the non-spinning shims require adjustment. The separation is equal to the frequency of the specimen tube spin rate or some integral multiple of that frequency. Thus, spinning side bands are readily identifiable.

#### General Method

Inadequate specimen preparation or incorrect instrumental adjustments and parameters may lead to poor resolution, decreased sensitivity, spectral artifacts, and erroneous data. It is preferable that the operator be familiar with the basic theory of NMR, the properties of the specimen, and the operating principles of the instruments. Strict adherence to the instruction manuals provided by the manufacturer and frequent checks of the performance of the instrument are essential.

The method and procedures discussed here refer specifically to  $1\text{H}$  (proton) and  $19\text{F}$  NMR. They are applicable, with modification, to other nuclei. The discussion presumes that the NMR spectra are obtained from liquid test substances or solutions in suitable solvents.

**Selection of Solvent**— In addition to having good solubility properties, suitable solvents do not exhibit resonance peaks that obscure resonance peaks of the specimen being analyzed. The most commonly used solvents for proton and carbon NMR are listed in Table 2. Deuterated solvents also provide the signal for the heteronuclear system lock. If solvent peaks might interfere with any signals from the specimen, then the isotopic purity of the solvent should be as high as possible. Deuterium ( $I = 1$ ) does not exhibit resonance under  $1\text{H}$  conditions but may cause  $J$ -coupling to be observed. The residual protons generate solvent peaks whose chemical shifts are shown in Table 2.

Table 2. Solvents Commonly Used for Proton NMR

Solvent	Residual Proton Signal, $\delta$ <sup>a</sup>
$\text{CCl}_4$ <sup>b</sup>	—
$\text{CS}_2$ <sup>b</sup>	—
$\text{SO}_2$ (liquid)	—
$(\text{CF}_3)_2\text{CO}$	—
$\text{CDCl}_3$	7.27
$\text{CD}_3\text{OD}$	3.35, 4.8 <sup>c</sup>
$(\text{CD}_3)_2\text{CO}$	2.05
$\text{D}_2\text{O}$	4.7 <sup>c</sup>
$\text{DMSO-d}_6$ <sup>d</sup>	2.50
$\text{C}_6\text{D}_6$	7.20
p-Dioxane-d <sub>8</sub>	3.55
$\text{CD}_3\text{CO}_2\text{D}$	2.05, 8.5 <sup>c</sup>
$\text{DMF-d}_7$ <sup>e</sup>	2.77, 2.93, 8.05

<sup>a</sup>  $\delta$  in ppm relative to tetramethylsilane arbitrarily taken as 0 $\delta$  or 0 ppm.

<sup>b</sup> Spectrophotometric grade.

<sup>c</sup> Highly variable; depends on solute and temperature.

<sup>d</sup> Dimethyl sulfoxide-d<sub>6</sub>.

<sup>e</sup> N,N-Dimethylformamide-d<sub>7</sub> per Aldrich, Alfa, Fluka, and Sigma catalogs.

Some solvents (e.g.,  $\text{D}_2\text{O}$  or  $\text{CD}_3\text{OD}$ ) enter into fast exchange reactions with protons and may eliminate resonance signals from  $-\text{COOH}$ ,  $-\text{OH}$ , and  $-\text{NH}_2$  structural groups. The protons in alcohols and amines do not take part in rapid exchange unless catalyzed by small concentrations of acid or base, except in the presence of  $\text{D}_2\text{O}$  and some other solvents (e.g.,  $\text{CD}_3\text{OD}$ ).

For  $19\text{F}$  NMR, most solvents used in proton NMR may be employed, the most common ones being  $\text{CHCl}_3$ ,  $\text{CCl}_4$ ,  $\text{H}_2\text{O}$ ,  $\text{CS}_2$ , aqueous acids and bases, and dimethylacetamide. In general, any nonfluorinated solvent may be used, provided that it is of spectral quality. Obviously, there is no interference from the protonated functional groups of the solvent. However, unless they are decoupled, protonated functional groups on the  $19\text{F}$ -containing specimen will provide  $J$ -coupling.

**Specimen Preparation**— Directions are usually given in individual monographs. The solute concentration depends on the objective of the experiment and on the type of instrument. Detection of minor contaminants may require higher concentrations. The solutions are prepared in separate vials and transferred to the NMR specimen tube. The volume required depends on the size of the specimen tube and on the geometry of the instrument. The level of the solution in the tube must be high enough to extend beyond the coils when the tube is

inserted in the instrument probe and spun.

The NMR specimen tubes must meet narrow tolerance specifications in diameter, wall thickness, concentricity, and camber. The most widely used tubes have a 5- or 10-mm outside diameter and a length of between 15 and 20 cm. Microtubes are available for the analysis of small amounts of specimen.

Procedure— The specimen tube is placed in a probe located in the magnetic field. The probe contains electronic circuitry including the radio-frequency coil(s), and is provided with attachments for the air supply that spins the specimen tubes.

Instrument adjustments are made before each experiment. The spinning rate of the specimen tube is adjusted so that spinning side bands do not interfere with the peaks of interest and the vortex does not extend beyond the coils in the probe. To optimize the instrument performance, the magnetic shim gradients on FT-NMR spectrometers are adjusted. In adjusting resolution on CW spectrometers, a good indicator is the definite "ringing" of the TMS peak. The phenomenon of ringing is the oscillation of the recorder trace after the magnetic field has passed through a resonance frequency. Ringing, evident on a number of the peaks in Figures 5 and 6, arises during rapid scans and decays exponentially to the baseline value.

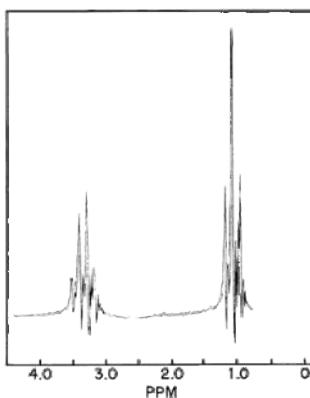


Fig. 6. Continuous wave proton spectrum of ethyl ether.

Figure 7 clearly indicates the absence, in an FT experiment, of the ringing phenomenon. Ringing will not appear because the spectrum obtained is the result of analysis of the FID by Fourier transformation and not a magnetic field or frequency sweep through the individual resonance positions.

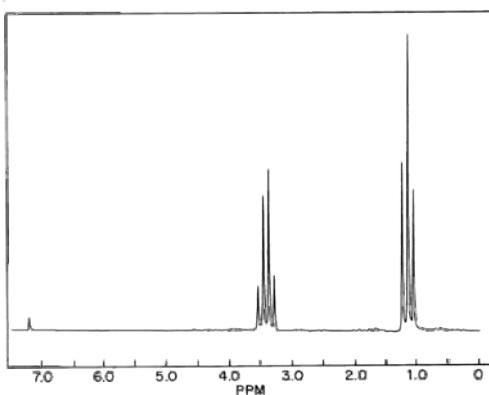


Fig. 7. Proton NMR spectrum of ethyl ether in deuterated chloroform.

With proton CW instruments the spectrum is scanned from 0 ppm to about 10 ppm with a scan time of about 1 to 5 minutes. The amplification is adjusted so that all peaks remain on scale. If the response is low at reasonable amplitude, the radio-frequency power is increased to obtain the highest possible peak response without peak broadening. After the initial scan, the presence of peaks downfield of 10 ppm is quickly checked by off-setting the instrument response by about 5 ppm. With CW instrumentation, it is common for the TMS peak to shift slightly during an extended scan. The extent of the shift is usually obtained by comparing the relative positions of another peak in the initial scan with the same peak in the offset scan.

The operation of an FT-NMR spectrometer is a much more elaborate experiment. The computer serves to control the spectrometer, to program the experiment, and to store and process the data. Programming the experiment involves setting values for a large number of variables including the spectral width to be examined, the duration ("width") of the excitation pulse, the time interval over which data will be acquired, the number of transients to be accumulated, and the delay between one acquisition and the next. The analysis time for one transient is in the order of seconds. The number of transients is a function of the specimen concentration, the type of nucleus, and the objective of the experiment. At the end of the experiment, the FID signal is stored in digitized form in the computer memory and is displayed on the video screen. The signal can be processed mathematically to enhance either the resolution or the sensitivity, and it can be Fourier-transformed into a frequency-domain spectrum. The instrument provides a plot of the spectrum. The integration routine, accessed through keyboard commands, results in a stepped-line plot. Considerably more accurate integrals are obtained if the signals or regions of interest are separately integrated.

FT-NMR spectrometers may yield qualitative and quantitative data from the same experiment, but this is seldom done in practice. In quantitative FT experiments, special precautions must be taken for the signal areas to be proportional to the number of protons. The delays between pulses must be long enough to allow complete relaxation of all excited nuclei. This results in a considerable increase in analysis time and in some loss of resolution. Qualitative analysis is usually performed in nonquantitative conditions, with the design of the experiment directed to fast analysis with maximum resolution or sensitivity.

#### Qualitative and Quantitative Analysis

NMR spectroscopy has been used for a wide range of applications such as structure elucidation; thermodynamic, kinetic, and mechanistic studies; and quantitative analysis. Some of these applications are beyond the scope of compendial methods.

All five characteristics of the signal—chemical shift, multiplicity, line width, coupling constants, and relative intensity—contribute analytical information.

Qualitative Applications— Comparison of a spectrum from the literature or from an authentic specimen with that of a test specimen may be used to confirm the identity of a compound and to detect the presence of impurities that generate extraneous signals. The NMR spectra of simple structures can be adequately described by the numeric value of the chemical shifts and coupling constants, and by the number of protons under each signal. (The software of modern instruments includes programs that generate simulated spectra using these data.) Experimental details, such as the solvent used, the specimen concentration, and the chemical shift reference, must also be provided.

For unknown specimens, NMR analysis, usually coupled with other analytical techniques, is a powerful tool for structure elucidation. Chemical shifts provide information on the chemical environment of the nuclei. Extensive literature is available with correlation charts and rules for predicting chemical shifts. The multiplicity of the signals provides important stereochemical information. Mutual signal splitting of functional groups indicates close proximity. The magnitude of the coupling constant,  $J$ , between residual protons on substituted

aromatic, olefinic, or cycloalkyl structures is used to identify the relative position of the substituents.

Several special techniques (double resonance, chemical exchange, use of shift reagents, two-dimensional analysis, etc.) are available to simplify some of the more complex spectra, to identify certain functional groups, and to determine coupling correlations.

Double resonance, or spin decoupling, is a technique that removes the coupling between nuclei and thus simplifies the spectrum and identifies the components in a coupling relationship. For example, in a simple two-proton system, generally designated an AX system (see Figure 4), each proton appears as a doublet. If a strong radio-frequency field is introduced at the frequency of X, while the normal radio-frequency field is maintained at the frequency that causes A to resonate, the coupling between A and X is removed (homonuclear decoupling). A is no longer split, but instead appears as a singlet. Routine  $^{13}\text{C}$  spectra are obtained under proton decoupling conditions that remove all heteronuclear  $^{13}\text{C}$ - $^1\text{H}$  couplings. As a result of this decoupling, the carbon signals appear as singlets, unless other nuclei that are not decoupled are present (e.g.,  $^{19}\text{F}$ ,  $^{31}\text{P}$ ).

Functional groups containing exchangeable protons bound to hetero-atoms such as  $-\text{OH}$ ,  $-\text{NH}_2$ , or  $-\text{COOH}$  groups may be identified by taking advantage of the rapid exchange of these protons with  $\text{D}_2\text{O}$ . To determine the presence and position of these groups, scan the test substance in  $\text{CDCl}_3$  or  $\text{DMSO-d}_6$ , then add a few drops of  $\text{D}_2\text{O}$  to the specimen tube, shake, and scan again. The resonance peaks from these groups collapse in the second scan and are replaced by the  $\text{HDO}$  singlet between 4.7 and 5.0 ppm.

This chemical exchange is an example of the effect of intermolecular and intramolecular rate processes on NMR spectra. If a proton can experience different environments by virtue of such a process (tautomerism, rotation about a bond, exchange equilibria, ring inversion, etc.), the appearance of the spectrum will be a function of the rate of the process. Slow processes (on an NMR time scale) result in more than one signal, fast processes average these signals to one line, and intermediate processes produce broad signals.

The software of modern FT-NMR spectrometers allows for sequences of pulses much more complex than the repetitive accumulation of transients described above. Such experiments include homonuclear or heteronuclear two-dimensional analysis, which determines the correlation of couplings and may simplify the interpretation of otherwise complex spectra.

**Quantitative Applications**— If appropriate instrument settings for quantitative analysis have been made, the areas (or intensities) of two signals are proportional to the total number of protons generating the signals.

$$\frac{A_1}{A_2} = \frac{N_1}{N_2} \quad (1)$$

If the two signals originate from two functional groups of the same molecule, the equation can be simplified to

$$\frac{A_1}{A_2} = \frac{n_1}{n_2} \quad (2)$$

in which  $n_1$  and  $n_2$  are the number of protons in the respective functional groups.

If the two signals originate from different molecular species,

$$\frac{A_1}{A_2} = \frac{n_1 m_1}{n_2 m_2} = \frac{n_1 W_1 / M_1}{n_2 W_2 / M_2} \quad (3)$$

where  $m_1$  and  $m_2$  are the numbers of moles;  $W_1$  and  $W_2$  are the masses; and  $M_1$  and  $M_2$  are the molecular weights of compounds 1 and 2, respectively.

Examination of Equations 2 and 3 shows that NMR quantitative analysis can be performed in an absolute or relative manner. In the absolute method, an internal standard is added to the specimen and a resonance peak area arising from the test substance is compared with a resonance peak area from the internal standard. If both test substance and internal standard are accurately weighed, the absolute purity of the substance may be calculated. A good internal standard has the following properties: it presents a reference resonance peak, preferably a singlet, at a field position removed from all specimen peaks; it is soluble in the analytical solvent; its proton equivalent weight, i.e., the molecular weight divided by the number of protons giving rise to the reference peak, is low; and it does not interact with the compound being tested. Typical examples of useful standards are 1,2,4,5-tetrachlorobenzene, 1,4-dinitrobenzene, benzyl benzoate, and maleic acid. The choice of a standard will be dictated by the spectrum of the specimen.

The relative method may be used to determine the molar fraction of an impurity in a test substance (or of the components in a mixture) as calculated by Equation 3.

Quantitative analysis, as well as detection of trace impurities, is markedly improved with modern instrumentation. Stronger magnetic fields and the ability to accumulate and/or average signals over long periods of time greatly enhance the sensitivity of the method.

**Absolute Method of Quantitation**— Where the individual monograph directs that the Absolute Method of Quantitation be employed, proceed as follows.  
**Solvent, Internal Standard, and NMR Reference**— Use as directed in the individual monograph.

**Test Preparation**— Transfer an accurately weighed quantity of the test substance, containing about 4.5 proton mEq, to a glass-stoppered, graduated centrifuge tube. Add about 4.5 proton mEq of Internal Standard, accurately weighed, and 3.0 mL of Solvent, insert the stopper, and shake. When dissolution is complete, add about 30  $\mu\text{L}$  (30 mg if a solid) of NMR Reference, provided that it does not interfere with subsequent measurements, and shake.

**Procedure**— Transfer an appropriate amount (0.4 to 0.8 mL) of Test Preparation to a standard 5-mm NMR spinning tube, and record the spectrum, adjusting the spin rate so that no spinning side bands interfere with the peaks of interest. Measure the area under each of the peaks specified in the individual monograph by integrating not fewer than five times.

Record the average area of the Internal Standard peak as AS and that of the Test Preparation peak as AU.

Calculate the quantity, in mg, of the analyte in the Test Preparation by the formula:

$$W_S (A_U / A_S) (E_U / E_S)$$

in which  $W_S$  is the weight, in mg, of Internal Standard taken; and  $E_U$  and  $E_S$  are the proton equivalent weights (i.e., the molecular weights divided by the number of protons giving rise to the reference peak) of the analyte and the Internal Standard, respectively.

**Relative Method of Quantitation**— Where the individual monograph directs that the Relative Method of Quantitation be employed, proceed as follows.  
**Solvent, NMR Reference, and Test Preparation**— Use as directed under Absolute Method of Quantitation.

**Procedure**— Transfer an appropriate amount (0.4 to 0.8 mL) of Test Preparation to a standard 5-mm NMR spinning tube, and record the spectrum, adjusting the spin rate so that no spinning side bands interfere with the peaks of interest. Measure the area or intensity under each of the peaks specified in the individual monograph by integrating not fewer than five times. Record the average area or intensity resulting from the resonances of the groups designated in the individual monograph as  $A_1$  and  $A_2$ .

Calculate the quantity, in mole percent, of the analyte in the Test Preparation by the formula:

$$100 (A_1 / n_1) / [(A_1 / n_1) + (A_2 / n_2)]$$

in which  $n_1$  and  $n_2$  are, respectively, the numbers of protons in the designated groups.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Gary E. Ritchie, M.Sc.</a> Scientific Fellow 1-301-816-8353	(GC05) General Chapters 05

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### 771 OPHTHALMIC OINTMENTS

**Added Substances**— Suitable substances may be added to ophthalmic ointments to increase stability or usefulness, unless proscribed in the individual monograph, provided they are harmless in the amounts administered and do not interfere with the therapeutic efficacy or with the responses to the specified assays and tests. No coloring agent may be added, solely for the purpose of coloring the finished preparation, to an article intended for ophthalmic use (see also [Added Substances under General Notices](#) and under [Antimicrobial Effectiveness Testing](#) (51)).

A suitable substance or mixture of substances to prevent the growth of microorganisms must be added to ophthalmic ointments that are packaged in multiple-use containers, regardless of the method of sterilization employed, unless otherwise directed in the individual monograph, or unless the formula itself is bacteriostatic. Such substances are used in concentrations that will prevent the growth of or kill microorganisms in the ophthalmic ointments (see also [Antimicrobial Effectiveness Testing](#) (51)) and [Antimicrobial Agents—Content](#) (341)). Sterilization processes are employed for the finished ointment or for all ingredients, if the ointment is manufactured under rigidly aseptic conditions, even though such substances are used (see also [Parenteral and Topical Preparations](#) in the section [Added Substances](#), under General Notices, and [Sterilization and Sterility Assurance of Compendial Articles](#) (121)). Ophthalmic ointments that are packaged in single-use containers are not required to contain antibacterial agents; however, they meet the requirements for [Sterility Tests](#) (71).

**Containers**— Containers, including the closures, for ophthalmic ointments do not interact physically or chemically with the preparation in any manner to alter the strength, quality, or purity beyond the official requirements under the ordinary or customary conditions of handling, shipment, storage, sale, and use.

**Metal Particles**— Follow the Procedure set forth under [Metal Particles in Ophthalmic Ointments](#) (751).

**Leakage**— Select 10 tubes of the Ointment, with seals applied when specified. Thoroughly clean and dry the exterior surfaces of each tube with an absorbent cloth. Place the tubes in a horizontal position on a sheet of absorbent blotting paper in an oven maintained at a temperature of  $60 \pm 3^\circ$  for 8 hours. No significant leakage occurs during or at the completion of the test (disregard traces of ointment presumed to originate externally from within the crimp of the tube or from the thread of the cap). If leakage is observed from one, but not more than one, of the tubes, repeat the test with 20 additional tubes of the Ointment. The requirement is met if no leakage is observed from the first 10 tubes tested, or if leakage is observed from not more than one of 30 tubes tested.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Desmond G. Hunt, Ph.D.</a> Scientist 1-301-816-8341	(GC05) General Chapters 05

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### 776 OPTICAL MICROSCOPY

Optical microscopy for particle characterization can generally be applied to particles 1  $\mu\text{m}$  and greater. The lower limit is imposed by the resolving power of the microscope. The upper limit is less definite and is determined by the increased difficulty associated with the characterization of larger particles. Various alternative techniques are available for particle characterization outside the applicable range of optical microscopy. Optical microscopy is particularly useful for characterizing particles that are not spherical. This method may also serve as a base for the calibration of faster and more routine methods that may be developed.

**Apparatus**— Use a microscope that is stable and protected from vibration. The microscope magnification (product of the objective magnification, ocular magnification, and additional magnifying components) must be sufficient to allow adequate characterization of the smallest particles to be classified in the test specimen. The greatest numerical aperture of the objective should be sought for each magnification range. Polarizing filters may be used in conjunction with suitable analyzers and retardation plates. Color filters of relatively narrow spectral transmission should be used with achromatic objectives, are preferable with apochromats, and are required for appropriate color rendition in photomicrography. Condensers corrected at least for spherical aberration should be used in the microscope substage and with the lamp. The numerical aperture of the substage condenser should match that of the objective under the conditions of use and is affected by the actual aperture of the condenser diaphragm and by the presence of immersion oils.

**Adjustment**— The precise alignment of all elements of the optical system and proper focusing are essential. The focusing of the elements should be done in accordance with the recommendations of the microscope manufacturer. Critical axial alignment is recommended.

**Illumination**— A requirement for good illumination is a uniform and adjustable intensity of light over the entire field of view; Kohler illumination is preferred. With colored particles, choose the color of the filters used so as to control the contrast and detail of the image.

**Visual Characterization**— The magnification and numerical aperture should be sufficiently high to allow adequate resolution of the images of the particles to be characterized. Determine the actual magnification using a calibrated stage micrometer to calibrate an ocular micrometer. Errors can be minimized if the magnification is sufficient that the image of the particle is at least 10 ocular divisions. Each objective must be calibrated separately. To calibrate the ocular scale, the stage micrometer scale and the ocular scale should be aligned. In this way, a precise determination of the distance between ocular stage divisions can be made. Several different magnifications may be necessary to characterize materials having a wide particle size distribution.

**Photographic Characterization**— If particle size is to be determined by photographic methods, take care to ensure that the object is sharply focused at the plane of the photographic emulsion. Determine the actual magnification by photographing a calibrated stage micrometer, using photographic film of sufficient speed, resolving power, and contrast. Exposure and processing should be identical for photographs of both the test specimen and the determination of magnification. The apparent size of a photographic image is influenced by the exposure, development, and printing processes as well as by the resolving power of the microscope.

**Preparation of the Mount**— The mounting medium will vary according to the physical properties of the test specimen. Sufficient but not excessive contrast between the specimen and the mounting medium is required to ensure adequate detail of the specimen edge. The particles should rest in one plane and be adequately dispersed to distinguish individual particles of interest. Furthermore, the particles must be representative of the distribution of sizes in the material and must not be altered during preparation of the mount. Care should be taken to ensure that this important requirement is met. Selection of the mounting medium must include a consideration of the analyte solubility.

**Crystallinity Characterization**— The crystallinity of a material may be characterized to determine compliance with the crystallinity requirement where stated in the individual monograph of a drug substance. Unless otherwise specified in the individual monograph, mount a few particles of the specimen in mineral oil on a clean glass slide. Examine the mixture using a polarizing microscope: the particles show birefringence (interference colors) and extinction positions when the microscope stage is revolved.

**Limit Test of Particle Size by Microscopy**— Weigh a suitable quantity of the powder to be examined (for example, 10 to 100 mg), and suspend it in 10 mL of a suitable medium in which the powder does not dissolve, adding, if necessary, a wetting agent. A homogeneous suspension of particles can be maintained by suspending the particles in a medium of similar or matching density and by providing adequate agitation. Introduce a portion of the homogeneous suspension into a suitable counting cell, and scan under a microscope an area corresponding to not less than 10  $\mu\text{g}$  of the powder to be examined. Count all the particles having a maximum dimension greater than the prescribed size limit. The size limit and the permitted number of particles exceeding the limit are defined for each substance.

**Particle Size Characterization**— The measurement of particle size varies in complexity depending on the shape of the particle, and the number of particles characterized must be sufficient to ensure an acceptable level of uncertainty in the measured parameters. Additional information on particle size measurement, sample size, and data analysis is available, for example, in ISO 9276. For spherical particles, size is defined by the diameter. For irregular particles, a variety of definitions of particle size exist. In general, for irregularly shaped particles, characterization of particle size must also include information on the type of diameter measured as well as information on particle shape. Several commonly used measurements of particle size are defined below (see [Figure 1](#)):

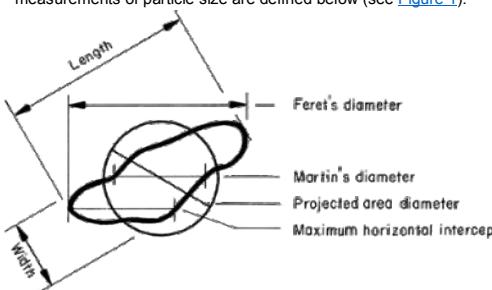


Fig. 1. Commonly used measurements of particle size.

**Feret's Diameter**— The distance between imaginary parallel lines tangent to a randomly oriented particle and perpendicular to the ocular scale.

**Martin's Diameter**— The diameter of the particle at the point that divides a randomly oriented particle into two equal projected areas.

**Projected Area Diameter**— The diameter of a circle that has the same projected area as the particle.

**Length**— The longest dimension from edge to edge of a particle oriented parallel to the ocular scale.

**Width**— The longest dimension of the particle measured at right angles to the length.

**Particle Shape Characterization**— For irregularly shaped particles, characterization of particle size must also include information on particle shape. The homogeneity of the powder should be checked using appropriate magnification. The following defines some commonly used descriptors of particle shape (see [Figure 2](#)):

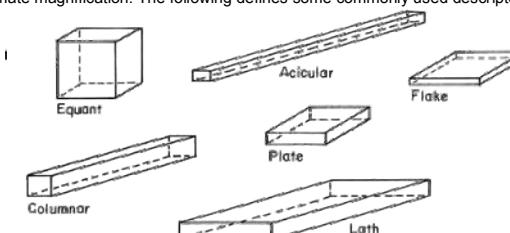


Fig. 2. Commonly used descriptions of particle shape.

**Acicular**— Slender, needle-like particle of similar width and thickness.

**Columnar**— Long, thin particle with a width and thickness that are greater than those of an acicular particle.

**Flake**— Thin, flat particle of similar length and width.

**Plate**— Flat particles of similar length and width but with greater thickness than flakes.

**Lath**— Long, thin, and blade-like particle.

**Equant**— Particles of similar length, width, and thickness; both cubical and spherical particles are included.

**General Observations**— A particle is generally considered to be the smallest discrete unit. A particle may be a liquid or semisolid droplet; a single crystal or polycrystalline; amorphous or an agglomerate. Particles may be associated. This degree of association may be described by the following terms:

**Lamellar**— Stacked plates.

**Aggregate**— Mass of adhered particles.

**Agglomerate**— Fused or cemented particles.

**Conglomerate**— Mixture of two or more types of particles.

**Spherulite**— Radial cluster.

**Drusy**— Particle covered with tiny particles.

Particle condition may be described by the following terms:

**Edges**— Angular, rounded, smooth, sharp, fractured.

**Optical**— Color (using proper color-balancing filters), transparent, translucent, opaque.

**Defects**— Occlusions, inclusions.

Surface characteristics may be described as:



Cracked— Partial split, break, or fissure.

Smooth— Free of irregularities, roughness, or projections.

Porous— Having openings or passageways.

Rough— Bumpy, uneven, not smooth.

Pitted— Small indentations.

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General Chapter	<a href="#">Antonio Hernandez-Cardoso, B.S.</a> Scientist, Latin American Specialist 1-301-816-8308	(GC05) General Chapters 05

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### 781 OPTICAL ROTATION

Many pharmaceutical substances are optically active in the sense that they rotate an incident plane of polarized light so that the transmitted light emerges at a measurable angle to the plane of the incident light. This property is characteristic of some crystals and of many pharmaceutical liquids or solutions of solids. Where the property is possessed by a liquid or by a solute in solution, it is generally the result of the presence of one or more asymmetric centers, usually a carbon atom with four different substituents. The number of optical isomers is  $2^n$ , where n is the number of asymmetric centers. Polarimetry, the measurement of optical rotation, of a pharmaceutical article may be the only convenient means for distinguishing optically active isomers from each other and thus is an important criterion of identity and purity.

Substances that may show optical rotatory power are chiral. Those that rotate light in a clockwise direction as viewed towards the light source are dextrorotatory, or (+) optical isomers. Those that rotate light in the opposite direction are called levorotatory or (-) optical isomers. (The symbols d- and l-, formerly used to indicate dextro- and levorotatory isomers, are no longer sanctioned owing to confusion with d- and l-, which refer to configuration relative to d-glyceraldehyde. The symbols R and S and  $\alpha$  and  $\beta$  are also used to indicate configuration, the arrangement of atoms or groups of atoms in space.)

The physicochemical properties of nonsuperimposable chiral substances rotating plane polarized light in opposite directions to the same extent, enantiomers, are identical, except for this property and in their reactions with other chiral substances. Enantiomers often exhibit profound differences in pharmacology and toxicology, owing to the fact that biological receptors and enzymes themselves are chiral. Many articles from natural sources, such as amino acids, proteins, alkaloids, antibiotics, glycosides, and sugars, exist as chiral compounds. Synthesis of such compounds from nonchiral materials results in equal numbers of the enantiomers, racemates. Racemates have a net null optical rotation, and their physical properties may differ from those of the component enantiomers. Use of stereoselective or stereospecific synthetic methods or separation of racemic mixtures can be used to obtain individual optical isomers.

Measurement of optical rotation is performed using a polarimeter.<sup>1</sup> The general equation used in polarimetry is:

$$[\alpha]_{\lambda}^t = \frac{100a}{lc}$$

where  $[\alpha]$  is the specific rotation at wavelength  $\lambda$ , t is the temperature, a is the observed rotation in degrees ( $^{\circ}$ ), l is the pathlength in decimeters, and c is the concentration of the analyte in g per 100 mL. Thus,  $[\alpha]$  is 100 times the measured value, in degrees ( $^{\circ}$ ), for a solution containing 1 g in 100 mL, measured in a cell having a pathlength of 1.0 decimeter under defined conditions of incident wavelength of light and temperature. For some Pharmacopeial articles, especially liquids such as essential oils, the optical rotation requirement is expressed in terms of the observed rotation, a, measured under conditions defined in the monograph.

Historically, polarimetry was performed using an instrument where the extent of optical rotation is estimated by visual matching of the intensity of split fields. For this reason, the d-line of the sodium lamp at the visible wavelength of 589 nm was most often employed. Specific rotation determined at the d-line is expressed by the symbol:

$$[\alpha]_D^{25} \text{ or } [\alpha]_D^{20}$$

and much of the data available are expressed in this form. Use of lower wavelengths, such as those available with the mercury lamp lines isolated by means of filters of maximum transmittance at approximately 578, 546, 436, 405, and 365 nm in a photoelectric polarimeter, has been found to provide advantages in sensitivity with a consequent reduction in the concentration of the test compound. In general, the observed optical rotation at 436 nm is about double and at 365 nm about three times that at 589 nm. Reduction in the concentration of the solute required for measurement may sometimes be accomplished by conversion of the substance under test to one that has a significantly higher optical rotation. Optical rotation is also affected by the solvent used for the measurement, and this is always specified.

It is now common practice to use other light sources, such as xenon or tungsten halogen, with appropriate filters, because these may offer advantages of cost, long life, and broad wavelength emission range, over traditional light sources.

Specific Rotation— The reference [Specific rotation](#) in a monograph signifies that specific rotation is to be calculated from observed optical rotations in the Test solution obtained as directed therein. Unless otherwise directed, measurements of optical rotation are made at 589 nm at  $25^{\circ}$ . Where a photoelectric polarimeter is used, a single measurement, corrected for the solvent blank, is made. Where a visual polarimeter is employed, the average of no fewer than five determinations, corrected for the reading of the same tube with a solvent blank, is used. Temperature, which applies to the solution or the liquid under test, should be maintained within  $0.5^{\circ}$  of the stated value. Use the same cell for sample and blank. Maintain the same angular orientation of the cell in each reading. Place the cell so that the light passes through it in the same direction each time. Unless otherwise specified, specific rotation is calculated on the dried basis where Loss on drying is specified in the monograph or on the anhydrous basis where Water is specified.

Optical rotation of solutions should be determined within 30 minutes of preparation. In the case of substances known to undergo racemization or mutarotation, care should be taken to standardize the time between adding the solute to the Solvent and introduction of the solution into the polarimeter tube.

Angular Rotation— The reference [Angular rotation](#) in a monograph signifies, unless otherwise directed, that the optical rotation of the neat liquid is measured in a 1.0-dm tube at 589 nm at  $25^{\circ}$ , corrected for the reading of the dry empty tube.

\* Suitable calibrators are available from the Office of Standard Reference Materials, National Institute of Standards and Technology (NIST), Gaithersburg, MD 20899, as current lots of



Standard Reference Materials, Dextrose and Sucrose. Alternatively, calibration may be checked using a Polarization Reference Standard, which consists of a plate of quartz mounted in a holder perpendicular to the light path. These standards are available, traceable to NIST, from Rudolph Research Analytical, 354 Route 206, Flanders, NJ 07836, or from Rudolph Instruments, Inc., 40 Pier Lane, Fairfield, NJ 07004-2113.

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General Chapter	<a href="#">Antonio Hernandez-Cardoso, B.S.</a> Scientist, Latin American Specialist 1-301-816-8308	(GC05) General Chapters 05

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## 785 OSMOLALITY AND OSMOLARITY

### INTRODUCTION

Osmotic pressure plays a critical role in all biological processes that involve diffusion of solutes or transfer of fluids through membranes. Osmosis occurs when solvent but not solute molecules cross a semipermeable membrane from regions of lower to higher concentrations to produce equilibrium. The knowledge of osmotic pressures is important for practitioners in determining whether a parenteral solution is hypo-osmotic, iso-osmotic, or hyperosmotic. A quantitative measure of osmotic pressure facilitates the dilution required to render a solution iso-osmotic relative to whole blood.

### OSMOTIC PRESSURE

The osmotic pressure of a solution depends on the number of particles in solution, and is therefore referred to as a colligative property. A particle can be a molecule or an ion or an aggregated species (e.g., a dimer) that can exist discretely in solution. A solution exhibits ideal behavior when no interaction occurs between solutes and solvent, except where solvent molecules are bound to solutes by hydrogen bonding or coordinate covalency. For such a solution containing a nondissociating solute, the osmotic pressure ( $\pi$ ) is directly proportional to its molality (number of moles of solute per kilogram of the solvent):

$$\pi = (P RT/1000)m,$$

where  $P$  is the density of the solvent at the temperature  $T$  (in the absolute scale);  $R$  is the universal gas constant; and  $m$  is the molality of the solution. For a real solution containing more than one solute, the osmotic pressure is given by the formula:

$$\pi = (P RT/1000)\sum \pi_i m_i \phi_{m,i}$$

where  $\pi_i$  is the number of particles formed by the dissociation of one molecule of the  $i$ th solute;  $\pi_i = 1$  for nonionic (nondissociating) solutes;  $m_i$  is the molality of the  $i$ th solute; and  $\phi_{m,i}$  is the molal osmotic coefficient of the  $i$ th solute. The molal osmotic coefficient takes into account the deviation of a solution from ideal behavior. Its value depends upon the concentration of the solute(s) in solution, its chemical properties, and ionic characteristics. The value of the molal osmotic coefficient of a solute can be determined experimentally by measuring the freezing point depression at different molal concentrations. At concentrations of pharmaceutical interest, the value of the molal osmotic coefficient is less than one. The molal osmotic coefficient decreases with the increase in concentration of the solute ([Table 1](#)).

### OSMOLALITY

The osmolality of a solution  $\bar{\pi}_m$  is given by

$$\bar{\pi}_m = \sum \pi_i m_i \phi_{m,i}$$

The osmolality of a real solution corresponds to the molality of an ideal solution containing nondissociating solutes and is expressed in osmoles or milliosmoles per kilogram of solvent (Osmol per kg or mOsmol per kg, respectively), a unit that is similar to the molality of the solution. Thus, osmolality is a measure of the osmotic pressure exerted by a real solution across a semipermeable membrane. Like osmotic pressure, other colligative properties of a solution, such as vapor pressure lowering, boiling point elevation, and freezing point depression, are also directly related to the osmolality of the solution. Indeed, the osmolality of a solution is typically determined most accurately and conveniently by measuring freezing point depression ( $\Delta T_f$ ):

$$\Delta T_f = k_f \bar{\pi}_m$$

where  $k_f$  is the molal cryoscopic constant, which is a property of the solvent. For water, the value of  $k_f$  is  $1.860^{\circ}$  per Osmol. That is, 1 Osmol of a solute added to 1 kg of water lowers the freezing point by  $1.860^{\circ}$ .

### OSMOLARITY

Osmolarity of a solution is a theoretical quantity expressed in osmoles per L (Osmol per L) of a solution and is widely used in clinical practice because it expresses osmoles as a function of volume. Osmolarity cannot be measured but is calculated theoretically from the experimentally measured value of osmolality.

Sometimes, osmolarity ( $\bar{\pi}_c$ ) is calculated theoretically from the molar concentrations:

$$\bar{\pi}_c = \sum \pi_i c_i$$

where  $\pi_i$  is as defined above, and  $c_i$  is the molar concentration of the  $i$ th solute in solution. For example, the osmolarity of a solution prepared by dissolving 1 g of vancomycin in 100 mL of 0.9% sodium chloride solution can be calculated as follows:

$$[3 \times 10 \text{ g/L}/1449.25(\text{mol. wt. of vancomycin}) + 2 \times 9 \text{ g/L}/58.44(\text{mol. wt. of sodium chloride})] \times 1000 = 329 \text{ mOsmol/L}$$

The results suggest that the solution is slightly hyperosmotic because the osmolality of blood ranges between 285 and 310 mOsmol per kg. However, the solution is found to be hypo-osmotic and has an experimentally determined osmolality of 255 mOsmol per kg.<sup>1</sup> The example illustrates that osmolarity values calculated theoretically from the concentration of a solution should be interpreted cautiously and may not represent the osmotic properties of infusion solutions.

The discrepancy between theoretical (osmolarity) and experimental (osmolality) results is, in part, due to the fact that osmotic pressure is related to osmolality and not osmolarity. More significantly, the discrepancy between experimental results and the theoretical calculation is due to the fact that the osmotic pressure of a real solution is less than that of an ideal solution because of interactions between solute molecules or between solute and solvent molecules in a solution. Such interactions reduce the pressure exerted by solute molecules on a semipermeable membrane, reducing experimental values of osmolality compared to theoretical values. This difference is related to the molal osmotic coefficient ( $\phi_{m,i}$ ). The example also illustrates the importance of determining the osmolality of a solution experimentally, rather than calculating the value theoretically.

### MEASUREMENT OF OSMOLALITY

The osmolality of a solution is commonly determined by the measurement of the freezing point depression of the solution.

Apparatus— The apparatus, an osmometer for freezing point depression measurement, consists of the following: a means of cooling the container used for the measurement; a resistor sensitive to temperature (thermistor), with an appropriate current- or potential-difference measurement device that may be graduated in temperature change or in osmolality; and a means of mixing the sample.

Osmometers that measure the vapor pressures of solutions are less frequently employed. They require a smaller volume of specimen (generally about 5  $\mu$ L), but the accuracy and precision of the resulting osmolality determination are comparable to those obtained by the use of osmometers that depend upon the observed freezing points of solutions.

Standard Solutions— Prepare Standard Solutions as specified in [Table 1](#), as necessary.

Table 1. Standard Solutions for Osmometer Calibration<sup>2</sup>

Standard Solutions (Weight in g of sodium chloride per kg of water)	Osmolality (mOsmol/kg) ( $\bar{m}$ )	Molal Osmotic Coefficient ( $\phi_m$ , NaCl)	Freezing Point Depression ( $^\circ$ ) $\Delta T_f$
3.087	100	0.9463	0.186
6.260	200	0.9337	0.372
9.463	300	0.9264	0.558
12.684	400	0.9215	0.744
15.916	500	0.9180	0.930
19.147	600	0.9157	1.116
22.380	700	0.9140	1.302

2. Adapted from the European Pharmacopoeia, 4th Edition, 2002, p. 50.

Test Solution— For a solid for injection, constitute with the appropriate diluent as specified in the instructions on the labeling. For solutions, use the sample as is. [note—A solution can be diluted to bring it within the range of measurement of the osmometer, if necessary, but the results must be expressed as that of the diluted solution and must NOT be multiplied by a dilution factor to calculate the osmolality of the original solution, unless otherwise indicated in the monograph. The molal osmotic coefficient is a function of concentration. Therefore, it changes with dilution.]

Procedure— First, calibrate the instrument by the manufacturer's instructions. Confirm the instrument calibration with at least two solutions from Table 1 such that the osmolalities of the Standard Solutions span the expected range of osmolality of the Test Solution. The instrument reading should be within  $\pm 2$  mOsmol/kg from the Standard Solution (over the standard range of 100 to 700 mOsmol/kg). Introduce an appropriate volume of each Standard Solution into the measurement cell as per the manufacturer's instructions, and start the cooling system. Usually, the mixing device is programmed to operate at a temperature below the lowest temperature expected from the freezing point depression. The apparatus indicates when the equilibrium is attained. Calibrate the osmometer using an appropriate adjustment device such that the reading corresponds to either the osmolality or freezing point depression value of the Standard Solution shown in Table 1. [note—Some instruments indicate osmolality and some others show freezing point depression.] Before each measurement, rinse the measurement cell at least twice with the solution to be tested. Repeat the procedure with each Test Solution. Read the osmolality of the Test Solution directly, or calculate it from the measured freezing point depression.

Assuming that the value of the osmotic coefficient is essentially the same whether the concentration is expressed in molality or molarity, the experimentally determined osmolality of a solution can be converted to osmolarity in the same manner in which the concentration of a solution is converted from molality to molarity. Unless a solution is very concentrated, the osmolarity of a solution ( $\bar{c}$ ) can be calculated from its experimentally determined osmolality ( $\bar{m}$ ):

$$\bar{c} = 1000 \bar{m} / (1000 / \rho + \sum w_i V_i)$$

where  $w_i$  is the weight in g; and  $V_i$  is the partial specific volume, in mL per g, of the  $i$ th solute. The partial specific volume of a solute is the change in volume of a solution when an additional 1 g of solute is dissolved in the solution. This volume can be determined by the measurement of densities of the solution before and after the addition of the solute. The partial specific volumes of salts are generally very small, around 0.1 mL per g. However, those of other solutes are generally higher. For example, the partial specific volumes of amino acids are in the range of 0.6–0.9 mL per g. It can be shown from the above equation correlating osmolarity with osmolality that,

$$\bar{c} = \bar{m} (\rho - c)$$

where  $\rho$  is the density of the solution, and  $c$  is the total solute concentration, both expressed in g per mL. Thus, alternatively, the osmolarity can also be calculated from experimentally determined osmolality from the measurement of density of the solution by a suitable method and the total weight of the solute, after correction for water content, dissolved per mL of the solution.

1 Kastango, E.S. and Hadaway, L. International Journal of Pharmaceutical Compounding 5, (2001) 465-469.

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#### 786 PARTICLE SIZE DISTRIBUTION ESTIMATION BY ANALYTICAL SIEVING

Sieving is one of the oldest methods of classifying powders and granules by particle size distribution. When using a woven sieve cloth, the sieving will essentially sort the particles by their intermediate size dimension (i.e., breadth or width). Mechanical sieving is most suitable where the majority of the particles are larger than about 75  $\mu$ m. For smaller particles, the light weight provides insufficient force during sieving to overcome the surface forces of cohesion and adhesion that cause the particles to stick to each other and to the sieve, and thus cause particles that would be expected to pass through the sieve to be retained. For such materials, other means of agitation such as air-jet sieving or sonic sifting may be more appropriate. Nevertheless, sieving can sometimes be used for some powders or granules having median particle sizes smaller than 75  $\mu$ m where the method can be validated. In pharmaceutical terms, sieving is usually the method of choice for classification of the coarser grades of single powders or granules. It is a particularly attractive method in that powders and granules are classified only on the basis of particle size, and in most cases the analysis can be carried out in the dry state.

Among the limitations of the sieving method are the need for an appreciable amount of sample (normally at least 25 g, depending on the density of the powder or granule, and the diameter of test sieves) and difficulty in sieving oily or other cohesive powders or granules that tend to clog the sieve openings. The method is essentially a two-dimensional estimate of size because passage through the sieve aperture is frequently more dependent on maximum width and thickness than on length.

This method is intended for estimation of the total particle size distribution of a single material. It is not intended for determination of the proportion of particles passing or retained on one or two sieves.

Estimate the particle size distribution as described under Dry Sieving Method, unless otherwise specified in the individual monograph. Where difficulty is experienced in reaching the endpoint (i.e., material does not readily pass through the sieves) or when it is necessary to use the finer end of the sieving range (below 75  $\mu$ m), serious consideration should be given

to the use of an alternative particle-sizing method.

Sieving should be carried out under conditions that do not cause the test sample to gain or lose moisture. The relative humidity of the environment in which the sieving is carried out should be controlled to prevent moisture uptake or loss by the sample. In the absence of evidence to the contrary, analytical test sieving is normally carried out at ambient humidity.

Any special conditions that apply to a particular material should be detailed in the individual monograph.

**Principles of Analytical Sieving**— Analytical test sieves are constructed from a woven-wire mesh, which is of simple weave that is assumed to give nearly square apertures and is sealed into the base of an open cylindrical container. The basic analytical method involves stacking the sieves on top of one another in ascending degrees of coarseness, and then placing the test powder on the top sieve.

The nest of sieves is subjected to a standardized period of agitation, and then the weight of material retained on each sieve is accurately determined. The test gives the weight percentage of powder in each sieve size range.

This sieving process for estimating the particle size distribution of a single pharmaceutical powder is generally intended for use where at least 80% of the particles are larger than 75  $\mu\text{m}$ . The size parameter involved in determining particle size distribution by analytical sieving is the length of the side of the minimum square aperture through which the particle will pass.

#### TEST SIEVES

Test sieves suitable for pharmacopeial tests conform to the most current edition of International Organization for Standardization Specification ISO 3310-1: Test Sieves—Technical Requirements and Testing (see [Table 1](#)). Unless otherwise specified in the monograph, use those ISO sieves listed as principal sizes in [Table 1](#). Unless otherwise specified in the monograph, use those ISO sieves listed in [Table 1](#) as recommended in the particular region.

Table 1. Sizes of Standard Sieve Series in Range of Interest

ISO Nominal Aperture			US Sieve No.	Recommended USP Sieves (mesh)	European Sieve No.	Japan Sieve No.
Principal Sizes	Supplementary Sizes					
R 20/3	R 20	R 40/3				
11.20 mm	11.20 mm	11.20 mm			11200	
	10.00 mm					
		9.50 mm				
		9.00 mm				
8.00 mm	8.00 mm	8.00 mm				
	7.10 mm					
		6.70 mm				
		6.30 mm				
5.60 mm	5.60 mm	5.60 mm			5600	3.5
	5.00 mm					
		4.75 mm				4
		4.50 mm				
4.00 mm	4.00 mm	4.00 mm	5	4000	4000	4.7
	3.55 mm					
		3.35 mm	6			5.5
		3.15 mm				
2.80 mm	2.80 mm	2.80 mm	7	2800	2800	6.5
	2.50 mm					
		2.36 mm	8			7.5
		2.24 mm				
2.00 mm	2.00 mm	2.00 mm	10	2000	2000	8.6
	1.80 mm					
		1.70 mm	12			10
		1.60 mm				
1.40 mm	1.40 mm	1.40 mm	14	1400	1400	12
	1.25 mm					
		1.18 mm	16			14
		1.12 mm				
1.00 mm	1.00 mm	1.00 mm	18	1000	1000	16
	900 $\mu\text{m}$					
		850 $\mu\text{m}$	20			18
		800 $\mu\text{m}$				
710 $\mu\text{m}$	710 $\mu\text{m}$	710 $\mu\text{m}$	25	710	710	22
	630 $\mu\text{m}$					
		600 $\mu\text{m}$	30			26
		560 $\mu\text{m}$				
500 $\mu\text{m}$	500 $\mu\text{m}$	500 $\mu\text{m}$	35	500	500	30
	450 $\mu\text{m}$					
		425 $\mu\text{m}$	40			36
		400 $\mu\text{m}$				
355 $\mu\text{m}$	355 $\mu\text{m}$	355 $\mu\text{m}$	45	355	355	42
	315 $\mu\text{m}$					



		300 $\mu\text{m}$	50			50
	280 $\mu\text{m}$					
250 $\mu\text{m}$	250 $\mu\text{m}$	250 $\mu\text{m}$	60	250	250	60
	224 $\mu\text{m}$					
		212 $\mu\text{m}$	70			70
	200 $\mu\text{m}$					
180 $\mu\text{m}$	180 $\mu\text{m}$	180 $\mu\text{m}$	80	180	180	83
	160 $\mu\text{m}$					
		150 $\mu\text{m}$	100			100
	140 $\mu\text{m}$					
125 $\mu\text{m}$	125 $\mu\text{m}$	125 $\mu\text{m}$	120	125	125	119
	112 $\mu\text{m}$					
		106 $\mu\text{m}$	140			140
	100 $\mu\text{m}$					
90 $\mu\text{m}$	90 $\mu\text{m}$	90 $\mu\text{m}$	170	90	90	166
	80 $\mu\text{m}$					
		75 $\mu\text{m}$	200			200
	71 $\mu\text{m}$					
63 $\mu\text{m}$	63 $\mu\text{m}$	63 $\mu\text{m}$	230	63	63	235
	56 $\mu\text{m}$					
		53 $\mu\text{m}$	270			282
	50 $\mu\text{m}$					
45 $\mu\text{m}$	45 $\mu\text{m}$	45 $\mu\text{m}$	325	45	45	330
	40 $\mu\text{m}$					
		38 $\mu\text{m}$			38	391

Sieves are selected to cover the entire range of particle sizes present in the test specimen. A nest of sieves having a  $\sqrt{2}$  progression of the area of the sieve openings is recommended. The nest of sieves is assembled with the coarsest screen at the top and the finest at the bottom. Use micrometers or millimeters in denoting test sieve openings. [note—Mesh numbers are provided in the table for conversion purposes only.] Test sieves are made from stainless steel or, less preferably, from brass or other suitable nonreactive wire.

Calibration and recalibration of test sieves is in accordance with the most current edition of ISO 3310-1. Sieves should be carefully examined for gross distortions and fractures, especially at their screen frame joints, before use. Sieves may be calibrated optically to estimate the average opening size, and opening variability, of the sieve mesh. Alternatively, for the evaluation of the effective opening of test sieves in the size range of 212 to 850  $\mu\text{m}$ , Standard Glass Spheres are available. Unless otherwise specified in the individual monograph, perform the sieve analysis at controlled room temperature and at ambient relative humidity.

**Cleaning Test Sieves**— Ideally, test sieves should be cleaned using only an air jet or a liquid stream. If some apertures remain blocked by test particles, careful gentle brushing may be used as a last resort.

**Test Specimen**— If the test specimen weight is not given in the monograph for a particular material, use a test specimen having a weight between 25 and 100 g, depending on the bulk density of the material, and test sieves having a 200-mm diameter. For 76-mm sieves, the amount of material that can be accommodated is approximately 1/7th that which can be accommodated on a 200-mm sieve. Determine the most appropriate weight for a given material by test sieving accurately weighed specimens of different weights, such as 25, 50, and 100 g, for the same time period on a mechanical shaker. [note—If the test results are similar for the 25-g and 50-g specimens, but the 100-g specimen shows a lower percentage through the finest sieve, the 100-g specimen size is too large.] Where only a specimen of 10 to 25 g is available, smaller diameter test sieves conforming to the same mesh specifications may be substituted, but the endpoint must be redetermined. The use of test samples having a smaller mass (e.g., down to 5 g) may be needed. For materials with low apparent particle density, or for materials mainly comprising particles with a highly isodiametrical shape, specimen weights below 5 g for a 200-mm screen may be necessary to avoid excessive blocking of the sieve. During validation of a particular sieve analysis method, it is expected that the problem of sieve blocking will have been addressed.

If the test material is prone to picking up or losing significant amounts of water with varying humidity, the test must be carried out in an appropriately controlled environment. Similarly, if the test material is known to develop an electrostatic charge, careful observation must be made to ensure that such charging is not influencing the analysis. An antistatic agent, such as colloidal silicon dioxide and/or aluminum oxide, may be added at a 0.5 percent (m/m) level to minimize this effect. If both of the above effects cannot be eliminated, an alternative particle-sizing technique must be selected.

**Agitation Methods**— Several different sieve and powder agitation devices are commercially available, all of which may be used to perform sieve analyses. However, the different methods of agitation may give different results for sieve analyses and endpoint determinations because of the different types and magnitude of the forces acting on the individual particles under test. Methods using mechanical agitation or electromagnetic agitation, and that can induce either a vertical oscillation or a horizontal circular motion, or tapping or a combination of both tapping and horizontal circular motion are available. Entrainment of the particles in an air stream may also be used. The results must indicate which agitation method was used and the agitation parameters used (if they can be varied), because changes in the agitation conditions will give different results for the sieve analysis and endpoint determinations, and may be sufficiently different to give a failing result under some circumstances.

**Endpoint Determination**— The test sieving analysis is complete when the weight on any of the test sieves does not change by more than 5% or 0.1 g (10% in the case of 76-mm sieves) of the previous weight on that sieve. If less than 5% of the total specimen weight is present on a given sieve, the endpoint for that sieve is increased to a weight change of not more than 20% of the previous weight on that sieve.

If more than 50% of the total specimen weight is found on any one sieve, unless this is indicated in the monograph, the test should be repeated, but with the addition to the sieve nest of a more coarse sieve, intermediate between that carrying the excessive weight and the next coarsest sieve in the original nest, i.e., addition of the ISO series sieve omitted from the nest of sieves.

#### SIEVING METHODS

##### Mechanical Agitation

**Dry Sieving Method**— Tare each test sieve to the nearest 0.1 g. Place an accurately weighed quantity of test specimen on the top (coarsest) sieve, and replace the lid. Agitate the nest of sieves for 5 minutes. Then carefully remove each from the nest without loss of material. Reweigh each sieve, and determine the weight of material on each sieve. Determine the weight of material in the collecting pan in a similar manner. Reassemble the nest of sieves, and agitate for 5 minutes. Remove and weigh each sieve as previously described. Repeat these steps until the endpoint criteria are met (see Endpoint Determination under Test Sieves). Upon completion of the analysis, reconcile the weights of material. Total losses must not exceed 5% of the weight of the original test specimen.

Repeat the analysis with a fresh specimen, but using a single sieving time equal to that of the combined times used above. Confirm that this sieving time conforms to the requirements



endpoint determination. When this endpoint has been validated for a specific material, then a single fixed time of sieving may be used for future analyses, providing the particle size distribution falls within normal variation.

If there is evidence that the particles retained on any sieve are aggregates rather than single particles, the use of mechanical dry sieving is unlikely to give good reproducibility, and a different particle size analysis method should be used.

#### Air Entrainment Methods

Air Jet and Sonic Sifter Sieving— Different types of commercial equipment that use a moving air current are available for sieving. A system that uses a single sieve at a time is referred to as air jet sieving. It uses the same general sieving methodology as that described under the Dry Sieving Method, but with a standardized air jet replacing the normal agitation mechanism. It requires sequential analyses on individual sieves starting with the finest sieve to obtain a particle size distribution. Air jet sieving often includes the use of finer test sieves than those used in ordinary dry sieving. This technique is more suitable where only oversize or undersize fractions are needed.

In the sonic sifting method, a nest of sieves is used, and the test specimen is carried in a vertically oscillating column of air that lifts the specimen and then carries it back against the mesh openings at a given number of pulses per minute. It may be necessary to lower the sample amount to 5 g, when sonic sifting is employed.

The air jet sieving and sonic sieving methods may be useful for powders or granules when mechanical sieving techniques are incapable of giving a meaningful analysis.

These methods are highly dependent upon proper dispersion of the powder in the air current. This requirement may be hard to achieve if the method is used at the lower end of the sieving range (i.e., below 75  $\mu$ m), when the particles tend to be more cohesive, and especially if there is any tendency for the material to develop an electrostatic charge. For the above reasons endpoint determination is particularly critical, and it is very important to confirm that the oversize material comprises single particles and is not composed of aggregates.

#### INTERPRETATION

The raw data must include the weight of test specimen, the total sieving time, and the precise sieving methodology and the set values for any variable parameters, in addition to the weights retained on the individual sieves and in the pan. It may be convenient to convert the raw data into a cumulative weight distribution, and if it is desired to express the distribution in terms of a cumulative weight undersize, the range of sieves used should include a sieve through which all the material passes. If there is evidence on any of the test sieves that the material remaining on it is composed of aggregates formed during the sieving process, the analysis is invalid.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Robert H. Lafaver, B.A.</a> Scientist 1-301-816-8335	(EGC05) Excipient General Chapters

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#### 788 PARTICULATE MATTER IN INJECTIONS

This general chapter is harmonized with the corresponding texts of the European Pharmacopoeia and/or the Japanese Pharmacopoeia. These pharmacopeias have undertaken not to make any unilateral change to this harmonized chapter. Portions of the present general chapter text that are national USP text, and therefore not part of the harmonized text, are marked with symbols (♦♦) to specify this fact.

Particulate matter in injections and parenteral infusions consists of mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions.

For the determination of particulate matter, two procedures, Method 1 (Light Obscuration Particle Count Test) and Method 2 (Microscopic Particle Count Test), are specified hereinafter. When examining injections and parenteral infusions for subvisible particles, Method 1 is preferably applied. However, it may be necessary to test some preparations by the Light Obscuration Particle Count Test followed by the Microscopic Particle Count Test to reach a conclusion on conformance to the requirements.

Not all parenteral preparations can be examined for subvisible particles by one or both of these methods. When Method 1 is not applicable, e.g., in the case of preparations having reduced clarity or increased viscosity, the test should be carried out according to Method 2. Emulsions, colloids, and liposomal preparations are examples. Similarly, products that produce air or gas bubbles when drawn into the sensor may also require microscopic particle count testing. If the viscosity of the preparation to be tested is sufficiently high so as to preclude its examination by either test method, a quantitative dilution with an appropriate diluent may be made to decrease viscosity, as necessary, to allow the analysis to be performed.

The results obtained in examining a discrete unit or group of units for particulate matter cannot be extrapolated with certainty to other units that remain untested. Thus, statistically sound sampling plans must be developed if valid inferences are to be drawn from observed data to characterize the level of particulate matter in a large group of units.

#### METHOD 1 LIGHT OBSCURATION PARTICLE COUNT TEST

Use a suitable apparatus based on the principle of light blockage that allows for an automatic determination of the size of particles and the number of particles according to size. The definition for particle-free water is provided in Reagent Specifications under Reagents, Indicators and Solutions.

The apparatus is calibrated using dispersions of spherical particles of known sizes between 10  $\mu$ m and 25  $\mu$ m. These standard particles are dispersed in particle-free water. Care must be taken to avoid aggregation of particles during dispersion. ♦System suitability can be verified by using the [USP Particle Count RS](#).♦

##### General Precautions

The test is carried out under conditions limiting particulate matter, preferably in a laminar flow cabinet.

Very carefully wash the glassware and filtration equipment used, except for the membrane filters, with a warm detergent solution, and rinse with abundant amounts of water to remove all traces of detergent. Immediately before use, rinse the equipment from top to bottom, outside and then inside, with particle-free water.

Take care not to introduce air bubbles into the preparation to be examined, especially when fractions of the preparation are being transferred to the container in which the determination is to be carried out.

In order to check that the environment is suitable for the test, that the glassware is properly cleaned, and that the water to be used is particle-free, the following test is carried out: determine the particulate matter in 5 samples of particle-free water, each of 5 mL, according to the method described below. If the number of particles of 10  $\mu$ m or greater size exceeds 25 for the combined 25 mL, the precautions taken for the test are not sufficient. The preparatory steps must be repeated until the environment, glassware, and water are suitable for the test.

##### Method

Mix the contents of the sample by slowly inverting the container 20 times successively. If necessary, cautiously remove the sealing closure. Clean the outer surfaces of the container opening using a jet of particle-free water and remove the closure, avoiding any contamination of the contents. Eliminate gas bubbles by appropriate measures such as allowing to stand for 2 minutes or sonicating.

For large-volume parenterals, single units are tested. For small-volume parenterals less than 25 mL in volume, the contents of 10 or more units are combined in a cleaned container to obtain a volume of not less than 25 mL; the test solution may be prepared by mixing the contents of a suitable number of vials and diluting to 25 mL with particle-free water or with an

appropriate particle-free solvent when particle-free water is not suitable. Small-volume parenterals having a volume of 25 mL or more may be tested individually.

Powders for parenteral use are reconstituted with particle-free water or with an appropriate particle-free solvent when particle-free water is not suitable.

The number of test specimens must be adequate to provide a statistically sound assessment. For large-volume parenterals or for small-volume parenterals having a volume of 25 mL or more, fewer than 10 units may be tested, using an appropriate sampling plan.

Remove four portions, not less than 5 mL each, and count the number of particles equal to or greater than 10  $\mu\text{m}$  and 25  $\mu\text{m}$ . Disregard the result obtained for the first portion, and calculate the mean number of particles for the preparation to be examined.

#### Evaluation

For preparations supplied in containers with a nominal volume of more than 100 mL, apply the criteria of Test 1.A.

For preparations supplied in containers with a nominal volume of less than 100 mL, apply the criteria of Test 1.B.

For preparations supplied in containers with a nominal volume of 100 mL, apply the criteria of Test 1.B. [note—Test 1.A is used in the Japanese Pharmacopeia.]

If the average number of particles exceeds the limits, test the preparation by the Microscopic Particle Count Test.

Test 1.A (Solutions for parenteral infusion or solutions for injection supplied in containers with a nominal content of more than 100 mL)—The preparation complies with the test if the average number of particles present in the units tested does not exceed 25 per mL equal to or greater than 10  $\mu\text{m}$  and does not exceed 3 per mL equal to or greater than 25  $\mu\text{m}$ .

Test 1.B (Solutions for parenteral infusion or solutions for injection supplied in containers with a nominal content of less than 100 mL)—The preparation complies with the test if the average number of particles present in the units tested does not exceed 6000 per container equal to or greater than 10  $\mu\text{m}$  and does not exceed 600 per container equal to or greater than 25  $\mu\text{m}$ .

### METHOD 2 MICROSCOPIC PARTICLE COUNT TEST

Use a suitable binocular microscope, a filter assembly for retaining particulate matter, and a membrane filter for examination.

The microscope is adjusted to  $100 \pm 10$  magnifications and is equipped with an ocular micrometer calibrated with an objective micrometer, a mechanical stage capable of holding and traversing the entire filtration area of the membrane filter, and two suitable illuminators to provide episcopic illumination in addition to oblique illumination.

The ocular micrometer is a circular diameter graticule (see [Figure 1](#))

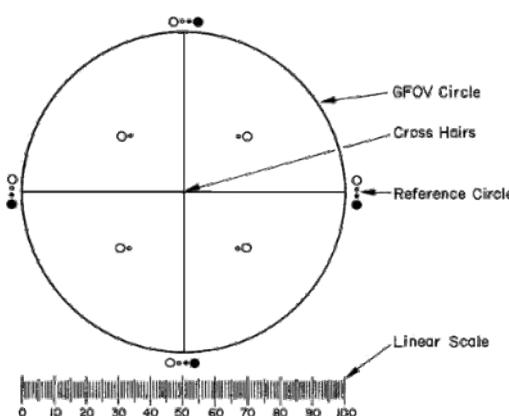


Fig. 1. Circular diameter graticule. The large circle divided by crosshairs into quadrants is designated the graticule field of view (GFOV). Transparent and black circles having 10- $\mu\text{m}$  and 25- $\mu\text{m}$  diameters at 100 $\times$  are provided as comparison scales for particle sizing.

and consists of a large circle divided by crosshairs into quadrants, transparent and black reference circles 10  $\mu\text{m}$  and 25  $\mu\text{m}$  in diameter at 100 magnifications, and a linear scale graduated in 10- $\mu\text{m}$  increments. It is calibrated using a stage micrometer that is certified by either a domestic or international standard institution. A relative error of the linear scale of the graticule within  $\pm 2\%$  is acceptable. The large circle is designated the graticule field of view (GFOV).

Two illuminators are required. One is an episcopic brightfield illuminator internal to the microscope, the other is an external, focusable auxiliary illuminator that can be adjusted to give reflected oblique illumination at an angle of 10° to 20°.

The filter assembly for retaining particulate matter consists of a filter holder made of glass or other suitable material, and is equipped with a vacuum source and a suitable membrane filter.

The membrane filter is of suitable size, black or dark gray in color, nongridded or gridded, and 1.0  $\mu\text{m}$  or finer in nominal pore size.

#### General Precautions

The test is carried out under conditions limiting particulate matter, preferably in a laminar flow cabinet.

Very carefully wash the glassware and filter assembly used, except for the membrane filter, with a warm detergent solution, and rinse with abundant amounts of water to remove all traces of detergent. Immediately before use, rinse both sides of the membrane filter and the equipment from top to bottom, outside and then inside, with particle-free water.

In order to check that the environment is suitable for the test, that the glassware and the membrane filter are properly cleaned, and that the water to be used is particle-free, the following test is carried out: determine the particulate matter of a 50-mL volume of particle-free water according to the method described below. If more than 20 particles 10  $\mu\text{m}$  or larger in size or if more than 5 particles 25  $\mu\text{m}$  or larger in size are present within the filtration area, the precautions taken for the test are not sufficient. The preparatory steps must be repeated until the environment, glassware, membrane filter, and water are suitable for the test.

#### Method

Mix the contents of the samples by slowly inverting the container 20 times successively. If necessary, cautiously remove the sealing closure. Clean the outer surfaces of the container opening using a jet of particle-free water and remove the closure, avoiding any contamination of the contents.

For large-volume parenterals, single units are tested. For small-volume parenterals less than 25 mL in volume, the contents of 10 or more units are combined in a cleaned container; the test solution may be prepared by mixing the contents of a suitable number of vials and diluting to 25 mL with particle-free water or with an appropriate particle-free solvent when particle-free water is not suitable. Small-volume parenterals having a volume of 25 mL or more may be tested individually.

Powders for parenteral use are constituted with particle-free water or with an appropriate particle-free solvent when particle-free water is not suitable.

The number of test specimens must be adequate to provide a statistically sound assessment. For large-volume parenterals or for small-volume parenterals having a volume of 25 mL or more, fewer than 10 units may be tested, using an appropriate sampling plan.



Wet the inside of the filter holder fitted with the membrane filter with several mL of particle-free water. Transfer to the filtration funnel the total volume of a solution pool or of a single unit, and apply a vacuum. If needed, add stepwise a portion of the solution until the entire volume is filtered. After the last addition of solution, begin rinsing the inner walls of the filter holder by using a jet of particle-free water. Maintain the vacuum until the surface of the membrane filter is free from liquid. Place the membrane filter in a Petri dish, and allow the membrane filter to air-dry with the cover slightly ajar. After the membrane filter has been dried, place the Petri dish on the stage of the microscope, scan the entire membrane filter under the reflected light from the illuminating device, and count the number of particles that are equal to or greater than 10  $\mu\text{m}$  and the number of particles that are equal to or greater than 25  $\mu\text{m}$ . Alternatively, partial membrane filter count and determination of the total filter count by calculation is allowed. Calculate the mean number of particles for the preparation to be examined.

The particle sizing process with the use of the circular diameter graticule is carried out by estimating the equivalent diameter of the particle in comparison with the 10  $\mu\text{m}$  and 25  $\mu\text{m}$  reference circles on the graticule. Thereby the particles are not moved from their initial locations within the graticule field of view and are not superimposed on the reference circles for comparison. The inner diameter of the transparent graticule reference circles is used to size white and transparent particles, while dark particles are sized by using the outer diameter of the black opaque graticule reference circles.

In performing the Microscopic Particle Count Test, do not attempt to size or enumerate amorphous, semiliquid, or otherwise morphologically indistinct materials that have the appearance of a stain or discoloration on the membrane filter. These materials show little or no surface relief and present a gelatinous or film-like appearance. In such cases, the interpretation of enumeration may be aided by testing a sample of the solution by the Light Obscuration Particle Count Test.

#### Evaluation

For preparations supplied in containers with a nominal volume of more than 100 mL, apply the criteria of Test 2.A.

For preparations supplied in containers with a nominal volume of less than 100 mL, apply the criteria of Test 2.B.

For preparations supplied in containers with a nominal volume of 100 mL, apply the criteria of Test 2.B. [note—Test 2.A is used in the Japanese Pharmacopeia.]

Test 2.A (Solutions for parenteral infusion or solutions for injection supplied in containers with a nominal content of more than 100 mL)—The preparation complies with the test if the average number of particles present in the units tested does not exceed 12 per mL equal to or greater than 10  $\mu\text{m}$  and does not exceed 2 per mL equal to or greater than 25  $\mu\text{m}$ .

Test 2.B (Solutions for parenteral infusion or solutions for injection supplied in containers with a nominal content of less than 100 mL)—The preparation complies with the test if the average number of particles present in the units tested does not exceed 3000 per container equal to or greater than 10  $\mu\text{m}$  and does not exceed 300 per container equal to or greater than 25  $\mu\text{m}$ .

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Desmond G. Hunt, Ph.D.</a> Scientist 1-301-816-8341	(PPI05) Parenteral Products-Industrial 05

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#### 789 PARTICULATE MATTER IN OPHTHALMIC SOLUTIONS

Particulate matter consists of mobile, randomly sourced, extraneous substances, other than gas bubbles, that cannot be quantitated by chemical analysis because of the small amount of material they represent and because of their heterogeneous composition. Ophthalmic solutions should be essentially free from particles that can be observed on visual inspection.

The tests described herein are physical tests performed for the purpose of enumerating extraneous particles within specific size ranges.

Every ophthalmic solution for which the monograph includes a test for Particulate matter is subject to the particulate matter limits set forth for the test being applied, unless otherwise specified in the individual monograph. When higher limits are appropriate, they will be specified in the individual monograph. Ophthalmic preparations that are suspensions, emulsions, or gels are exempt from these requirements, as are medical devices. Refer to the specific monograph when a question of test applicability occurs.

Light obscuration and microscopic procedures for the determination of particulate matter in ophthalmic solutions are identical to those for injections; therefore, where appropriate, [Particulate Matter in Injections](#) 788 is cross-referenced. This chapter provides a test approach in two stages. The ophthalmic solution is first tested by the light obscuration procedure (stage 1). If it fails to meet the prescribed limits, it must pass the microscopic procedure (stage 2) with its own set of test limits. Where for technical reasons the ophthalmic solution cannot be tested by light obscuration, microscopic testing may be used exclusively. Documentation is required, demonstrating that the light obscuration procedure is incapable of testing the ophthalmic solution or that it produces invalid results.

It is expected that most articles will meet the requirements on the basis of the light obscuration test alone; however, it may be necessary to test some articles by the light obscuration test followed by the microscopic test to reach a conclusion on conformance to requirements. Any product that is not a pure solution having a clarity and a viscosity approximating those of water may provide erroneous data when analyzed by the light obscuration counting method. Such materials may be analyzed by the microscopic counting method. In some instances, the viscosity of a material to be tested may be sufficiently high so as to preclude its analysis by either test method. In this event, a quantitative dilution with an appropriate diluent may be made to decrease viscosity, as necessary, to allow the analysis to be performed.

In the tests described below, the results obtained by examining a discrete unit or group of units for particulate matter cannot be extrapolated with certainty to other units that remain untested. Thus, sampling plans based on known operational factors must be developed if valid inferences are to be drawn from observed data to characterize the level of particulate matter in a large group of units. Sampling plans need to be based on consideration of product volume, particle numbers historically found to be present in comparison to limits, particle size distribution of particles present, and variability of particle counts between units.

#### LIGHT OBSCURATION PARTICLE COUNT TEST

This test applies to ophthalmic solutions, including solutions constituted from sterile solids, for which a test for Particulate matter is specified in the individual monograph. The test counts suspended particles that are solid or liquid.

Test Apparatus, Instrument Standardization, Test Environment, Test Procedure, and Calculations— Proceed as directed for Light Obscuration Particle Count Test under [Particulate Matter in Injections](#) 788.

Interpretation— The ophthalmic solution meets the requirements of the test if the average number of particles present in the units tested does not exceed the appropriate value listed in [Table 1](#). If the average number of particles exceeds the limit, test the article by the Microscopic Particle Count Test.

Table 1. Light Obscuration Test Particle Count

	Diameter	
	$\geq 10 \mu\text{m}$	$\geq 25 \mu\text{m}$
Number of particles	50 per mL	5 per mL

#### MICROSCOPIC PARTICLE COUNT TEST

Some articles cannot be tested meaningfully by light obscuration. In such cases, individual monographs clearly specify that only a microscopic particle count is to be performed. The



Microscopic particle count test enumerates subvisible, essentially solid, particulate matter in ophthalmic solutions, after collection on a microporous membrane filter. Some ophthalmic solutions, such as solutions that do not filter readily because of their high viscosity, may be exempted from analysis using the microscopic test.

When performing the microscopic test, do not attempt to size or enumerate amorphous, semiliquid, or otherwise morphologically indistinct materials that have the appearance of a stain or discoloration on the membrane surface. These materials show little or no surface relief and present a gelatinous or film-like appearance. Because in solution this material consists of units on the order of 1  $\mu\text{m}$  or less, which may be counted only after aggregation or deformation on an analytical membrane, interpretation of enumeration may be aided by testing a sample of the solution by the light obscuration particle count method.

Test Apparatus, Test Environment, Test Procedure, and Enumeration of Particles— Proceed as directed for Microscopic Particle Count Test under [Particulate Matter in Injections](#) (788).

Interpretation— The ophthalmic solution meets the requirements of the test if the average number of particles present in the units tested does not exceed the appropriate value listed in [Table 2](#).

Table 2. Microscopic Method Particle Count

	Diameter		
	$\geq 10 \mu\text{m}$	$\geq 25 \mu\text{m}$	$\geq 50 \mu\text{m}$
Number of particles	50 per mL	5 per mL	2 per mL

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Desmond G. Hunt, Ph.D.</a> Scientist 1-301-816-8341	(PPI05) Parenteral Products-Industrial 05

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(791) pH

For compendial purposes, pH is defined as the value given by a suitable, properly standardized, potentiometric instrument (pH meter) capable of reproducing pH values to 0.02 pH unit using an indicator electrode sensitive to hydrogen-ion activity, the glass electrode, and a suitable reference electrode. The instrument should be capable of sensing the potential across the electrode pair and, for pH standardization purposes, applying an adjustable potential to the circuit by manipulation of "standardization," "zero," "asymmetry," or "calibration" control, and should be able to control the change in millivolts per unit change in pH reading through a "temperature" and/or "slope" control. Measurements are made at  $25 \pm 2^\circ$ , unless otherwise specified in the individual monograph or herein.

The pH scale is defined by the equation:

$$\text{pH} = \text{pHs} + (E - ES) / k$$

in which E and ES are the measured potentials where the galvanic cell contains the solution under test, represented by pH, and the appropriate Buffer Solution for Standardization, represented by pHs, respectively. The value of k is the change in potential per unit change in pH and is theoretically  $[0.05916 + 0.000198(t - 25^\circ)]$  volts at any temperature t.

It should be emphasized that the definitions of pH, the pH scale, and the values assigned to the Buffer Solutions for Standardization are for the purpose of establishing a practical, operational system so that results may be compared between laboratories. The pH values thus measured do not correspond exactly to those obtained by the definition,  $\text{pH} = -\log a\text{H}^+$ . So long as the solution being measured is sufficiently similar in composition to the buffer used for standardization, the operational pH corresponds fairly closely to the theoretical pH. Although no claim is made with respect to the suitability of the system for measuring hydrogen-ion activity or concentration, the values obtained are closely related to the activity of the hydrogen-ion in aqueous solutions.

Where a pH meter is standardized by use of an aqueous buffer and then used to measure the "pH" of a nonaqueous solution or suspension, the ionization constant of the acid or base, the dielectric constant of the medium, the liquid-junction potential (which may give rise to errors of approximately 1 pH unit), and the hydrogen-ion response of the glass electrode are all changed. For these reasons, the values so obtained with solutions that are only partially aqueous in character can be regarded only as apparent pH values.

#### BUFFER SOLUTIONS FOR STANDARDIZATION OF THE pH METER

Buffer Solutions for Standardization are to be prepared as directed in the accompanying table.\* Buffer salts of requisite purity can be obtained from the National Institute of Science and Technology. Solutions may be stored in hard glass or polyethylene bottles fitted with a tight closure or carbon dioxide-absorbing tube (soda lime). Fresh solutions should be prepared at intervals not to exceed 3 months using carbon dioxide-free water. The table indicates the pH of the buffer solutions as a function of temperature. The instructions presented here are for the preparation of solutions having the designated molal (m) concentrations. For convenience, and to facilitate their preparation, however, instructions are given in terms of dilution to a 1000-mL volume rather than specifying the use of 1000 g of solvent, which is the basis of the molality system of solution concentration. The indicated quantities cannot be computed simply without additional information.

#### pH Values of Buffer Solutions for Standardization

Temperature, $^\circ\text{C}$	Potassium Tetraoxalate, 0.05 m	Potassium Biphthalate, 0.05 m	Equimolar Phosphate, 0.05 m	Sodium Tetraborate, 0.01 m	Calcium Hydroxide, Saturated at $25^\circ$
10	1.67	4.00	6.92	9.33	13.00
15	1.67	4.00	6.90	9.28	12.81
20	1.68	4.00	6.88	9.23	12.63
25	1.68	4.01	6.86	9.18	12.45
30	1.68	4.02	6.85	9.14	12.29
35	1.69	4.02	6.84	9.10	12.13
40	1.69	4.04	6.84	9.07	11.98
45	1.70	4.05	6.83	9.04	11.84
50	1.71	4.06	6.83	9.01	11.71
55	1.72	4.08	6.83	8.99	11.57
60	1.72	4.09	6.84	8.96	11.45

Potassium Tetraoxalate, 0.05 m— Dissolve 12.61 g of  $\text{KH}_3(\text{C}_2\text{O}_4)_2 \cdot 2\text{H}_2\text{O}$  in water to make 1000 mL.

Potassium Biphthalate, 0.05 m— Dissolve 10.12 g of  $\text{KHC}_8\text{H}_4\text{O}_4$ , previously dried at  $110^\circ$  for 1 hour, in water to make 1000 mL.



Equimolar Phosphate, 0.05 m— Dissolve 3.53 g of Na<sub>2</sub>HPO<sub>4</sub> and 3.39 g of KH<sub>2</sub>PO<sub>4</sub>, each previously dried at 120° for 2 hours, in water to make 1000 mL.

Sodium Tetraborate, 0.01 m— Dissolve 3.80 g of Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>·10H<sub>2</sub>O in water to make 1000 mL. Protect from absorption of carbon dioxide.

Calcium Hydroxide, saturated at 25°— Shake an excess of calcium hydroxide with water, and decant at 25° before use. Protect from absorption of carbon dioxide.

Because of variations in the nature and operation of the available pH meters, it is not practicable to give universally applicable directions for the potentiometric determinations of pH. The general principles to be followed in carrying out the instructions provided for each instrument by its manufacturer are set forth in the following paragraphs. Examine the electrodes and, if present, the salt bridge prior to use. If necessary, replenish the salt bridge solution, and observe other precautions indicated by the instrument or electrode manufacturer.

To standardize the pH meter, select two Buffer Solutions for Standardization whose difference in pH does not exceed 4 units and such that the expected pH of the material under test falls between them. Fill the cell with one of the Buffer Solutions for Standardization at the temperature at which the test material is to be measured. Set the "temperature" control at the temperature of the solution, and adjust the calibration control to make the observed pH value identical with that tabulated. Rinse the electrodes and cell with several portions of the second Buffer Solution for Standardization, then fill the cell with it, at the same temperature as the material to be measured. The pH of the second buffer solution is within ±0.07 pH unit of the tabulated value. If a larger deviation is noted, examine the electrodes and, if they are faulty, replace them. Adjust the "slope" or "temperature" control to make the observed pH value identical with that tabulated. Repeat the standardization until both Buffer Solutions for Standardization give observed pH values within 0.02 pH unit of the tabulated value without further adjustment of the controls. When the system is functioning satisfactorily, rinse the electrodes and cell several times with a few portions of the test material, fill the cell with the test material, and read the pH value. Use carbon dioxide-free water (see Water in the section [Reagents, Indicators, and Solutions](#)) for solution or dilution of test material in pH determinations. In all pH measurements, allow a sufficient time for stabilization.

Where approximate pH values suffice, indicators and test papers (see Indicators and Indicator Test Papers, in the section [Reagents, Indicators, and Solutions](#)) may be suitable.

For a discussion of buffers, and for the composition of standard buffer solutions called for in compendial tests and assays, see Buffer Solutions in the section [Reagents, Indicators, and Solutions](#).

\* Commercially available buffer solutions for pH meter standardization, standardized by methods traceable to the National Institute of Standards and Technology (NIST), labeled with a pH value accurate to 0.01 pH unit may be used. For standardization solutions having a pH lower than 4, a labeled accuracy of 0.02 is acceptable. Solutions prepared from ACS reagent grade materials or other suitable materials, in the stated quantities, may be used provided the pH of the resultant solution is the same as that of the solution prepared from the NIST certified material.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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#### 795 PHARMACEUTICAL COMPOUNDING—NONSTERILE PREPARATIONS

For the purposes of this chapter, the pharmacist or other licensed health care professional responsible for preparing the compounded preparations is referred to as "compounder".

Compounding is an integral part of pharmacy practice and is essential to the provision of health care. The purpose of this chapter and applicable monographs on formulation is to help define what constitutes good compounding practices and to provide general information to enhance the compounder's ability in the compounding facility to extemporaneously compound preparations that are of acceptable strength, quality, and purity.

Compounding is different from manufacturing, which is guided by GMPs (see [Good Manufacturing Practices for Bulk Pharmaceutical Excipients](#)). Some of the characteristics or criteria that differentiate compounding from manufacturing include the existence of specific practitioner–patient–compounder relationships; the quantity of medication prepared in anticipation of receiving a prescription or a prescription order; and the conditions of sale, which are limited to specific prescription orders.

The pharmacist's responsibilities in compounding drug preparations are to dispense the finished preparation in accordance with a prescription or a prescriber's order or intent and to dispense those preparations in compliance with the requirements established by the Boards of Pharmacy and other regulatory agencies. Compounders must be familiar with statutes and regulations that govern compounding because these requirements vary from state to state.

The compounder is responsible for compounding preparations of acceptable strength, quality, and purity with appropriate packaging and labeling in accordance with good compounding practices (see [Good Compounding Practices](#)), official standards, and relevant scientific data and information. Compounders engaging in compounding should have to continually expand their compounding knowledge by participating in seminars, studying appropriate literature, and consulting colleagues.

#### RESPONSIBILITY OF THE COMPOUNDER

The compounder is responsible for ensuring that the quality is built into the compounded preparations of products, with key factors including at least the following general principles.

(See also [Good Compounding Practices](#).)

1. Personnel are capable and qualified to perform their assigned duties.
2. Ingredients used in compounding have their expected identity, quality, and purity.
3. Compounded preparations are of acceptable strength, quality, and purity, with appropriate packaging and labeling, and prepared in accordance with good compounding practices, official standards, and relevant scientific data and information.
4. Critical processes are validated to ensure that procedures, when used, will consistently result in the expected qualities in the finished preparation.
5. The compounding environment is suitable for its intended purpose.
6. Appropriate stability evaluation is performed or determined from the literature for establishing reliable beyond-use dating to ensure that the finished preparations have their expected potency, purity, quality, and characteristics, at least until the labeled beyond-use date.
7. There is assurance that processes are always carried out as intended or specified and are under control.
8. Compounding conditions and procedures are adequate for preventing errors.
9. Adequate procedures and records exist for investigating and correcting failures or problems in compounding, testing, or in the preparation itself.

#### COMPOUNDING ENVIRONMENT

##### Facilities

Areas designated for compounding have adequate space for the orderly placement of equipment and materials to prevent mixups between ingredients, containers, labels, in-process materials, and finished preparations. The compounding area is also to be designed, arranged, used, and maintained to prevent adventitious cross-contamination. Areas used for sterile preparations are to be separated and distinct from the nonsterile compounding area (see Environmental Quality and Control under [Pharmaceutical Compounding—Sterile Preparations](#)). The entire compounding area is to be well-lighted. Heating, ventilation, and air conditioning systems are to be controlled to avoid decomposition of chemicals (see Storage Temperature under Preservation, Packaging, Storage, and Labeling in the General Notices and Requirements and the manufacturers' labeled storage conditions). Storage areas provide an environment suitably controlled to ensure quality and stability of bulk chemicals and finished preparations.



Potable water is to be supplied for hand and equipment washing. This water meets the standards prescribed in the EPA's National Primary Drinking Water Regulations (40 CFR Part 141). Purified Water must be used for compounding nonsterile drug preparations when formulations indicate the inclusion of water. Purified Water must also be used for rinsing equipment and utensils. In those cases when a water is used to prepare a sterile preparation, Water for Injection, Sterile Water for Injection, or Bacteriostatic Water for Injection must be used (see [Water for Pharmaceutical Purposes](#) (1231) and [Pharmaceutical Compounding—Sterile Preparations](#) (797)).

Compounding areas are to be maintained in a clean and sanitary condition. Adequate washing facilities are to be provided, including hot and cold water, soap or detergent, and air dryers or single-service towels. Sewage, trash, and other refuse in the compounding area is to be disposed of in a safe, sanitary, and timely manner. Equipment is to be thoroughly cleaned promptly after use to avoid cross-contamination of ingredients and preparations. Special precautions are to be taken to clean equipment and compounding areas meticulously after compounding preparations that contain allergenic ingredients (e.g., sulfonamides or penicillins).

#### Equipment

Equipment is to be of appropriate design and size for compounding and suitable for the intended uses. The types and sizes of equipment will depend on the dosage forms and the quantities compounded (see [Weights and Balances](#) (41), [Prescription Balances and Volumetric Apparatus](#) (1176), and equipment manufacturers' instruction manuals). All equipment is to be constructed so that surfaces that contact pharmaceutical components, in-process materials, or finished preparations are not reactive, additive, or adsorptive to avoid altering the safety, identity, strength, quality, or purity of the preparation. The use of micropipets, electronic or analytical balances, or triturations or dilutions shall be considered when needed quantities are too small to accurately measure with standard equipment required by a state Board of Pharmacy. Equipment and accessories used in compounding are to be inspected, maintained, cleaned, and validated at appropriate intervals to ensure the accuracy and reliability of their performance.

#### STABILITY OF COMPOUNDED PREPARATIONS

"Stability" is defined as the extent to which a preparation retains, within specified limits, and throughout its period of storage and use, the same properties and characteristics that it possessed at the time of compounding. See the table Criteria for Acceptable Levels of Stability under [Stability Considerations in Dispensing Practice](#) (1191).

The compounder must avoid formulation ingredients and processing conditions that would result in a potentially toxic or ineffective preparation. The compounder's knowledge of the chemical reactions by which drugs degrade provides a means for establishing conditions under which the rate of degradation is minimized. The factors that influence the stability of compounded preparations are generally the same as those for manufactured drug products (see [Factors Affecting Product Stability and Responsibility of the Pharmacist](#) under [Stability Considerations in Dispensing Practice](#) (1191)).

#### Primary Packaging

Compounded preparations should be packaged in containers meeting USP standards (see [Containers under Preservation, Packaging, Storage, and Labeling in the General Notices and Requirements](#), [Containers—Plastics](#) (661), and [Containers—Performance Testing](#) (671)). The container used depends on the physical and chemical properties of the compounded preparation. Container-drug interaction is to be considered with substances such as phenolic compounds and sorptive materials (e.g., polypeptides and proteins).

#### Sterility

Assurance of sterility in a compounded sterile preparation is mandatory. Compounding and packaging of sterile drugs, such as ophthalmic solutions, will require strict adherence to guidelines presented in the general test chapter [Pharmaceutical Compounding—Sterile Preparations](#) (797) and in the manufacturers' labeling instructions.

#### Stability Criteria and Beyond-Use Dating

The beyond-use date is the date after which a compounded preparation is not to be used and is determined from the date the preparation is compounded. Because compounded preparations are intended for administration immediately or following short-term storage, their beyond-use dates may be assigned based on criteria different from those applied to assigning expiration dates to manufactured drug products.

Compounders are to consult and apply drug-specific and general stability documentation and literature when available, and are to consider the nature of the drug and its degradation mechanism, the container in which it is packaged, the expected storage conditions, and the intended duration of therapy when assigning a beyond-use date (see [Expiration Date and Beyond-Use Date under Labeling in the General Notices and Requirements](#)). Beyond-use dates are to be assigned conservatively. When using manufactured solid dosage forms to prepare a solution or aqueous suspension, the compounder is also to consider factors such as hydrolysis and the freeze-thaw property of the final preparation before assigning a beyond-use date. In assigning a beyond-use date for a compounded drug preparation, in addition to using all available stability information, the compounder is also to use his or her pharmaceutical education and experience.

When a manufactured product is used as the source of active ingredient for a nonsterile compounded preparation, the product expiration date cannot be used to extrapolate directly a beyond-use date for the compounded preparation. However, a compounder may refer to the literature or to the manufacturer for stability information. The compounder may also refer to applicable publications to obtain stability, compatibility, and degradation information on ingredients. All stability data must be carefully interpreted in relation to the actual compounded formulation.

At all steps in the compounding, dispensing, and storage process, the compounder is to observe the compounded drug preparation for signs of instability. For more specific details of some of the common physical signs of deterioration, see [Observing Products for Evidence of Instability](#) under [Stability Considerations in Dispensing Practice](#) (1191). However, excessive chemical degradation and other drug concentration loss due to reactions may be invisible more often than they are visible.

In the absence of stability information that is applicable to a specific drug and preparation, the following maximum beyond-use dates are recommended for nonsterile compounded drug preparations<sup>4</sup> that are packaged in tight, light-resistant containers and stored at controlled room temperature unless otherwise indicated (see [Preservation, Packaging, Storage, and Labeling in the General Notices and Requirements](#)).

#### For Nonaqueous Liquids and Solid Formulations—

Where the Manufactured Drug Product is the Source of Active Ingredient— The beyond-use date is not later than 25% of the time remaining until the product's expiration date or 6 months, whichever is earlier.

Where a USP or NF Substance is the Source of Active Ingredient— The beyond-use date is not later than 6 months.

For Water-Containing Formulations (prepared from ingredients in solid form)— The beyond-use date is not later than 14 days for liquid preparations when stored at cold temperatures between 2° and 8° (36° and 46° F).

For All Other Formulations— The beyond-use date is not later than the intended duration of therapy or 30 days, whichever is earlier. These beyond-use date limits may be exceeded when there is supporting valid scientific stability information that is directly applicable to the specific preparation (i.e., the same drug concentration range, pH, excipients, vehicle, water content, etc.). See also the beyond-use dating information in the Labeling section under [Repackaging Into Single-Unit Containers and Unit-Dose Containers for Nonsterile Solid and Liquid Dosage Forms](#) (681).

#### Beyond-Use Labeling

Federal law requires that manufactured drug products be labeled with an expiration date. Some state laws may require a beyond-use date. The label on the container or package of an official compounded preparation must bear a beyond-use date. Good compounding practice dictates beyond-use labeling for all compounded preparations.

#### DEFINITIONS

For purposes of this chapter, the following terms shall have these meanings.

preparation is a drug dosage form, a dietary supplement, or a finished device. It is the finished or partially finished preparation of one or more substances formulated for use on or for



the patient or consumer (see General Notices and Requirements).

official substance includes an active drug entity, a dietary supplement, or a pharmaceutic ingredient (see also NF 23) or a component of a finished device.

active ingredient usually refers to chemicals, substances, or other components of articles intended for use in the diagnosis, cure, mitigation, treatment, or prevention of diseases in humans or other animals or for use as dietary supplements.

added substances are ingredients that are necessary to prepare the preparation but are not intended or expected to cause a human pharmacologic response if administered alone in the amount or concentration contained in a single dose of the compounded preparation. The term added substances is usually used synonymously with the terms inactive ingredients, excipients, and pharmaceutic ingredients.

#### INGREDIENT SELECTION

##### Sources

Official compounded preparations are prepared from ingredients that meet requirements of the compendial monograph for those individual ingredients for which monographs are provided.

A USP or an NF grade substance is the preferred source of ingredients for compounding all other preparations. If that is not available, or when food, cosmetics, or other substances are or must be used, then the use of another high-quality source, such as analytical reagent (AR), certified American Chemical Society (ACS), or Food Chemicals Codex (FCC) grade, is an option for professional judgment. For any substance used in compounding not purchased from a registered drug manufacturer, the compounder must establish purity and safety by reasonable means, which may include lot analysis, manufacturer reputation, or reliability of source.

A manufactured drug product may be a source of active ingredient. Only manufactured drugs from containers labeled with a batch control number and a future expiration date are acceptable as a potential source of active ingredients. When compounding with manufactured drug products, the compounder must consider all ingredients present in the drug product relative to the intended use of the compounded preparation.

A compounder may not compound a drug preparation that appears on the FDA list of drug products withdrawn or removed from the market for safety reasons.

##### Compounding Nondrug Requirements

If the preparation is intended for use as a dietary or nutritional supplement (to supplement the diet) or cosmetic (e.g., to beautify), then the compounder must adhere to [Good Compounding Practices \(1075\)](#) and to this chapter, and must comply with any federal and state requirements.

#### CHECKLIST FOR ACCEPTABLE STRENGTH, QUALITY, AND PURITY

The following questions are to be considered carefully before compounding.

1. Have the physical and chemical properties and medicinal, dietary, and pharmaceutical uses of the drug substances been reviewed?
2. Are the quantity and quality of each active ingredient identifiable?
3. Will the active ingredients be effectively absorbed, locally or systemically according to the prescribed purpose, from the preparation and route of administration?
4. Are there added substances (see Definitions), confirmed or potentially present from manufactured products, that may be expected to cause an allergic reaction, irritation, toxicity, or undesirable organoleptic response from the patient? Are there added substances (see Definitions), confirmed or potentially present, that may be unfavorable (e.g., unsuitable pH or inadequate solubility)?
5. Were all calculations and measurements confirmed to ensure that the preparation will be compounded accurately (see [Pharmaceutical Calculations in Prescription Compounding \(1160\)](#))?

#### COMPOUNDED PREPARATIONS

The term compounded preparations includes the terms compounded dosage forms, compounded drugs, and compounded formulations, and means finished forms that are prepared by or under the direct supervision of a licensed compounder.

When controlled substances are used, check with state and federal authorities concerning their policies. Unless otherwise indicated or appropriate, compounded preparations are to be prepared to ensure that each preparation shall contain not less than 90.0% and not more than 110.0% of the theoretically calculated and labeled quantity of active ingredient per unit weight or volume and not less than 90.0% and not more than 110.0% of the theoretically calculated weight or volume per unit of the preparation. Compounded preparations include, but are not restricted to, the following pharmaceutical dosage forms described under [Pharmaceutical Dosage Forms \(1151\)](#).

##### Capsules, Powders, Lozenges, and Tablets

When compounding these dosage forms, the compounder is to prepare an amount of the total formulation sufficient to allow the prescribed amount or quantity to be accurately dispensed. Selected practices and precautions for compounding these dosage forms include the following:

- reducing solid ingredients to the smallest reasonable particle size;
- implementing appropriate checks to ensure that all ingredients are blended to achieve a homogeneous mixture;
- monitoring humidity if moisture might cause hydrolysis, dosage form adhesion to containers, or softening or partial dissolution of capsule shells;
- accurately performing weighings to ensure that each unit shall be not less than 90% and not more than 110% of the theoretically calculated weight for each unit [note—Preparations classified as dietary supplements are required by the U.S. Food and Drug regulations to be not less than 100% of the declared potency.]; and
- packaging dosage units according to container specifications for capsules and tablets of the specific active ingredient unless specified otherwise in individual monographs (see [Containers—Glass \(660\)](#) and [Containers—Plastic \(661\)](#)).

##### Emulsions, Solutions, and Suspensions

When compounding these dosage forms, the compounder is to prepare a 2% to 3% excess amount of the total formulation to allow the prescribed amount to be accurately dispensed. Selected practices and precautions for compounding these dosage forms include the following:

- For single-unit containers, the weight of each filled container, corrected for tare weight, shall be the equivalent of not less than 100% and not more than 110% of the labeled volume.
- Aqueous suspensions are prepared by levigating the powder mixture to a smooth paste with an appropriate wetting agent. This paste is converted to a free-flowing fluid by adding adequate vehicle. Successive portions of the vehicle are used to wash the mortar, or other vessel, to transfer the suspension quantitatively to a calibrated dispensing bottle or graduate. The preparation may be homogenized to ensure a uniform final dispersion.
  - Reducing solid ingredients to the smallest reasonable particle size.
- Solutions shall contain no visible undissolved matter when dispensed. [note—An exception may occur with supersaturated solutions such as Potassium Iodide Oral Solution.]
  - Emulsions and suspensions are labeled, "Shake well before using."

##### Suppositories

When compounding suppositories, the compounder is to prepare an excess amount of total formulation to allow the prescribed quantity to be accurately dispensed. Selected practices and precautions for compounding these dosage forms include the following:

- not using ingredients that are caustic or irritating, and thoroughly comminute solids that are abrasive to the mucous membranes;



- selecting a base that allows active ingredients to provide the intended local or systemic therapeutic effect;
- reducing solid ingredients to the smallest reasonable particle size; and
- weighing a representative number of suppositories to ensure that each is not less than 90% and not more than 110% of the average weight of all suppositories in the batch.

#### Creams, Topical Gels, Ointments, and Pastes

When compounding semisolid dosage forms, the compounder is to prepare an excess amount of total formulation to allow the prescribed quantity to be accurately dispensed. Selected practices and precautions for compounding these dosage forms include the following:

- not using ingredients that are caustic, irritating, or allergenic to the skin or other application sites unless they are necessary for a treatment;
- selecting a base or vehicle that allows active ingredients to provide the intended local or systemic therapeutic effect;
- reducing solid ingredients to the smallest reasonable particle size;
- geometrically incorporating the active ingredients with the added substances to achieve a uniform liquid or solid dispersion in the dosage form; and
- observing the uniformity of the dispersion by spreading a thin film of finished formulation on a flat transparent surface (e.g., clear glass ointment slab).

#### COMPOUNDING PROCESS

The compounders are to consider using the following steps to minimize error and maximize the prescriber's intent.

1. Judge the suitability of the prescription to be compounded in terms of its safety and intended use. Determine what legal limitations, if any, are applicable.
2. Perform necessary calculations to establish the amounts of ingredients needed (see [Pharmaceutical Calculations in Prescription Compounding \(1160\)](#)).
3. Identify equipment needed.
4. Don the proper attire and wash hands.
5. Clean the compounding area and needed equipment.
6. Only one prescription should be compounded at one time in a specified compounding area.
7. Assemble all necessary materials to compound the prescription.
8. Compound the preparation following the formulation record or prescription (see Compounding Records and Documents below), according to the art and science of pharmacy.
9. Assess weight variation, adequacy of mixing, clarity, odor, color, consistency, and pH as appropriate.
10. Annotate the compounding log, and describe the appearance of the formulation.
11. Label the prescription containers to include the following items: a) the name of the preparation; b) the internal identification number; c) the beyond-use date (see Beyond-Use Labeling); d) the initials of the compounder who prepared the label; e) any storage requirements; and f) any other statements required by law.
12. Sign and date the prescription affirming that all procedures were carried out to ensure uniformity, identity, strength, quantity, and purity.
13. Thoroughly and promptly clean all equipment, and store properly.

#### COMPOUNDING RECORDS AND DOCUMENTS

All compounders who dispense prescriptions must comply with the record keeping requirements of their individual states. If the compounder compounds a preparation according to the manufacturer's labeling instructions, then further documentation is not required. All other compounded preparations require further documentation. Such compounding documents are to list the ingredients and the quantity of each in the order of the compounding process.

The objective of the documentation is to allow another compounder to reproduce the identical prescription at a future date. The formulation record provides a consistent source document for preparing the preparation (recipe), and the compounding record documents the actual ingredients in the preparation and the person responsible for the compounding activity. These records are to be retained for the same period of time that is required for any prescription under state law. The record may be a copy of the prescription in written or machine readable form that includes a formulation record, a compounding record, and a Material Safety Data Sheets (MSDS) file.

##### Formulation Record

The formulation record is a file of individually compounded preparations. This record must list the name, strength, and dosage form of the preparation compounded, all ingredients and their quantities, equipment needed to prepare the preparation, when appropriate, and mixing instructions. Mixing instructions should include the order of mixing, mixing temperatures or other environmental controls, such as the duration of mixing, and other factors pertinent to the replication of the preparation as compounded. The formulation record must include an assigned beyond-use date, the container used in dispensing, the storage requirements, and any quality control procedures.

##### Compounding Record

The compounding record contains documentation of the name and strength of the compounded preparation, the formulation record reference for the preparation, and the sources and lot numbers of ingredients. The compounding record also includes information on the total number of dosage units compounded, the name of the person who prepared the preparation and the name of the compounder who approved the preparation, the date of preparation, the assigned internal identification number or the prescription number and an assigned beyond-use date, and the prescription number. For all compounded preparations, results of quality control procedures are to be recorded (e.g., weight range of filled capsules). When compounding problems occur with preparations prepared according to USP compounding monographs, the compounder must complete a USP Monograph Experience Reporting Form, and submit the form to USP for evaluation.

##### MSDS File

MSDS are to be readily accessible to all employees working with drug substances or bulk chemicals located on the compounding facility premises. Employees are to be instructed on how to retrieve and interpret needed information.

#### QUALITY CONTROL

The safety, quality, and performance of compounded preparations depend on correct ingredients and calculations, accurate and precise measurements, appropriate formulation conditions and procedures, and prudent pharmaceutical judgment. As a final check, the compounder is to review each procedure in the compounding process. To ensure accuracy and completeness, the compounder is to observe the finished preparation to ensure that it appears as expected and is to investigate any discrepancies and take appropriate corrective action before the prescription is dispensed to the patient (see the Checklist for Acceptable Strength, Quality, and Purity, the appropriate pharmaceutical dosage form under Compounded Preparations, and the steps under Compounding Process).

#### VERIFICATION

Compounding procedures that are routinely performed, including batch compounding, shall be completed and verified according to written procedures. The act of verification of a compounding procedure involves checking to ensure that calculations, weighing and measuring, order of mixing, and compounding techniques were appropriate and accurately performed.

#### PATIENT COUNSELING

The patient or the patient's agent should be counseled about proper use, storage, and evidence of instability in the compounded preparation at the time of dispensing (see Responsibility of the Pharmacist under [Stability Considerations in Dispensing Practice \(1191\)](#)).



1 For guidelines applicable to dating sterile compounded preparations, see Storage and Beyond-Use Dating under [Pharmaceutical Compounding—Sterile Preparations](#) (797).

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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## 797 PHARMACEUTICAL COMPOUNDING—STERILE PREPARATIONS

### INTRODUCTION

The objective of this chapter is to describe conditions and practices to prevent harm, including death, to patients that could result from (1) microbial contamination (nonsterility), (2) excessive bacterial endotoxins, (3) variability in the intended strength of correct ingredients that exceeds either monograph limits for official articles (see “official” and “article” in the General Notices and Requirements) or 10% for nonofficial articles, (4) unintended chemical and physical contaminants, and (5) ingredients of inappropriate quality in compounded sterile preparations (CSPs). Contaminated CSPs are potentially most hazardous to patients when administered into body cavities, central nervous and vascular systems, eyes, and joints, and when used as baths for live organs and tissues. When CSPs contain excessive bacterial endotoxins (see [Bacterial Endotoxins Test](#) (85)), they are potentially most hazardous to patients when administered into the central nervous system.

Despite the extensive attention in this chapter to the provision, maintenance, and evaluation of air quality, the avoidance of direct or physical contact contamination is paramount. It is generally acknowledged that direct or physical contact of critical sites of CSPs with contaminants, especially microbial sources, poses the greatest probability of risk to patients. Therefore, compounding personnel must be meticulously conscientious in precluding contact contamination of CSPs both within and outside ISO Class 5 (see [Table 1](#)) areas.

To achieve the above five conditions and practices, this chapter provides minimum practice and quality standards for CSPs of drugs and nutrients based on current scientific information and best sterile compounding practices. The use of technologies, techniques, materials, and procedures other than those described in this chapter is not prohibited so long as they have been proven to be equivalent or superior with statistical significance to those described herein. The standards in this chapter do not pertain to the clinical administration of CSPs to patients via application, implantation, infusion, inhalation, injection, insertion, instillation, and irrigation, which are the routes of administration. Four specific categories of CSPs are described in this chapter: low-risk level, medium-risk level, and high-risk level, and immediate use. Sterile compounding differs from nonsterile compounding (see [Pharmaceutical Compounding—Nonsterile Preparations](#) (795) and [Good Compounding Practices](#) (1075)) primarily by requiring the maintenance of sterility when compounding exclusively with sterile ingredients and components (i.e., with immediate-use CSPs, low-risk level CSPs, and medium-risk level CSPs) and the achievement of sterility when compounding with nonsterile ingredients and components (i.e., with high-risk level CSPs). Some differences between standards for sterile compounding in this chapter and those for nonsterile compounding in [Pharmaceutical Compounding—Nonsterile Preparations](#) (795) include, but are not limited to, ISO-classified air environments (see [Table 1](#)); personnel garbing and gloving; personnel training and testing in principles and practices of aseptic manipulations and sterilization; environmental quality specifications and monitoring; and disinfection of gloves and surfaces of ISO Class 5 (see [Table 1](#)) sources.

Table 1. ISO Classification of Particulate Matter in Room Air (limits are in particles of 0.5  $\mu$ m and larger per cubic meter [current ISO] and cubic feet [former Federal Standard No. 209E, FS 209E]).\*

Class Name		Particle Count	
ISO Class	U.S. FS 209E	ISO, m <sup>3</sup>	FS 209E, ft <sup>3</sup>
3	Class 1	35.2	1
4	Class 10	352	10
5	Class 100	3,520	100
6	Class 1,000	35,200	1,000
7	Class 10,000	352,000	10,000
8	Class 100,000	3,520,000	100,000

\* Adapted from former Federal Standard No. 209E, General Services Administration, Washington, DC, 20407 (September 11, 1992) and ISO 14644-1:1999, Cleanrooms and associated controlled environments—Part 1: Classification of air cleanliness. For example, 3,520 particles of 0.5  $\mu$ m per m<sup>3</sup> or larger (ISO Class 5) is equivalent to 100 particles per ft<sup>3</sup> (Class 100) (1 m<sup>3</sup> = 35.2 ft<sup>3</sup>).

The standards in this chapter are intended to apply to all persons who prepare CSPs and all places where CSPs are prepared (e.g., hospitals and other healthcare institutions, patient treatment clinics, pharmacies, physicians' practice facilities, and other locations and facilities in which CSPs are prepared, stored, and transported). Persons who perform sterile compounding include pharmacists, nurses, pharmacy technicians, and physicians. These terms recognize that most sterile compounding is performed by or under the supervision of pharmacists in pharmacies and also that this chapter applies to all healthcare personnel who prepare, store, and transport CSPs. For the purposes of this chapter, CSPs include any of the following:

1. Compounded biologics, diagnostics, drugs, nutrients, and radiopharmaceuticals, including but not limited to the following dosage forms that must be sterile when they are administered to patients: aqueous bronchial and nasal inhalations, baths and soaks for live organs and tissues, injections (e.g., colloidal dispersions, emulsions, solutions, suspensions), irrigations for wounds and body cavities, ophthalmic drops and ointments, and tissue implants.
2. Manufactured sterile products that are either prepared strictly according to the instructions appearing in manufacturers' approved labeling (product package inserts) or prepared differently than published in such labeling. [note—The FDA states that “Compounding does not include mixing, reconstituting, or similar acts that are performed in accordance with the directions contained in approved labeling provided by the product's manufacturer and other manufacturer directions consistent with that labeling” [21 USC 321 (k) and (m)]. However, the FDA-approved labeling (product package insert) rarely describes environmental quality (e.g., ISO Class air designation, exposure durations to non-ISO classified air, personnel garbing and gloving, and other aseptic precautions by which sterile products are to be prepared for administration). Beyond-use exposure and storage dates or times (see General Notices and Requirements and [Pharmaceutical Compounding—Nonsterile Preparations](#) (795)) for sterile products that have been either opened or prepared for administration are not specified in all package inserts for all sterile products. Furthermore, when such durations are specified, they may refer to chemical stability and not necessarily to microbiological purity or safety.]

### ORGANIZATION OF THIS CHAPTER

The sections in this chapter are organized to facilitate the practitioner's understanding of the fundamental accuracy and quality practices for preparing CSPs. They provide a foundation for the development and implementation of essential procedures for the safe preparation of low-risk, medium-risk, and high-risk level CSPs and immediate-use CSPs, which are classified according to the potential for microbial, chemical, and physical contamination. The chapter is divided into the following main sections:

- Definitions
- Responsibility of Compounding Personnel
- CSP Microbial Contamination Risk Levels
- Personnel Training and Evaluation in Aseptic Manipulation Skills



- Immediate-Use CSPs
- Single-Dose and Multiple-Dose Containers
  - Hazardous Drugs as CSPs
  - Radiopharmaceuticals as CSPs
  - Allergen Extracts as CSPs
- Verification of Compounding Accuracy and Sterility
  - Environmental Quality and Control
- Suggested Standard Operating Procedures (SOPs)
  - Elements of Quality Control
- Verification of Automated Compounding Devices (ACDs) for Parenteral Nutrition Compounding
  - Finished Preparation Release Checks and Tests
  - Storage and Beyond-Use Dating
- Maintaining Sterility, Purity, and Stability of Dispensed and Distributed CSPs
  - Patient or Caregiver Training
- Patient Monitoring and Adverse Events Reporting
  - Quality Assurance (QA) Program
  - Abbreviations and Acronyms
  - Appendices I–V

The requirements and recommendations in this chapter are summarized in Appendix I. A list of abbreviations and acronyms is included at the end of the main text, before the Appendices.

All personnel who prepare CSPs shall be responsible for understanding these fundamental practices and precautions, for developing and implementing appropriate procedures, and for continually evaluating these procedures and the quality of final CSPs to prevent harm.

#### DEFINITIONS

**Ante-Area**— An ISO Class 8 (see [Table 1](#)) or better area where personnel hand hygiene and garbing procedures, staging of components, order entry, CSP labeling, and other high-particulate-generating activities are performed. It is also a transition area that (1) provides assurance that pressure relationships are constantly maintained so that air flows from clean to dirty areas and (2) reduces the need for the heating, ventilating, and air-conditioning (HVAC) control system to respond to large disturbances.<sup>1</sup>

**Aseptic Processing** (see [Microbiological Evaluation of Clean Rooms and Other Controlled Environments](#) [\(1116\)](#))—A mode of processing pharmaceutical and medical products that involves the separate sterilization of the product and of the package (containers—closures or packaging material for medical devices) and the transfer of the product into the container and its closure under at least ISO Class 5 (see [Table 1](#)) conditions.

**Beyond-Use Date (BUD)** (see General Notices and Requirements and [Pharmaceutical Compounding—Nonsterile Preparations](#) [\(795\)](#))—For the purpose of this chapter, the date or time after which a CSP shall not be stored or transported. The date is determined from the date or time the preparation is compounded.

**Biological Safety Cabinet (BSC)**— A ventilated cabinet for CSPs, personnel, product, and environmental protection having an open front with inward airflow for personnel protection, downward high-efficiency particulate air (HEPA)-filtered laminar airflow for product protection, and HEPA-filtered exhausted air for environmental protection.

**Buffer Area**— An area where the primary engineering control (PEC) is physically located. Activities that occur in this area include the preparation and staging of components and supplies used when compounding CSPs.

**Clean Room** (see [Microbiological Evaluation of Clean Rooms and Other Controlled Environments](#) [\(1116\)](#) and also the definition of Buffer Area)—A room in which the concentration of airborne particles is controlled to meet a specified airborne particulate cleanliness class. Microorganisms in the environment are monitored so that a microbial level for air, surface, and personnel gear are not exceeded for a specified cleanliness class.

**Compounding Aseptic Containment Isolator (CACI)**— A compounding aseptic isolator (CAI) designed to provide worker protection from exposure to undesirable levels of airborne drug throughout the compounding and material transfer processes and to provide an aseptic environment for compounding sterile preparations. Air exchange with the surrounding environment should not occur unless the air is first passed through a microbial retentive filter (HEPA minimum) system capable of containing airborne concentrations of the physical size and state of the drug being compounded. Where volatile hazardous drugs are prepared, the exhaust air from the isolator should be appropriately removed by properly designed building ventilation.

**Compounding Aseptic Isolator (CAI)**— A form of isolator specifically designed for compounding pharmaceutical ingredients or preparations. It is designed to maintain an aseptic compounding environment within the isolator throughout the compounding and material transfer processes. Air exchange into the isolator from the surrounding environment should not occur unless the air has first passed through a microbially retentive filter (HEPA minimum).<sup>2</sup>

**Critical Area**— An ISO Class 5 (see [Table 1](#)) environment.

**Critical Site**— A location that includes any component or fluid pathway surfaces (e.g., vial septa, injection ports, beakers) or openings (e.g., opened ampuls, needle hubs) exposed and at risk of direct contact with air (e.g., ambient room or HEPA filtered), moisture (e.g., oral and mucosal secretions), or touch contamination. Risk of microbial particulate contamination of the critical site increases with the size of the openings and exposure time.

**Direct Compounding Area (DCA)**— A critical area within the ISO Class 5 (see [Table 1](#)) primary engineering control (PEC) where critical sites are exposed to unidirectional HEPA-filtered air, also known as first air.

**Disinfectant**— An agent that frees from infection, usually a chemical agent but sometimes a physical one, and that destroys disease-causing pathogens or other harmful microorganisms but may not kill bacterial and fungal spores. It refers to substances applied to inanimate objects.

**First Air**— The air exiting the HEPA filter in a unidirectional air stream that is essentially particle free.

**Hazardous Drugs**— Drugs are classified as hazardous if studies in animals or humans indicate that exposures to them have a potential for causing cancer, development or reproductive toxicity, or harm to organs. (See current NIOSH publication.)

**Labeling** [see General Notices and Requirements and 21 USC 321 (k) and (m)]—A term that designates all labels and other written, printed, or graphic matter on an immediate container of an article or preparation or on, or in, any package or wrapper in which it is enclosed, except any outer shipping container. The term “label” designates that part of the labeling on the immediate container.

**Media-Fill Test** (see [Microbiological Evaluation of Clean Rooms and Other Controlled Environments](#) [\(1116\)](#))—A test used to qualify aseptic technique of compounding personnel or processes and to ensure that the processes used are able to produce sterile product without microbial contamination. During this test, a microbiological growth medium such as Soybean–Casein Digest Medium is substituted for the actual drug product to simulate admixture compounding.<sup>3</sup> The issues to consider in the development of a media-fill test are media-fill procedures, media selection, fill volume, incubation, time and temperature, inspection of filled units, documentation, interpretation of results, and possible corrective actions required.

**Multiple-Dose Container** (see General Notices and Requirements and Containers for Injections under [Injections](#) [\(1\)](#))—A multiple-unit container for articles or preparations intended for parenteral administration only and usually containing antimicrobial preservatives. The beyond-use date (BUD) for an opened or entered (e.g., needle-punctured) multiple-dose container with antimicrobial preservatives is 28 days (see [Antimicrobial Effectiveness Testing](#) [\(51\)](#)), unless otherwise specified by the manufacturer.

**Negative Pressure Room**— A room that is at a lower pressure than the adjacent spaces and, therefore, the net flow of air is into the room.<sup>1</sup>



Pharmacy Bulk Package (see Containers for Injections under [Injections \(1\)](#))—A container of a sterile preparation for parenteral use that contains many single doses. The contents are intended for use in a pharmacy admixture program and are restricted to the preparation of admixtures for infusion or, through a sterile transfer device, for the filling of empty sterile syringes. The closure shall be penetrated only one time after constitution with a suitable sterile transfer device or dispensing set, which allows measured dispensing of the contents.

The pharmacy bulk package is to be used only in a suitable work area such as a laminar flow hood (or an equivalent clean air compounding area).

Where a container is offered as a pharmacy bulk package, the label shall (a) state prominently "Pharmacy Bulk Package—Not for Direct Infusion," (b) contain or refer to information on proper techniques to help ensure safe use of the product, and (c) bear a statement limiting the time frame in which the container may be used once it has been entered, provided it is held under the labeled storage conditions.

Primary Engineering Control (PEC)—A device or room that provides an ISO Class 5 (see [Table 1](#)) environment for the exposure of critical sites when compounding CSPs. Such devices include, but may not be limited to, laminar airflow workbenches (LAFWs), biological safety cabinets (BSCs), compounding aseptic isolators (CAIs), and compounding aseptic containment isolators (CACIs).

Preparation—A preparation, or a CSP, that is a sterile drug or nutrient compounded in a licensed pharmacy or other healthcare-related facility pursuant to the order of a licensed prescriber; the article may or may not contain sterile products.

Product—A commercially manufactured sterile drug or nutrient that has been evaluated for safety and efficacy by the FDA. Products are accompanied by full prescribing information, which is commonly known as the FDA-approved manufacturer's labeling or product package insert.

Positive Pressure Room—A room that is at a higher pressure than the adjacent spaces and, therefore, the net airflow is out of the room.<sup>1</sup>

Single-Dose Container (see General Notices and Requirements and Containers for Injections under [Injections \(1\)](#))—A single-dose container is a single-unit container for articles (see General Notices and Requirements) or preparations intended for parenteral administration only. It is intended for a single use. A single-dose container is labeled as such. Examples of single-dose containers include prefilled syringes, cartridges, fusion-sealed containers, and closure-sealed containers when so labeled.

Segregated Compounding Area—A designated space, either a demarcated area or room, that is restricted to preparing low-risk level CSPs with 12-hour or less BUD. Such area shall contain a device that provides unidirectional airflow of ISO Class 5 (see [Table 1](#)) air quality for preparation of CSPs and shall be void of activities and materials that are extraneous to sterile compounding.

Sterilizing Grade Membranes—Membranes that are documented to retain 100% of a culture of 107 microorganisms of a strain of *Brevundimonas (Pseudomonas) diminuta* per square centimeter of membrane surface under a pressure of not less than 30 psi (2.0 bar). Such filter membranes are nominally at 0.22-μm or 0.2-μm nominal pore size, depending on the manufacturer's practice.

Sterilization by Filtration—Passage of a fluid or solution through a sterilizing grade membrane to produce a sterile effluent.

Terminal Sterilization—The application of a lethal process (e.g., steam under pressure or autoclaving) to sealed containers for the purpose of achieving a predetermined sterility assurance level of usually less than 10<sup>-6</sup>, or a probability of less than one in one million of a nonsterile unit.<sup>3</sup>

Unidirectional Flow (see footnote 3)—An airflow moving in a single direction in a robust and uniform manner and at sufficient speed to reproducibly sweep particles away from the critical processing or testing area.

#### RESPONSIBILITY OF COMPOUNDING PERSONNEL

Compounding personnel are responsible for ensuring that CSPs are accurately identified, measured, diluted, and mixed and are correctly purified, sterilized, packaged, sealed, labeled, stored, dispensed, and distributed. These performance responsibilities include maintaining appropriate cleanliness conditions and providing labeling and supplementary instructions for the proper clinical administration of CSPs.

Compounding supervisors shall ensure, through either direct measurement or appropriate information sources, that specific CSPs maintain their labeled strength within monograph limits for USP articles, or within 10% if not specified, until their BUDs. All CSPs are prepared in a manner that maintains sterility and minimizes the introduction of particulate matter.

A written quality assurance procedure includes the following in-process checks that are applied, as appropriate, to specific CSPs: accuracy and precision of measuring and weighing; the requirement for sterility; methods of sterilization and purification; safe limits and ranges for strength of ingredients, bacterial endotoxins, and particulate matter; pH; labeling accuracy and completeness; BUD assignment; and packaging and storage requirements. The dispenser shall, when appropriate and practicable, obtain and evaluate results of testing for identity, strength, purity, and sterility before a CSP is dispensed. Qualified licensed healthcare professionals who supervise compounding and dispensing of CSPs shall ensure that the following objectives are achieved:

1. Compounding personnel are adequately skilled, educated, instructed, and trained to correctly perform and document the following activities in their sterile compounding duties:
  - a. perform antiseptic hand cleansing and disinfection of nonsterile compounding surfaces;
  - b. select and appropriately don protective garb;
  - c. maintain or achieve sterility of CSPs in ISO Class 5 (see [Table 1](#)) PEC devices and protect personnel and compounding environments from contamination by radioactive, cytotoxic, and chemotoxic drugs (see Hazardous Drugs as CSPs and Radiopharmaceuticals as CSPs);
  - d. identify, weigh, and measure ingredients; and
  - e. manipulate sterile products aseptically, sterilize high-risk level CSPs, and label and quality inspect CSPs.
2. Ingredients have their correct identity, quality, and purity.
3. Opened or partially used packages of ingredients for subsequent use in CSPs are properly stored under restricted access conditions in the compounding facility. Such packages cannot be used when visual inspection detects unauthorized breaks in the container, closure, and seal; when the contents do not possess the expected appearance, aroma, and texture; when the contents do not pass identification tests specified by the compounding facility; and when either the BUD or expiration date has been exceeded.
4. Water-containing CSPs that are nonsterile during any phase of the compounding procedure are sterilized within 6 hours after completing the preparation in order to minimize the generation of bacterial endotoxins.
5. Sterilization methods achieve sterility of CSPs while maintaining the labeled strength of active ingredients and the physical integrity of packaging.
6. Measuring, mixing, sterilizing, and purifying devices are clean, appropriately accurate, and effective for their intended use.
7. Potential harm from added substances and differences in rate and extent of bioavailability of active ingredients for other than oral route of administration are carefully evaluated before such CSPs are dispensed and administered.
  8. Packaging selected for CSPs is appropriate to preserve the sterility and strength until the BUD.
9. While being used, the compounding environment maintains the sterility or the presterilization purity, whichever is appropriate, of the CSP.
10. Labels on CSPs list the names and amounts or concentrations of active ingredients, and the labels or labeling of injections (see Preservation, Packaging, Storage, and Labeling in the General Notices and Requirements) list the names and amounts or concentrations of all ingredients (see [Injections \(1\)](#)). Before being dispensed or administered, the clarity of solutions is visually confirmed; also, the identity and amounts of ingredients, procedures to prepare and sterilize CSPs, and specific release criteria are reviewed to ensure their accuracy and completeness.
11. BUDs are assigned on the basis of direct testing or extrapolation from reliable literature sources and other documentation (see Stability Criteria and Beyond-Use Dating under [Pharmaceutical Compounding—Nonsterile Preparations \(795\)](#)).
12. Procedures for measuring, mixing, dilution, purification, sterilization, packaging, and labeling conform to the correct sequence and quality established for the specified CSP.
  13. Deficiencies in compounding, labeling, packaging, and quality testing and inspection can be rapidly identified and corrected.
14. When time and personnel availability so permit, compounding manipulations and procedures are separated from postcompounding quality inspection and review before CSPs are dispensed.

This chapter emphasizes the need to maintain high standards for the quality and control of processes, components, and environments and for the skill and knowledge of personnel



who prepare CSPs. The rigor of in-process quality-control checks and of postcompounding quality inspection and testing increases with the potential hazard of the route of administration. For example, nonsterility, excessive bacterial endotoxin contamination, large errors in strength of correct ingredients, and incorrect ingredients in CSPs are potentially more dangerous to patients when the CSPs are administered into the vascular and central nervous systems than when administered by most other routes.

#### CSP MICROBIAL CONTAMINATION RISK LEVELS

The three contamination categories for CSPs described in this section are assigned primarily according to the potential for microbial contamination during the compounding of low-risk level CSPs and medium-risk level CSPs or the potential for not sterilizing high-risk level CSPs, any of which would subject patients to risk of harm, including death. High-risk level

CSPs must be sterilized before being administered to patients. The appropriate risk level—low, medium, or high—is assigned according to the corresponding probability of contaminating a CSP with (1) microbial contamination (e.g., microbial organisms, spores, endotoxins) and (2) chemical and physical contamination (e.g., foreign chemicals, physical matter). Potential sources of contamination include, but are not limited to, solid and liquid matter from compounding personnel and objects; nonsterile components employed and incorporated before terminal sterilization; inappropriate conditions within the restricted compounding environment; prolonged presterilization procedures with aqueous preparations; and nonsterile dosage forms used to compound CSPs.

The characteristics described below for low-, medium-, and high-risk level CSPs are intended as a guide to the breadth and depth of care necessary in compounding, but they are neither exhaustive nor prescriptive. The licensed healthcare professionals who supervise compounding are responsible for determining the procedural and environmental quality practices and attributes that are necessary for the risk level they assign to specific CSPs.

These risk levels apply to the quality of CSPs immediately after the final aseptic mixing or filling or immediately after the final sterilization, unless precluded by the specific characteristics of the preparation. Upon subsequent storage and shipping of freshly finished CSPs, an increase in the risks of chemical degradation of ingredients, contamination from physical damage to packaging, and permeability of plastic and elastomeric packaging is expected. In such cases, compounding personnel are responsible for considering the potential additional risks to the integrity of CSPs when assigning BUDs. The pre-administration storage duration and temperature limits specified in the following subsections apply in the absence of direct sterility testing results that justify different limits for specific CSPs.

##### Low-Risk Level CSPs

CSPs compounded under all the following conditions are at a low risk of contamination.

##### Low-Risk Conditions—

1. The CSPs are compounded with aseptic manipulations entirely within ISO Class 5 (see [Table 1](#)) or better air quality using only sterile ingredients, products, components, and devices.
2. The compounding involves only transfer, measuring, and mixing manipulations using not more than three commercially manufactured packages of sterile products and not more than two entries into any one sterile container or package (e.g., bag, vial) of sterile product or administration container/device to prepare the CSP.
3. Manipulations are limited to aseptically opening ampuls, penetrating disinfected stoppers on vials with sterile needles and syringes, and transferring sterile liquids in sterile syringes to sterile administration devices, package containers of other sterile products, and containers for storage and dispensing.
4. For a low-risk level preparation, in the absence of passing a sterility test (see [Sterility Tests \(71\)](#)), the storage periods cannot exceed the following time periods: before administration, the CSPs are properly stored and are exposed for not more than 48 hours at controlled room temperature (see General Notices and Requirements), for not more than 14 days at a cold temperature (see General Notices and Requirements), and for 45 days in solid frozen state between  $-25^{\circ}$  and  $-10^{\circ}$ .

##### Examples of Low-Risk Compounding—

1. Single-volume transfers of sterile dosage forms from ampuls, bottles, bags, and vials using sterile syringes with sterile needles, other administration devices, and other sterile containers. The solution content of ampuls should be passed through a sterile filter to remove any particles.
2. Simple aseptic measuring and transferring with not more than three packages of manufactured sterile products, including an infusion or diluent solution to compound drug admixtures and nutritional solutions.

**Low-Risk Level CSPs with 12-Hour or Less BUD**— If the PEC is a CAI or CACI that does not meet the requirements described in Placement of Primary Engineering Controls or is a laminar airflow workbench (LAFW) or a biological safety cabinet (BSC) that cannot be located within an ISO Class 7 (see [Table 1](#)) buffer area, then only low-risk level nonhazardous and radiopharmaceutical CSPs pursuant to a physician's order for a specific patient may be prepared, and administration of such CSPs shall commence within 12 hours of preparation or as recommended in the manufacturers' package insert, whichever is less. Low-risk level CSPs with a 12-hour or less BUD shall meet all of the following four criteria:

1. PECs (LAFWs, BSCs, CAIs, CACIs,) shall be certified and maintain ISO Class 5 (see [Table 1](#)) as described in Facility Design and Environmental Controls for exposure of critical sites and shall be in a segregated compounding area restricted to sterile compounding activities that minimize the risk of CSP contamination.
2. The segregated compounding area shall not be in a location that has unsealed windows or doors that connect to the outdoors or high traffic flow, or that is adjacent to construction sites, warehouses, or food preparation. Note that this list is not intended to be all inclusive.
3. Personnel shall follow the procedures described in Personnel Cleansing and Garbing and Additional Personnel Requirements prior to compounding. Sinks should not be located adjacent to the ISO Class 5 (see [Table 1](#)) PEC. Sinks should be separated from the immediate area of the ISO Class 5 (see [Table 1](#)) PEC device.
4. The specifications in Cleaning and Disinfecting the Sterile Compounding Areas, Personnel Training and Competency Evaluation of Garbing, Aseptic Work Practices and Cleaning/Disinfection Procedures, and Viable and Nonviable Environmental Sampling (ES) Testing shall be followed as described in the chapter.

Compounding personnel must recognize that the absence of an ISO Class 7 (see [Table 1](#)) buffer area environment in a general uncontrolled environment increases the potential of microbial contamination, and administration durations of microbially contaminated CSPs exceeding a few hours increase the potential for clinically significant microbial colonization, and thus for patient harm, especially in critically ill or immunocompromised patients.

##### Quality Assurance— Quality assurance practices include, but are not limited to the following:

1. Routine disinfection and air quality testing of the direct compounding environment to minimize microbial surface contamination and maintain ISO Class 5 (see [Table 1](#)) air quality.
2. Visual confirmation that compounding personnel are properly donning and wearing appropriate items and types of protective garments, including eye protection and face masks.
3. Review of all orders and packages of ingredients to ensure that the correct identity and amounts of ingredients were compounded.
4. Visual inspection of CSPs to ensure the absence of particulate matter in solutions, the absence of leakage from vials and bags, and the accuracy and thoroughness of labeling.

**Media-Fill Test Procedure**— This test or an equivalent test is performed at least annually by each person authorized to compound in a low-risk level environment under conditions that closely simulate the most challenging or stressful conditions encountered during compounding of low-risk level CSPs. Once begun, this test is completed without interruption. Example of test procedure: within an ISO Class 5 (see [Table 1](#)) air quality environment, three sets of four 5-mL aliquots of sterile Soybean–Casein Digest Medium (also known as trypticase soy broth or trypticase soy agar [TSA]) are transferred with the same sterile 10-mL syringe and vented needle combination into separate sealed, empty, sterile 30-mL clear vials (i.e., four 5-mL aliquots into each of three 30-mL vials). Sterile adhesive seals are aseptically affixed to the rubber closures on the three filled vials, then the vials are incubated at  $20^{\circ}$  to  $25^{\circ}$  or at  $30^{\circ}$  to  $35^{\circ}$  for a minimum of 14 days. If two temperatures are used for incubation of media-filled samples, then these filled containers should be incubated for at least 7 days at each temperature (see [Microbiological Evaluation of Clean Rooms and Other Controlled Environments \(116\)](#)). Inspect for microbial growth over 14 days as described in Personnel Training and Competency Evaluation of Garbing, Aseptic Work Practices and Cleaning/Disinfection Procedures.

## Medium-Risk Level CSPs

When CSPs are compounded aseptically under Low-Risk Conditions and one or more of the following conditions exists, such CSPs are at a medium risk of contamination.

## Medium-Risk Conditions—

1. Multiple individual or small doses of sterile products are combined or pooled to prepare a CSP that will be administered either to multiple patients or to one patient on multiple occasions.
2. The compounding process includes complex aseptic manipulations other than the single-volume transfer.
3. The compounding process requires unusually long duration, such as that required to complete dissolution or homogeneous mixing.
4. For a medium-risk preparation, in the absence of passing a sterility test (see [Sterility Tests \(71\)](#)), the storage periods cannot exceed the following time periods: before administration, the CSPs are properly stored and are exposed for not more than 30 hours at controlled room temperature (see General Notices and Requirements), for not more than 9 days at a cold temperature (see General Notices and Requirements), and for 45 days in solid frozen state between  $-25^{\circ}$  and  $-10^{\circ}$ .

## Examples of Medium-Risk Compounding—

1. Compounding of total parenteral nutrition fluids using manual or automated devices during which there are multiple injections, detachments, and attachments of nutrient source products to the device or machine to deliver all nutritional components to a final sterile container.
2. Filling of reservoirs of injection and infusion devices with more than three sterile drug products and evacuation of air from those reservoirs before the filled device is dispensed.
3. Transfer of volumes from multiple ampuls or vials into one or more final sterile containers.

Quality Assurance— Quality assurance procedures for medium-risk level CSPs include all those for low-risk level CSPs, as well as a more challenging media-fill test passed annually or more frequently.

Media-Fill Test Procedure— This test or an equivalent test is performed at least annually under conditions that closely simulate the most challenging or stressful conditions encountered during compounding. Once begun, this test is completed without interruption. Example of test procedure: within an ISO Class 5 (see [Table 1](#)) air quality environment, six 100-mL aliquots of sterile Soybean–Casein Digest Medium are aseptically transferred by gravity through separate tubing sets into separate evacuated sterile containers. The six containers are then arranged as three pairs, and a sterile 10-mL syringe and 18-gauge needle combination is used to exchange two 5-mL aliquots of medium from one container to the other container in the pair. For example, after a 5-mL aliquot from the first container is added to the second container in the pair, the second container is agitated for 10 seconds, then a 5-mL aliquot is removed and returned to the first container in the pair. The first container is then agitated for 10 seconds, and the next 5-mL aliquot is transferred from it back to the second container in the pair. Following the two 5-mL aliquot exchanges in each pair of containers, a 5-mL aliquot of medium from each container is aseptically injected into a sealed, empty, sterile 10-mL clear vial, using a sterile 10-mL syringe and vented needle. Sterile adhesive seals are aseptically affixed to the rubber closures on the three filled vials, then the vials are incubated at  $20^{\circ}$  to  $25^{\circ}$  or at  $30^{\circ}$  to  $35^{\circ}$  for a minimum of 14 days. If two temperatures are used for incubation of media-filled samples, then these filled containers should be incubated for at least 7 days at each temperature (see [Microbiological Evaluation of Clean Rooms and Other Controlled Environments \(116\)](#)). Inspect for microbial growth over 14 days as described in Personnel Training and Competency Evaluation of Garbing, Aseptic Work Practices and Cleaning/Disinfection Procedures.

## High-Risk Level CSPs

CSPs compounded under any of the following conditions are either contaminated or at a high risk to become contaminated.

## High-Risk Conditions—

1. Nonsterile ingredients, including manufactured products not intended for sterile routes of administration (e.g., oral), are incorporated or a nonsterile device is employed before terminal sterilization.
2. Any of the following are exposed to air quality worse than ISO Class 5 (see [Table 1](#)) for more than 1 hour (see Immediate-Use CSPs):
  - sterile contents of commercially manufactured products,
  - CSPs that lack effective antimicrobial preservatives, and
  - sterile surfaces of devices and containers for the preparation, transfer, sterilization, and packaging of CSPs.
3. Compounding personnel are improperly garbed and gloved (see Personnel Cleansing and Use of Barrier Protective Equipment).
4. Nonsterile water-containing preparations are stored for more than 6 hours before being sterilized.
5. It is assumed, and not verified by examination of labeling and documentation from suppliers or by direct determination, that the chemical purity and content strength of ingredients meet their original or compendial specifications in unopened or in opened packages of bulk ingredients (see Ingredient Selection under [Pharmaceutical Compounding—Nonsterile Preparations \(795\)](#)).

For a sterilized high-risk level preparation, in the absence of passing a sterility test, the storage periods cannot exceed the following time periods: before administration, the CSPs are properly stored and are exposed for not more than 24 hours at controlled room temperature (see General Notices and Requirements), for not more than 3 days at a cold temperature (see General Notices and Requirements), and for 45 days in solid frozen state between  $-25^{\circ}$  and  $-10^{\circ}$ . [note—Sterility tests for autoclaved CSPs are not required unless they are prepared in batches of more than 25 units.]

All nonsterile measuring, mixing, and purifying devices are rinsed thoroughly with sterile, pyrogen-free water, and then thoroughly drained or dried immediately before use for high-risk compounding. All high-risk level CSP solutions subjected to terminal sterilization are prefilled by passing through a filter with a nominal pore size not larger than 1.2  $\mu\text{m}$  preceding or during filling into their final containers to remove particulate matter. Sterilization of high-risk level CSPs by filtration shall be performed with a sterile 0.2- $\mu\text{m}$  or 0.22- $\mu\text{m}$  nominal pore size filter entirely within an ISO Class 5 (see [Table 1](#)) or superior air quality environment.

## Examples of High-Risk Conditions—

1. Dissolving nonsterile bulk drug and nutrient powders to make solutions that will be terminally sterilized.
2. Exposing the sterile ingredients and components used to prepare and package CSPs to room air quality worse than ISO Class 5 (see [Table 1](#)) for more than 1 hour (see Immediate-Use CSPs).
3. Measuring and mixing sterile ingredients in nonsterile devices before sterilization is performed.
4. Assuming, without appropriate evidence or direct determination, that packages of bulk ingredients contain at least 95% by weight of their active chemical moiety and have not been contaminated or adulterated between uses.

Quality Assurance— Quality assurance procedures for high-risk level CSPs include all those for low-risk level CSPs. In addition, a media-fill test that represents high-risk level compounding is performed semiannually by each person authorized to compound high-risk level CSPs.

Media-Fill Test Procedure for CSPs Sterilized by Filtration— This test or an equivalent test is performed under conditions that closely simulate the most challenging or stressful conditions encountered when compounding high-risk level CSPs. Once begun, this test is completed without interruption. Example of test procedure (in the following sequence):

1. Dissolve 3 g of nonsterile commercially available Soybean–Casein Digest Medium in 100 mL of nonbacteriostatic water to make a 3% nonsterile solution.
2. Draw 25 mL of the medium into each of three 30-mL sterile syringes. Transfer 5 mL from each syringe into separate sterile 10-mL vials. These vials are the positive controls to generate exponential microbial growth, which is indicated by visible turbidity upon incubation.
3. Under aseptic conditions and using aseptic techniques, affix a sterile 0.2- $\mu\text{m}$  or 0.22- $\mu\text{m}$  nominal pore size filter unit and a 20-gauge needle to each syringe. Inject the next 10 mL from each syringe into three separate 10-mL sterile vials. Repeat the process for three more vials. Label all vials, affix sterile adhesive seals to the closure of the nine vials, and incubate them at  $20^{\circ}$  to  $25^{\circ}$  or at  $30^{\circ}$  to  $35^{\circ}$  for a minimum of 14 days. If two temperatures are used for incubation of media-filled samples, then these filled containers



should be incubated for at least 7 days at each temperature (see [Microbiological Evaluation of Clean Rooms and Other Controlled Environments](#) (116)). Inspect for microbial growth over 14 days as described in Personnel Training and Competency Evaluation of Garbing, Aseptic Work Practices and Cleaning/Disinfection Procedures.

#### PERSONNEL TRAINING AND EVALUATION IN ASEPTIC MANIPULATION SKILLS

Personnel who prepare CSPs shall be trained conscientiously and skillfully by expert personnel and through audio-video instructional sources and professional publications in the theoretical principles and practical skills of aseptic manipulations and in achieving and maintaining ISO Class 5 (see [Table 1](#)) environmental conditions before they begin to prepare CSPs. Compounding personnel shall perform didactic review and pass written and media-fill testing of aseptic manipulative skills initially, at least annually thereafter for low- and medium-risk level compounding, and semiannually for high-risk level compounding. Compounding personnel who fail written tests or whose media-fill test vials result in gross microbial colonization shall be immediately re-instructed and re-evaluated by expert compounding personnel to ensure correction of all aseptic practice deficiencies.

**Media-Fill Challenge Testing**— The skill of personnel to aseptically prepare CSPs may be evaluated using sterile fluid bacterial culture media-fill verification<sup>3</sup> (i.e., sterile bacterial culture medium transfer via a sterile syringe and needle). Media-fill testing is used to assess the quality of the aseptic skill of compounding personnel. Media-fill tests represent the most challenging or stressful conditions actually encountered by the personnel being evaluated when they prepare particular risk level CSPs and when sterilizing high-risk level CSPs. Media-fill challenge tests that simulate high-risk level compounding are also used to verify the capability of the compounding environment and process to produce a sterile preparation.

Commercially available sterile fluid culture media, such as Soybean–Casein Digest Medium (see [Sterility Tests](#) (71)), shall be able to promote exponential colonization of bacteria that are most likely to be transmitted to CSPs from the compounding personnel and environment. Media-filled vials are generally incubated at 20° to 25° or at 30° to 35° for a minimum of 14 days. If two temperatures are used for incubation of media-filled samples, then these filled containers should be incubated for at least 7 days at each temperature (see [Microbiological Evaluation of Clean Rooms and Other Controlled Environments](#) (116)). Failure is indicated by visible turbidity in the medium on or before 14 days.

#### IMMEDIATE-USE CSPS

The immediate-use provision is intended only for those situations where there is a need for emergency or immediate patient administration of a CSP. Such situations may include cardiopulmonary resuscitation, emergency room treatment, preparation of diagnostic agents, or critical therapy where the preparation of the CSP under conditions described for Low-Risk Level CSPs subjects the patient to additional risk due to delays in therapy. Immediate-use CSPs are not intended for storage for anticipated needs or batch compounding.

Preparations that are medium-risk level and high-risk level CSPs shall not be prepared as immediate-use CSPs.

Immediate-use CSPs are exempt from the requirements described for Low-Risk Level CSPs only when all of the following criteria are met:

1. The compounding process involves simple transfer of not more than three commercially manufactured packages of sterile nonhazardous products or diagnostic radiopharmaceutical products from the manufacturers' original containers and not more than two entries into any one container or package (e.g., bag, vial) of sterile infusion solution or administration container/device. For example, anti-neoplastics shall not be prepared as immediate-use CSPs because they are hazardous drugs.
2. Unless required for the preparation, the compounding procedure is a continuous process not to exceed 1 hour.
3. During preparation, aseptic technique is followed and, if not immediately administered, the finished CSP is under continuous supervision to minimize the potential for contact with nonsterile surfaces, introduction of particulate matter or biological fluids, mix-ups with other CSPs, and direct contact of outside surfaces.
4. Administration begins not later than 1 hour following the start of the preparation of the CSP.
5. Unless immediately and completely administered by the person who prepared it or immediate and complete administration is witnessed by the preparer, the CSP shall bear a label listing patient identification information, the names and amounts of all ingredients, the name or initials of the person who prepared the CSP, and the exact 1-hour BUD and time.
6. If administration has not begun within 1 hour following the start of preparing the CSP, the CSP shall be promptly, properly, and safely discarded.

Compounding in worse than ISO Class 5 (see [Table 1](#)) conditions increases the likelihood of microbial contamination, and administration durations of microbially contaminated CSPs exceeding a few hours increase the potential for clinically significant microbial colonization and thus for patient harm, especially in critically ill or immunocompromised patients.

#### SINGLE-DOSE AND MULTIPLE-DOSE CONTAINERS

Opened or needle-punctured single-dose containers, such as bags, bottles, syringes, and vials of sterile products and CSPs shall be used within 1 hour if opened in worse than ISO Class 5 (see [Table 1](#)) air quality (see [Immediate-Use CSPs](#)), and any remaining contents must be discarded. Single-dose vials exposed to ISO Class 5 (see [Table 1](#)) or cleaner air may be used up to 6 hours after initial needle puncture. Opened single-dose ampuls shall not be stored for any time period. Multiple-dose containers (e.g., vials) are formulated for removal of portions on multiple occasions because they usually contain antimicrobial preservatives. The BUD after initially entering or opening (e.g., needle-punctured) multiple-dose containers is 28 days (see [Antimicrobial Effectiveness Testing](#) (51)) unless otherwise specified by the manufacturer.

#### HAZARDOUS DRUGS AS CSPS

Although the potential therapeutic benefits of compounded sterile hazardous drug preparations generally outweigh the risks of their adverse effects in ill patients, exposed healthcare workers risk similar adverse effects with no therapeutic benefit. Occupational exposure to hazardous drugs can result in (1) acute effects, such as skin rashes; (2) chronic effects, including adverse reproductive events; and (3) possibly cancer (see Appendix A of NIOSH Publication no. 2004-165).

Hazardous drugs shall be prepared for administration only under conditions that protect the healthcare workers and other personnel in the preparation and storage areas. Hazardous drugs shall be stored separately from other inventory in a manner to prevent contamination and personnel exposure. Many hazardous drugs have sufficient vapor pressures that allow volatilization at room temperature; thus storage is preferably within a containment area such as a negative pressure room. The storage area should have sufficient general exhaust ventilation, at least 12 air changes per hour (ACPH)<sup>4</sup> to dilute and remove any airborne contaminants.

Hazardous drugs shall be handled with caution at all times using appropriate chemotherapy gloves during receiving, distribution, stocking, inventorying, preparation for administration, and disposal. Hazardous drugs shall be prepared in an ISO Class 5 (see [Table 1](#)) environment with protective engineering controls in place and following aseptic practices specified for the appropriate contamination risk levels defined in this chapter. Access shall be limited to areas where drugs are stored and prepared to protect persons not involved in drug preparation.

All hazardous drugs shall be prepared in a BSC<sup>5</sup> or a CACI that meets or exceeds the standards for CACI in this chapter. The ISO Class 5 (see [Table 1](#)) BSC or CACI shall be placed in an ISO Class 7 (see [Table 1](#)) area that is physically separated (i.e., a different area from other preparation areas) and optimally has not less than 0.01-inch water column negative pressure to adjacent positive pressure ISO Class 7 (see [Table 1](#)) or better ante-areas, thus providing inward airflow to contain any airborne drug. A pressure indicator shall be installed that can be readily monitored for correct room pressurization. The BSC and CACI optimally should be 100% vented to the outside air through HEPA filtration.

If a CACI that meets the requirements of this chapter is used outside of a buffer area, the compounding area shall maintain a minimum negative pressure of 0.01-inch water column and have a minimum of 12 ACPHs.

When closed-system vial-transfer devices (CSTDs) (i.e., vial-transfer systems that allow no venting or exposure of hazardous substance to the environment) are used, they shall be used within the ISO Class 5 (see [Table 1](#)) environment of a BSC or CACI. The use of a CSTD is preferred because of their inherent closed system process. In facilities that prepare a low volume of hazardous drugs, the use of two tiers of containment (e.g., CSTD within a BSC or CACI that is located in a non-negative pressure room) is acceptable.

Appropriate personnel protective equipment (PPE) shall be worn when compounding in a BSC or CACI and when using CSTD devices. PPE should include gowns, face masks, eye protection, hair covers, shoe covers or dedicated shoes, double gloving with sterile chemo-type gloves, and compliance with manufacturers' recommendations when using a CACI.

All personnel who compound hazardous drugs shall be fully trained in the storage, handling, and disposal of these drugs. This training shall occur prior to preparing or handling



hazardous CSPs, and its effectiveness shall be verified by testing specific hazardous drugs preparation techniques. Such verification shall be documented for each person at least annually. This training shall include didactic overview of hazardous drugs, including mutagenic, teratogenic, and carcinogenic properties, and it shall include ongoing training for each new hazardous drug that enters the marketplace. Compounding personnel of reproductive capability shall confirm in writing that they understand the risks of handling hazardous drugs.

The training shall include at least the following: (1) safe aseptic manipulation practices; (2) negative pressure techniques when utilizing a BSC or CACI; (3) correct use of CSTD devices; (4) containment, cleanup, and disposal procedures for breakages and spills; and (5) treatment of personnel contact and inhalation exposure.

note—Because standards of assay and unacceptable quantities of contamination of each drug have not been established in the literature, the following paragraph is a recommendation only. Future standards will be adopted as these assay methods are developed and proven.

In order to ensure containment, especially in operations preparing large volumes of hazardous drugs, environmental sampling to detect uncontaminated hazardous drugs should be performed routinely (e.g., initially as a benchmark and at least every 6 months or more often as needed to verify containment). This sampling should include surface wipe sampling of the working area of BSCs and CACIs; counter tops where finished preparations are placed; areas adjacent to BSCs and CACIs, including the floor directly under the working area; and patient administration areas. Common marker hazardous drugs that can be assayed include cyclophosphamide, ifosfamide, methotrexate, and fluorouracil. If any measurable contamination (cyclophosphamide levels greater than 1.00 ng per cm<sup>2</sup> have been found to cause human uptake) is found by any of these quality assurance procedures, practitioners shall make the decision to identify, document, and contain the cause of contamination. Such action may include retraining, thorough cleaning (utilizing high-pH soap and water), and improving engineering controls. Examples of improving engineering controls are (1) venting BSCs or CACIs 100% to the outside, (2) implementing a CSTD, or (3) re-assessing types of BSCs or CACIs.

Disposal of all hazardous drug wastes shall comply with all applicable federal and state regulations. All personnel who perform routine custodial waste removal and cleaning activities in storage and preparation areas for hazardous drugs shall be trained in appropriate procedures to protect themselves and prevent contamination.

#### RADIOPHARMACEUTICALS AS CSPs

In the case of production of radiopharmaceuticals for positron emission tomography (PET), general test chapter [Radiopharmaceuticals for Positron Emission Tomography—Compounding](#) (823) supersedes this chapter. Upon release of a PET radiopharmaceutical as a finished drug product from a production facility, the further handling, manipulation, or use of the product will be considered compounding, and the content of this section and chapter is applicable.

For the purposes of this chapter, radiopharmaceuticals compounded from sterile components in closed sterile containers and with a volume of 100 mL or less for a single-dose injection or not more than 30 mL taken from a multiple-dose container (see [Injections](#) (1)) shall be designated as, and conform to, the standards for Low-Risk Level CSPs.

These radiopharmaceuticals shall be compounded using appropriately shielded vials and syringes in a properly functioning and certified ISO Class 5 (see [Table 1](#)) PEC located in an ISO Class 8 (see [Table 1](#)) or cleaner air environment to permit compliance with special handling, shielding, and negative air flow requirements.

Radiopharmaceutical vials designed for multi-use, compounded with technetium-99m, exposed to ISO Class 5 (see [Table 1](#)) environment, and punctured by needles with no direct contact contamination may be used up to the time indicated by manufacturers' recommendations. Storage and transport of properly shielded vials of radiopharmaceutical CSPs may occur in a limited access ambient environment without a specific ISO class designation.

Technetium-99m/molybdenum-99 generator systems shall be stored and eluted (operated) under conditions recommended by manufacturers and applicable state and federal regulations. Such generator systems shall be eluted in an ISO Class 8 (see [Table 1](#)) or cleaner air environment to permit special handling, shielding, and air flow requirements. To limit acute and chronic radiation exposure of inspecting personnel to a level that is as low as reasonably achievable (ALARA), direct visual inspection of radiopharmaceutical CSPs containing high concentrations of doses of radioactivity shall be conducted in accordance with ALARA.

Radiopharmaceuticals prepared as Low-Risk Level CSPs with 12-Hour or Less BUD shall be prepared in a segregated compounding area. A line of demarcation defining the segregated compounding area shall be established. Materials and garb exposed in a patient care and treatment area shall not cross a line of demarcation into the segregated compounding area.

#### ALLERGEN EXTRACTS AS CSPs

Allergen extracts as CSPs are single-dose and multiple-dose intradermal or subcutaneous injections that are prepared by specially trained physicians and personnel under their direct supervision. Allergen extracts as CSPs are not subject to the personnel, environmental, and storage requirements for all CSP Microbial Contamination Risk Levels in this chapter only when all of the following criteria are met:

1. The compounding process involves simple transfer via sterile needles and syringes of commercial sterile allergen products and appropriate sterile added substances (e.g., glycerin, phenol in sodium chloride injection).
2. All allergen extracts as CSPs shall contain appropriate substances in effective concentrations to prevent the growth of microorganisms. Nonpreserved allergen extracts shall comply with the appropriate CSP risk level requirements in the chapter.
3. Before beginning compounding activities, personnel perform a thorough hand-cleansing procedure by removing debris from under fingernails using a nail cleaner under running warm water followed by vigorous hand and arm washing to the elbows for at least 30 seconds with either nonantimicrobial or antimicrobial soap and water.
  4. Compounding personnel don hair covers, facial hair covers, gowns, and face masks.
  5. Compounding personnel perform antiseptic hand cleansing with an alcohol-based surgical hand scrub with persistent activity.
  6. Compounding personnel don powder-free sterile gloves that are compatible with sterile 70% isopropyl alcohol (IPA) before beginning compounding manipulations.
  7. Compounding personnel disinfect their gloves intermittently with sterile 70% IPA when preparing multiple allergen extracts as CSPs.
8. Ampul necks and vial stoppers on packages of manufactured sterile ingredients are disinfected by careful wiping with sterile 70% IPA swabs to ensure that the critical sites are wet for at least 10 seconds and allowed to dry before they are used to compound allergen extracts as CSPs.
9. The aseptic compounding manipulations minimize direct contact contamination (e.g., from glove fingertips, blood, nasal and oral secretions, shed skin and cosmetics, other nonsterile materials) of critical sites (e.g., needles, opened ampuls, vial stoppers).
10. The label of each multiple-dose vial (MDV) of allergen extracts as CSPs lists the name of one specific patient and a BUD and storage temperature range that is assigned based on manufacturers' recommendations or peer-reviewed publications.
11. Single-dose allergen extracts as CSPs shall not be stored for subsequent additional use.

Personnel who compound allergen extracts as CSPs must be aware of greater potential risk of microbial and foreign material contamination when allergen extracts as CSPs are compounded in compliance with the foregoing criteria instead of the more rigorous standards in this chapter for CSP Microbial Contamination Risk Levels. Although contaminated allergen extracts as CSPs can pose health risks to patients when they are injected intradermally or subcutaneously, these risks are substantially greater if the extract is inadvertently injected intravenously.

#### VERIFICATION OF COMPOUNDING ACCURACY AND STERILITY

The compounding procedures and sterilization methods for CSPs correspond to correctly designed and verified written documentation in the compounding facility. Verification requires planned testing, monitoring, and documentation to demonstrate adherence to environmental quality requirements, personnel practices, and procedures critical to achieving and maintaining sterility, accuracy, and purity of finished CSPs. For example, sterility testing (see Test for Sterility of the Product To Be Examined under [Sterility Tests](#) (71)) may be applied to specimens of low- and medium-risk level CSPs, and standard self-contained biological indicators (BI) shall be added to nondispensable specimens of high-risk level CSPs before terminal sterilization for subsequent evaluation to determine whether the sterilization cycle was adequate (see [Biological Indicators for Sterilization](#) (1035)). Packaged and labeled CSPs shall be visually inspected for physical integrity and expected appearance, including final fill amount. The accuracy of identities, concentrations, amounts, and purities of ingredients in CSPs shall be confirmed by reviewing labels on packages, observing and documenting correct measurements with approved and correctly standardized devices, and reviewing information in labeling and certificates of analysis provided by suppliers. When the correct identity, purity, strength, and sterility of ingredients and components of CSPs



cannot be confirmed (in cases of, for example, unlabeled syringes, opened ampuls, punctured stoppers of vials and bags, containers of ingredients with incomplete labeling), such ingredients and components shall be discarded immediately.

Some individual ingredients, such as bulk drug substances, are not labeled with expiration dates when they are stable indefinitely in their commercial packages under their labeled storage conditions. However, despite retaining full chemical stability, such ingredients may gain or lose moisture during storage and use. Changes in moisture content may require testing (see [Loss on Drying \(731\)](#)) to determine the correct amount to weigh for accurate content of active chemical moieties in CSPs (see [Pharmaceutical Calculations in Prescription Compounding \(1160\)](#)).

Although not required, a quantitative stability-indicating chemical assay is recommended to ensure compounding accuracy of CSPs, especially those that contain drug ingredients with a narrow therapeutic plasma concentration range.

#### Sterilization Methods

The licensed healthcare professionals who supervise compounding shall be responsible for determining that the selected sterilization method (see [Methods of Sterilization under Sterilization and Sterility Assurance of Compendial Articles \(1211\)](#)) both sterilizes and maintains the strength, purity, quality, and packaging integrity of CSPs. The selected sterilization process is obtained from experience and appropriate information sources (e.g., see [Sterilization and Sterility Assurance of Compendial Articles \(1211\)](#))—and, preferably, verified wherever possible—to achieve sterility in the particular CSPs. General guidelines for matching CSPs and components to appropriate sterilization methods include the following:

1. CSPs have been ascertained to remain physically and chemically stable when subjected to the selected sterilization method.
2. Glass and metal devices may be covered tightly with aluminum foil, then exposed to dry heat in an oven at a mean temperature of 250° for 30 minutes to achieve sterility and depyrogenation (see [Dry-Heat Sterilization under Sterilization and Sterility Assurance of Compendial Articles \(1211\)](#) and [Bacterial Endotoxins Test \(85\)](#)). Such items are either used immediately or stored until use in an environment suitable for compounding Low-Risk Level CSPs and Medium-Risk Level CSPs.
3. Personnel ascertain from appropriate information sources that the sterile microporous membrane filter used to sterilize CSP solutions, during either compounding or administration, is chemically and physically compatible with the CSP.

#### sterilization of high-risk level csp by filtration

Commercially available sterile filters shall be approved for human-use applications in sterilizing pharmaceutical fluids. Sterile filters used to sterilize CSPs shall be pyrogen free and have a nominal pore size of 0.2 or 0.22 µm. They shall be certified by the manufacturer to retain at least 107 microorganisms of a strain of *Brevundimonas (Pseudomonas) diminuta* on each square centimeter of upstream filter surface area under conditions similar to those in which the CSPs will be sterilized (see [High-Risk Conditions in High-Risk Level CSPs](#)).

The compounding supervisor shall ensure, directly or from appropriate documentation, that the filters are chemically and physically stable at the pressure and temperature conditions to be used, that they have enough capacity to filter the required volumes, and that they will achieve sterility and maintain prefiltration pharmaceutical quality, including strength of ingredients of the specific CSP. The filter dimensions and liquid material to be sterile-filtered shall permit the sterilization process to be completed rapidly, without the replacement of the filter during the process. When CSPs are known to contain excessive particulate matter, a prefILTER of larger nominal pore size membrane is placed upstream from the sterilizing filter to remove gross particulate contaminants in order to maximize the efficiency of the sterilizing filter.

Filter units used to sterilize CSPs shall also be subjected to manufacturers' recommended integrity test, such as the bubble point test.

Compounding personnel shall ascertain that selected filters will achieve sterilization of the particular CSPs being sterilized. Large deviations from usual or expected chemical and physical properties of CSPs (e.g., water-miscible alcohols) may cause undetectable damage to filter integrity and shrinkage of microorganisms to sizes smaller than filter nominal pore size.

#### sterilization of high-risk level csp by steam

The process of thermal sterilization employing saturated steam under pressure, or autoclaving, is the preferred method to terminally sterilize aqueous preparations that have been verified to maintain their full chemical and physical stability under the conditions employed (see [Steam Sterilization under Sterilization and Sterility Assurance of Compendial Articles \(1211\)](#)). To achieve sterility, all materials are to be exposed to steam at 121° under a pressure of about 1 atmosphere or 15 psi for the duration verified by testing to achieve sterility of the items, which is usually 20 to 60 minutes for CSPs. An allowance shall be made for the time required for the material to reach 121° before the sterilization exposure duration is timed.

Not directly exposing items to pressurized steam may result in survival of microbial organisms and spores. Before their sterilization, plastic, glass, and metal devices are tightly wrapped in low-particle-shedding paper or fabrics or sealed in envelopes that prevent poststerilization microbial penetration. Immediately before filling ampuls and vials that will be steam sterilized, solutions are passed through a filter having a nominal pore size not larger than 1.2 µm for removal of particulate matter. Sealed containers shall be able to generate steam internally; thus, stoppered and crimped empty vials shall contain a small amount of moisture to generate steam.

The description of steam sterilization conditions and duration for specific CSPs shall be included in written documentation in the compounding facility. The effectiveness of steam sterilization shall be verified using appropriate BIs of *Bacillus stearothermophilus* (see [Biological Indicators \(1035\)](#)) and other confirmation methods such as temperature-sensing devices (see [Sterilization and Sterility Assurance of Compendial Articles \(1211\)](#) and [Sterility Tests \(71\)](#)).

#### sterilization of high-risk level csp by dry heat

Dry heat sterilization is usually done as a batch process in an oven designed for sterilization. Heated filtered air shall be evenly distributed throughout the chamber by a blower device. The oven should be equipped with a system for controlling temperature and exposure period. Sterilization by dry heat requires higher temperatures and longer exposure times than does sterilization by steam. Dry heat shall be used only for those materials that cannot be sterilized by steam, when either the moisture would damage the material or the material is impermeable. During sterilization, sufficient space shall be left between materials to allow for good circulation of the hot air. The description of dry heat sterilization conditions and duration for specific CSPs shall be included in written documentation in the compounding facility. The effectiveness of dry heat sterilization shall be verified using appropriate BIs of *Bacillus subtilis* (see [Biological Indicators \(1035\)](#)) and other confirmation methods such as temperature-sensing devices (see [Sterilization and Sterility Assurance of Compendial Articles \(1211\)](#) and [Sterility Tests \(71\)](#)). [note—Dry heat sterilization may be performed at a lower temperature than may be effective for depyrogenation].

#### Depyrogenation by Dry Heat

Dry heat depyrogenation shall be used to render glassware or containers such as vials free from pyrogens as well as viable microbes. A typical cycle would be 30 minutes at 250°. The description of the dry heat depyrogenation cycle and duration for specific load items shall be included in written documentation in the compounding facility. The effectiveness of the dry heat depyrogenation cycle shall be verified using endotoxin challenge vials (ECVs). The bacterial endotoxin test should be performed on the ECVs to verify that the cycle is capable of achieving a 3-log reduction in endotoxin (see [Sterilization and Sterility Assurance of Compendial Articles \(1211\)](#) and [Bacterial Endotoxins Test \(85\)](#)).

#### ENVIRONMENTAL QUALITY AND CONTROL

Achieving and maintaining sterility and overall freedom from contamination of a CSP is dependent on the quality status of the components incorporated, the process utilized, personnel performance, and the environmental conditions under which the process is performed. The standards required for the environmental conditions depend on the amount of exposure of the CSP to the immediate environment anticipated during processing. The quality and control of environmental conditions for each risk level of operation are explained in this section.

In addition, operations using nonsterile components require the use of a method of preparation designed to produce a sterile preparation.

#### Exposure of Critical Sites

Maintaining the sterility and cleanliness (i.e., freedom from sterile foreign materials) of critical sites is a primary safeguard for CSPs. Critical sites are locations that include any component or fluid pathway surfaces (e.g., vial septa, injection ports, beakers) or openings (e.g., opened ampuls, needle hubs) exposed and at risk of direct contact with air (e.g.,



...lient room or HEPA filtered), moisture (e.g., oral and mucosal secretions), or touch contamination. The risk of, or potential for, critical sites to be contaminated with microorganisms and foreign matter increases with increasing exposed area of the critical sites, the density or concentration of contaminants, and exposure duration to worse than ISO Class 5 (see [Table 1](#)) air. Examples include an opened ampul or vial stopper on a 10-mL or larger vial or an injection port on a package of intravenous solution having an area larger than the point of a needle or the tip of a syringe.

The nature of a critical site also affects the risk of contamination. The relatively rough, permeable surface of an elastomeric closure retains microorganisms and other contaminants after swabbing with a sterile 70% IPA pad more readily than does the smoother glass surface of the neck of an ampul. Therefore, the surface disinfection can be expected to be more effective for an ampul.

Protection of critical sites by precluding physical contact and airborne contamination shall be given the highest priority in sterile compounding practice. Airborne contaminants, especially those generated by sterile compounding personnel, are much more likely to reach critical sites than are contaminants that are adhering to the floor or other surfaces below the work level. Furthermore, large and high-density particles that are generated and introduced by compounding manipulations and personnel have the potential to settle on critical sites even when those critical sites are exposed within ISO Class 5 (see [Table 1](#)) air.

#### ISO Class 5 Air Sources, Buffer Areas, and Ante-Areas

The most common sources of ISO Class 5 (see [Table 1](#)) air quality for exposure of critical sites are horizontal and vertical LAFWs, CAIs, and CACIs. A clean room (see [Microbiological Evaluation of Clean Rooms and Other Controlled Environments](#) (1116)) is a compounding environment that is supplied with HEPA or HEPA-filtered air that meets ISO Class 7 (see [Table 1](#)), the access to which is limited to personnel trained and authorized to perform sterile compounding and facility cleaning. A buffer area is an area that provides at least ISO Class 7 (see [Table 1](#)) air quality.

[Figure 1](#) is a conceptual representation of the placement of an ISO Class 5 (see [Table 1](#)) PEC in a segregated compounding area used for low-risk level CSPs with 12-hour or less BUD. This plan depicts the most critical operation area located within the PEC in a designated area (see definition of Segregated Compounding Area) separated from activities not essential to the preparation of CSPs. Placement of devices (e.g., computers, printers) and objects (e.g., carts, cabinets) that are not essential to compounding in the segregated area should be restricted or limited, depending on their effect on air quality in the ISO Class 5 (see [Table 1](#)) PEC.

Conceptual representation of USP Chapter <797> facility requirements

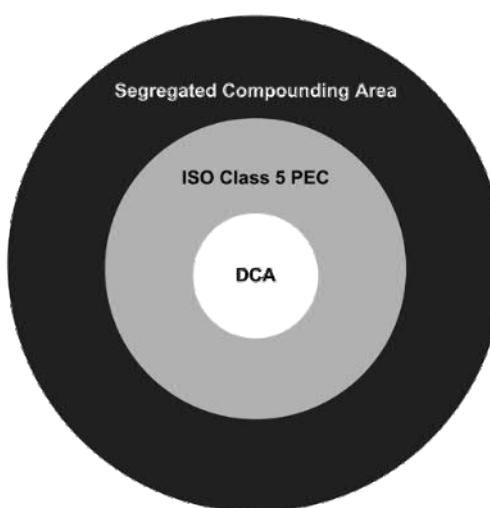


Figure 1. Conceptual representation of the placement of an ISO Class 5 PEC in a segregated compounding area used for low-risk level CSPs with 12-hour or less BUD.

[Figure 2](#) is a conceptual representation of the arrangement of a facility for preparation of CSPs categorized as low-, medium-, and high-risk level. The quality of the environmental air increases with movement from the outer boundary to the direct compounding area (DCA). Placement of devices in ante-areas and buffer areas is dictated by their effect on the designated environmental quality of atmospheres and surfaces, which shall be verified by monitoring (see Viable and Nonviable Environmental Sampling (ES) Testing). It is the responsibility of each compounding facility to ensure that each source of ISO Class 5 (see [Table 1](#)) environment for exposure of critical sites and sterilization by filtration is properly located, operated, maintained, monitored, and verified.

Conceptual representation of USP Chapter <797> facility requirements

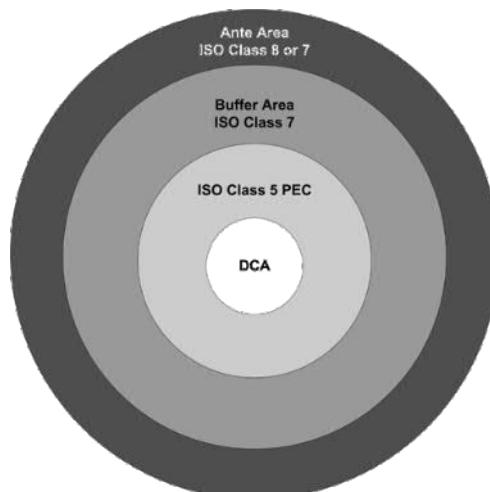




Figure 2. Conceptual representation of the arrangement of a facility for preparation of CSPs categorized as low-, medium-, and high-risk level.

Placement of devices (e.g., computers, printers) and objects (e.g., carts, cabinets) that are not essential to compounding in buffer areas is dictated by their effect on the required environmental quality of air atmospheres and surfaces, which shall be verified by monitoring (see Viable and Nonviable Environmental Sampling (ES) Testing). It is the responsibility of each compounding facility to ensure that each source of ISO Class 5 (see [Table 1](#)) environment for exposure of critical sites and sterilization by filtration is properly located, operated, maintained, monitored, and verified.

#### Facility Design and Environmental Controls

Compounding facilities are physically designed and environmentally controlled to minimize airborne contamination from contacting critical sites. These facilities shall also provide a comfortable and well-lighted working environment, which typically includes a temperature of 20° or cooler, to maintain comfortable conditions for compounding personnel to perform flawlessly when attired in the required aseptic compounding garb. PECs typically include, but are not limited to, LAFWs, BSCs, CAIs, and CACIs, which provide an ISO Class 5 (see [Table 1](#)) environment for the exposure of critical sites. PECs shall maintain ISO Class 5 (see [Table 1](#)) or better conditions for 0.5-μm particles (dynamic operating conditions) while compounding CSPs. Secondary engineering controls such as buffer areas and ante-areas generally serve as a core for the location of the PEC. Buffer areas are designed to maintain at least ISO Class 7 (see [Table 1](#)) conditions for 0.5-μm particles under dynamic conditions and ISO Class 8 (see [Table 1](#)) conditions for 0.5-μm and larger particles under dynamic conditions for the ante-areas. Airborne contamination control is achieved in the PEC through the use of HEPA filters. The airflow in the PEC shall be unidirectional (laminar flow), and because of the particle collection efficiency of the filter, the "first air" at the face of the filter is, for the purposes of aseptic compounding, free from airborne particulate contamination. HEPA-filtered air shall be supplied in critical areas (ISO Class 5, see [Table 1](#)) at a velocity sufficient to sweep particles away from the compounding area and maintain unidirectional airflow during operations. Proper design and control prevents turbulence and stagnant air in the critical area. In situ air pattern analysis via smoke studies shall be conducted at the critical area to demonstrate unidirectional airflow and sweeping action over and away from the product under dynamic conditions.

The principles of HEPA-filtered unidirectional airflow in the work environment shall be understood and practiced in the compounding process in order to achieve the desired environmental conditions. Policies and procedures for maintaining and working within the PEC area shall be written and followed. The policies and procedures will be determined by the scope and risk levels of the aseptic compounding activities utilized during the preparation of the CSPs. The CSP work environment is designed to have the cleanest work surfaces (PEC) located in a buffer area. The buffer area shall maintain at least ISO Class 7 (see [Table 1](#)) conditions for 0.5-μm and larger particles under dynamic operating conditions. The room shall be segregated from surrounding, unclassified spaces to reduce the risk of contaminants being blown, dragged, or otherwise introduced into the filtered unidirectional airflow

environment, and this segregation shall be continuously monitored. For rooms providing a physical separation through the use of walls, doors, and pass-throughs, a minimum differential positive pressure of 0.02- to 0.05-inch water column is required. For buffer areas not physically separated from the ante-areas, the principle of displacement airflow shall be employed. This concept utilizes a low pressure differential, high airflow principle. Using displacement airflow typically requires an air velocity of 40 ft per minute or more from the buffer area across the line of demarcation into the ante-area.

The displacement concept shall not be used for high-risk compounding.<sup>8</sup> The PEC shall be placed within a buffer area in such a manner as to avoid conditions that could adversely affect their operation. For example, strong air currents from opened doors, personnel traffic, or air streams from the HVAC systems can disrupt the unidirectional airflow in open-faced workbenches. The operators may also create disruptions in airflow by their own movements and by the placement of objects onto the work surface. The PEC shall be placed out of the traffic flow and in a manner to avoid disruption from the HVAC system and room cross-drafts. Room air exchanges are typically expressed as ACPHs. Adequate HEPA-filtered airflow supplied to the buffer area and ante-area is required to maintain cleanliness classification during operational activity through the number of ACPHs. Factors that should be considered when determining air-change requirements include number of personnel working in the room and compounding processes that generate particulates, as well as temperature effects. An ISO Class 7 (see [Table 1](#)) buffer area and ante-area supplied with HEPA-filtered air shall receive an ACPH of not less than 30. The PEC is a good augmentation to generating air changes in the air supply of an area but cannot be the sole source of HEPA-filtered air. If the area has an ISO Class 5 (see [Table 1](#)) recirculating device, a minimum of 15 ACPHs through the area supply HEPA filters is adequate, providing the combined ACPH is not less than 30. More air changes may be required, depending on the number of personnel and processes. HEPA-filtered supply air shall be introduced at the ceiling, and returns should be mounted low on the wall, creating a general top-down dilution of area air with HEPA-filtered make-up air. Ceiling-mounted returns are not recommended. All HEPA filters should be efficiency tested using the most penetrating particle size and should be leak tested at the factory and then leak tested again in situ after installation.<sup>2</sup>

Activities and tasks carried out within the buffer area shall be limited to only those necessary when working within a controlled environment. Only the furniture, equipment, supplies, and other material required for the compounding activities to be performed shall be brought into the area, and they shall be nonpermeable, nonshedding, cleanable, and resistant to disinfectants. Whenever such items are brought into the area, they shall first be cleaned and disinfected. Whenever possible, equipment and other items used in the buffer area shall not be taken out of the area except for calibration, servicing, or other activities associated with the proper maintenance of the item.

The surfaces of ceilings, walls, floors, fixtures, shelving, counters, and cabinets in the buffer area shall be smooth, impervious, free from cracks and crevices, and nonshedding, thereby promoting cleanability and minimizing spaces in which microorganisms and other contaminants may accumulate. The surfaces shall be resistant to damage by disinfectant agents. Junctures of ceilings to walls shall be coved or caulked to avoid cracks and crevices where dirt can accumulate. If ceilings consist of inlaid panels, the panels shall be impregnated with a polymer to render them impervious and hydrophobic, and they shall be caulked around each perimeter to seal them to the support frame. Walls may be constructed of flexible material (e.g., heavy gauge polymer), panels locked together and sealed, or of epoxy-coated gypsum board. Preferably, floors are overlaid with wide sheet vinyl flooring with heat-welded seams and coving to the sidewall. Dust-collecting overhangs, such as ceiling utility pipes, and ledges, such as windowsills, should be avoided. The exterior lens surface of ceiling lighting fixtures should be smooth, mounted flush, and sealed. Any other penetrations through the ceiling or walls shall be sealed. The buffer area shall not contain sources of water (sinks) or floor drains. Work surfaces shall be constructed of smooth, impervious materials, such as stainless steel or molded plastic, so that they are easily cleaned and disinfected. Carts should be of stainless steel wire, nonporous plastic, or sheet metal construction with good quality, cleanable casters to promote mobility. Storage shelving, counters, and cabinets shall be smooth, impervious, free from cracks and crevices, nonshedding, cleanable, and disinfectable; their number, design, and manner of installation shall promote effective cleaning and disinfection.

#### Placement of Primary Engineering Controls

PECs (LAFWs, BSCs, CAIs, and CACIs) shall be located within a restricted access ISO Class 7 (see [Table 1](#)) buffer area (see [Figure 1](#)), with the following CAI/CACI exceptions below:

- Only authorized personnel and materials required for compounding and cleaning shall be permitted in the buffer area.
- Presterilization procedures for high-risk level CSPs, such as weighing and mixing, shall be completed in no worse than an ISO Class 8 (see [Table 1](#)) environment.
- PECs shall be located out of traffic patterns and away from room air currents that could disrupt the intended airflow patterns.

CAIs and CACIs shall be placed in an ISO Class 7 (see [Table 1](#)) buffer area unless they meet all of the following conditions:

- The isolator shall provide isolation from the room and maintain ISO Class 5 (see [Table 1](#)) during dynamic operating conditions, including transferring ingredients, components, and devices into and out of the isolator and during preparation of CSPs.
- Particle counts sampled approximately 6 to 12 inches upstream of the critical exposure site shall maintain ISO Class 5 (see [Table 1](#)) levels during compounding operations.
- Not more than 3520 particles (0.5 μm and larger) per m<sup>3</sup> shall be counted during material transfer, with the particle counter probe located as near to the transfer door as possible without obstructing the transfer.<sup>8</sup>

It is incumbent on the compounding personnel to obtain documentation from the manufacturer that the CAI/CACI will meet this standard when located in environments where the background particle counts exceed ISO Class 8 (see [Table 1](#)) for 0.5-μm and larger particles. When isolators are used for sterile compounding, the recovery time to achieve ISO Class 5 (see [Table 1](#)) air quality shall be documented and internal procedures developed to ensure that adequate recovery time is allowed after material transfer before and during compounding operations.

If the PEC is a CAI or CACI that does not meet the requirements above or is a LAFW or BSC that cannot be located within an ISO Class 7 (see [Table 1](#)) buffer area, then only low-risk level nonhazardous and radiopharmaceutical CSPs pursuant to a physician order for a specific patient may be prepared, and administration of the CSP shall commence within 12 hours of preparation or as recommended in the manufacturer's package insert, whichever is less.

#### Viable and Nonviable Environmental Sampling (ES) Testing

The ES program should provide information to staff and leadership to demonstrate that the PEC is maintaining an environment within the compounding area that consistently ensures



Acceptably low viable and nonviable particle levels. The compounding area includes the ISO Class 5 (see [Table 1](#)) PEC (LAFWs, BSCs, CAIs, and CACIs), buffer areas, ante-areas, and segregated compounding areas.

Environmental sampling shall occur as part a comprehensive quality management program and shall occur minimally under any of the following conditions:

- as part of the commissioning and certification of new facilities and equipment;
- following any servicing of facilities and equipment;
- as part of the re-certification of facilities and equipment (i.e., every 6 months);
- in response to identified problems with end products or staff technique; or
- in response to issues with CSPs, observed compounding personnel work practices, or patient-related infections (where the CSP is being considered as a potential source of the infection).

environmental nonviable particle testing program

A program to sample nonviable airborne particles differs from that for viable particles in that it is intended to directly measure the performance of the engineering controls used to create the various levels of air cleanliness, for example, ISO Class 5, 7, or 8 (see [Table 1](#)).

Engineering Control Performance Verification— PECs (LAFWs, BSCs, CAIs, and CACIs) and secondary engineering controls (buffer and ante-areas) are essential components of the overall contamination control strategy for aseptic compounding. As such, it is imperative that they perform as designed and that the resulting levels of contamination be within acceptable limits. Certification procedures such as those outlined in Certification Guide for Sterile Compounding Facilities (CAG-003-2006)<sup>8</sup> shall be performed by a qualified individual no less than every 6 months and whenever the device or room is relocated or altered or major service to the facility is performed.

Total Particle Counts— Certification that each ISO classified area, for example, ISO Class 5, 7, and 8 (see [Table 1](#)), is within established guidelines shall be performed no less than every 6 months and whenever the LAFW, BSC, CAI, or CACI is relocated or the physical structure of the buffer area or ante-area has been altered. Testing shall be performed by qualified operators using current, state-of-the-art electronic equipment with results of the following:

- ISO Class 5: not more than 3520 particles 0.5  $\mu$ m and larger size per cubic meter of air for any LAFW, BSC, CAI, and CACI;
- ISO Class 7: not more than 352,000 particles of 0.5  $\mu$ m size and larger per cubic meter of air for any buffer area;
- ISO Class 8: not more than 3,520,000 particles or 0.5  $\mu$ m size and larger per cubic meter of air for any ante-area.

All certification records shall be maintained and reviewed by supervising personnel or other designated employees to ensure that the controlled environments comply with the proper air cleanliness, room pressures, and ACPHs.

pressure differential monitoring

A pressure gauge or velocity meter shall be installed to monitor the pressure differential or airflow between the buffer area and the ante-area and between the ante-area and the general environment outside the compounding area. The results shall be reviewed and documented on a log at least every work shift (minimum frequency shall be at least daily) or by a continuous recording device. The pressure between the ISO Class 7 (see [Table 1](#)) and the general pharmacy area shall not be less than 5 Pa (0.02 inch water column). In facilities where low- and medium-risk level CSPs are prepared, differential airflow shall maintain a minimum velocity of 0.2 meters per second (40 feet per minute) between buffer area and ante-area.

environmental viable airborne particle testing program

The risk of contaminating a CSP prepared under low-risk level and medium-risk level conditions is highly dependent on proper hand hygiene and garbing practices, compounding personnel aseptic technique, and the presence of surface contamination, assuming that all work is performed in a certified and properly functioning ISO Class 5 (see [Table 1](#)) PEC and secondary engineering controls, ISO Class 7 (see [Table 1](#)) buffer area, and ISO Class 8 (see [Table 1](#)) ante-area. High-risk level CSPs pose the greatest threat to patients because compounding personnel are tasked with the requirement of processing nonsterile components and devices in order to achieve sterility.

A sampling program in conjunction with an observational audit is designed to evaluate the competency of compounding personnel work practices, allowing for the implementation of corrective actions on an ongoing basis (see Personnel Training and Competency Evaluation of Garbing, Aseptic Work Practices and Cleaning/Disinfection Procedures).

**Sampling Plan**— An appropriate environmental sampling plan shall be developed for airborne viable particles based on a risk assessment of compounding activities performed.

Selected sampling sites shall include locations within each ISO Class 5 (see [Table 1](#)) environment and in the ISO Class 7 and 8 (see [Table 1](#)) areas and in the segregated compounding areas at greatest risk of contamination (e.g., work areas near the ISO Class 5 [see [Table 1](#)] environment, counters near doors, pass-through boxes). The plan shall include sample location, method of collection, frequency of sampling, volume of air sampled, and time of day as related to activity in the compounding area and action levels.

Review of the data generated during a sampling event may detect elevated amounts of airborne microbial bioburden; such changes may be indicative of adverse changes within the environment. It is recommended that compounding personnel refer to [Microbiological Evaluation of Clean Rooms and Other Controlled Environments](#) (1116) and the CDC's "Guidelines for Environmental Infection Control in Healthcare Facilities, 2003" for more information.

**Growth Medium**— A general microbiological growth medium such as Soybean–Casein Digest Medium shall be used to support the growth of bacteria. Malt extract agar or some other media that support the growth of fungi shall be used in high-risk level compounding environments. Media used for surface sampling must be supplemented with additives to neutralize the effects of disinfecting agents (e.g., TSA with lecithin and polysorbate 80).

**Viable Air Sampling**— Evaluation of airborne microorganisms using volumetric collection methods in the controlled air environments (LAFWs, CAIs, clean room or buffer areas, and ante-areas) shall be performed by properly trained individuals for all compounding risk levels.

Impaction shall be the preferred method of volumetric air sampling. Use of settling plates for qualitative air sampling may not be able to determine adequately the quality of air in the controlled environment. The settling of particles by gravity onto culture plates depends on the particle size and may be influenced by air movement. Consequently, the number of colony-forming units (cfu) on a settling plate may not always relate to the concentrations of viable particles in the sampled environment.

For low-, medium-, and high-risk level compounding, air sampling shall be performed at locations that are prone to contamination during compounding activities and during other activities such as staging, labeling, gowning, and cleaning. Locations shall include zones of air backwash turbulence within LAFW and other areas where air backwash turbulence may enter the compounding area (doorways, in and around ISO Class 5 [see [Table 1](#)] PEC and environments). Consideration should be given to the overall effect the chosen sampling method will have on the unidirectional airflow within a compounding environment.

For low-risk level CSPs with 12-hour or less BUD prepared in a PEC (LAFWs, BSCs, CAIs) that maintains an ISO Class 5 (see [Table 1](#)), air sampling shall be performed at locations inside the ISO Class 5 (see [Table 1](#)) environment and other areas that are in close proximity to the ISO Class 5 (see [Table 1](#)) environment during the certification of the PEC.

**Air Sampling Devices**— There are a number of manufacturers of electronic air sampling equipment. It is important that personnel refer to the manufacturer's recommended procedures when using the equipment to perform volumetric air sampling procedures. The instructions in the manufacturer's user's manual for verification and use of electric air samplers that actively collect volumes of air for evaluation must be followed. A sufficient volume of air (400 to 1000 liters) shall be tested at each location in order to maximize sensitivity. The volumetric air sampling devices need to be serviced and calibrated as recommended by the manufacturer.

It is recommended that compounding personnel also refer to Methodology and Instrumentation for Quantitation of Viable Airborne Microorganisms under [Microbiological Evaluation of Clean Rooms and Other Controlled Environments](#) (1116), which provides more information on the use of volumetric air samplers and volume of air that should be sampled to detect environmental bioburden excursions.

**Air Sampling Frequency and Process**— Air sampling shall be performed at least semiannually (i.e., every 6 months) as part of the re-certification of facilities and equipment. If compounding occurs in multiple locations within an institution (e.g., main pharmacy, satellites), environmental sampling is required for each individual compounding area. A sufficient volume of air shall be sampled and the manufacturer's guidelines for use of the electronic air sampling equipment followed. Any facility construction or equipment servicing may require that air sampling be performed during these events.



...cubation Period— At the end of the designated sampling or exposure period for air sampling activities, the microbial growth media plates are recovered and their covers secured (e.g., taped), and they are inverted and incubated at a temperature and for a time period conducive to multiplication of microorganisms. TSA should be incubated at 30° to 35° for 48 to 72 hours. Malt extract agar or other suitable fungal media should be incubated at 26° to 30° for 5 to 7 days. The number of discrete colonies of microorganisms are counted and reported as cfu and documented on an environmental sampling form. Counts from air sampling need to be transformed into cfu per cubic meter of air and evaluated for adverse trends.

Action Levels, Documentation, and Data Evaluation— The value of viable microbial sampling of the air in the compounding environment is realized when the data are used to identify and correct an unacceptable situation. Sampling data shall be collected and reviewed on a periodic basis as a means of evaluating the overall control of the compounding environment.

If an activity consistently shows elevated levels of microbial growth, competent microbiology personnel shall be consulted.

Any cfu count that exceeds its respective action level (see [Table 2](#)) should prompt a re-evaluation of the adequacy of personnel work practices, cleaning procedures, operational procedures, and air filtration efficiency within the aseptic compounding location. An investigation into the source of the contamination shall be conducted. Sources could include HVAC systems, damaged HEPA filters, and changes in personnel garbing or work practices. The source of the problem shall be eliminated, the affected area cleaned, and resampling performed.

Counts of cfu are to be used as an approximate measure of the environmental microbial bioburden. Action levels are determined on the basis of cfu data gathered at each sampling location and trended over time. The numbers in [Table 2](#) should be used only as guidelines. Regardless of the number of cfu identified in the pharmacy, further corrective actions will be dictated by the identification of microorganisms recovered (at least the genus level) by an appropriate credentialed laboratory of any microbial bioburden captured as a cfu using an impaction air sampler. Highly pathogenic microorganisms (e.g., Gram-negative rods, coagulase positive staphylococcus, molds and yeasts) can be potentially fatal to patients receiving CSPs and must be immediately remedied, regardless of cfu count, with the assistance of a competent microbiologist, infection control professional, or industrial hygienist.

Table 2. Recommended Action Levels for Microbial Contamination\*  
†(cfu per cubic meter [1000 liters] of air per plate)

Classification	Air Sample†
ISO Class 5	> 1
ISO Class 7	> 10
ISO Class 8 or worse	> 100

\* Guidance for Industry—Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice—US HHS, FDA September 2004.

#### Additional Personnel Requirements

Food, drinks, and materials exposed in patient care and treatment areas shall not enter ante-areas, buffer areas, or segregated compounding areas where components and ingredients of CSPs are present. When compounding activities require the manipulation of a patient's blood-derived or other biological material (e.g., radiolabeling a patient's or donor's white blood cells), the manipulations shall be clearly separated from routine material-handling procedures and equipment used in CSP preparation activities, and they shall be controlled by specific SOPs in order to avoid any cross-contamination. Packaged compounding supplies and components, such as needles, syringes, tubing sets, and small- and large-volume parenterals, should be uncartoned and wiped down with a disinfectant that does not leave a residue (e.g., sterile 70% IPA), when possible in an ante-area of ISO Class 8 (see [Table 1](#)) air quality, before being passed into the buffer areas. Personnel hand hygiene and garbing procedures are also performed in the ante-area, which may contain a sink that enables hands-free use with a closed system of soap dispensing to minimize the risk of extrinsic contamination. There shall be some demarcation designation that separates the ante-area from the buffer area. Adequate provision for performing antiseptic hand cleansing using an alcohol-based surgical hand scrub with persistent activity followed by the donning of sterile gloves should be provided after entry into the buffer area.

#### Cleaning and Disinfecting the Compounding Area

Environmental contact is a major source of microbial contamination of CSPs. Consequently, scrupulous attention to cleaning and disinfecting the sterile compounding areas is required to minimize this as a source of CSP contamination.

The cleaning and disinfecting practices and frequencies in this section apply to ISO Class 5 (see [Table 1](#)) compounding areas for exposure of critical sites as well as buffer areas, ante-areas, and segregated compounding areas. Compounding personnel are responsible for ensuring that the frequency of cleaning is in accordance with the requirements stated in [Table 3](#) and determining the cleaning and disinfecting products to be used (see Appendix II). Any organizational or institutional policies regarding disinfectant selection should be considered by compounding personnel. All cleaning and disinfecting practices and policies for the compounding of CSPs shall be included in written SOPs and shall be followed by all compounding personnel.

The selection and use of disinfectants in healthcare facilities is guided by several properties, such as microbicidal activity, inactivation by organic matter, residue, and shelf life (see Appendix II). In general, highly toxic disinfectants, such as glutaraldehyde, are not used on housekeeping surfaces (e.g., floors, countertops). Many disinfectants registered by the EPA are one-step disinfectants. This means that the disinfectant has been formulated to be effective in the presence of light to moderate soiling without a pre-cleaning step.

Surfaces in LAFWs, BSCs, CAIs, and CACIs, which are intimate to the exposure of critical sites, require disinfecting more frequently than do housekeeping surfaces such as walls and ceilings. Disinfecting sterile compounding areas shall occur on a regular basis at the intervals noted in [Table 3](#) when spills occur, when the surfaces are visibly soiled, and when microbial contamination is known to have been or is suspected of having been introduced into the compounding areas.

When the surface to be disinfected has heavy soiling, a cleaning step is recommended prior to the application of the disinfectant. Trained compounding personnel are responsible for developing, implementing, and practicing the procedures for cleaning and disinfecting the DCAs written in the SOPs. Cleaning and disinfecting shall occur before compounding is performed. Items shall be removed from all areas to be cleaned, and surfaces shall be cleaned by removing loose material and residue from spills; for example, water-soluble solid residues are removed with sterile water (for injection or irrigation) and low-shedding wipes. This shall be followed by wiping with a residue-free disinfecting agent such as sterile 70% IPA, which is allowed to dry before compounding begins.

Cleaning and disinfecting surfaces in the LAFWs, BSCs, CAIs, and CACIs are the most critical practices before the preparation of CSPs. Consequently, such surfaces shall be cleaned and disinfected frequently, including at the beginning of each work shift, before each batch preparation is started, every 30 minutes during continuous compounding periods of individual CSPs, when there are spills, and when surface contamination is known or suspected from procedural breaches.

Work surfaces in the ISO Class 7 (see [Table 1](#)) buffer areas and ISO Class 8 (see [Table 1](#)) ante-areas as well as segregated compounding areas shall be cleaned and disinfected at least daily, and dust and debris shall be removed when necessary from storage sites for compounding ingredients and supplies using a method that does not degrade the ISO Class 7 or 8 (see [Table 1](#)) air quality (see [Disinfectants and Antiseptics](#) (1072)).

Table 3. Minimum Frequency of Cleaning and Disinfecting Compounding Areas

Site	Minimum Frequency
ISO Class 5 (see <a href="#">Table 1</a> ) Primary Engineering Control (e.g., LAFW, BSC, CAI, CACI)	At the beginning of each shift, before each batch, not longer than 30 minutes following the previous surface disinfection when ongoing compounding activities are occurring, after spills, and when surface contamination is known or suspected
Counters and easily cleanable work surfaces	Daily
Floors	Daily
Walls	Monthly
Ceilings	Monthly
Storage shelving	Monthly

Floors in the buffer or clean area, ante-area, and segregated compounding area are cleaned by mopping with a cleaning and disinfecting agent once daily at a time when no aseptic operations are in progress. Mopping shall be performed by trained personnel using approved agents and procedures described in the written SOPs. It is incumbent on compounding personnel to ensure that such cleaning is performed properly. In the buffer or clean area, ante-area, and segregated compounding area, walls, ceilings, and shelving shall be cleaned



disinfected monthly. Cleaning and disinfecting agents are to be used with careful consideration of compatibilities, effectiveness, and inappropriate or toxic residues (see Appendix II). Their schedules of use and methods of application shall be in accordance with written SOPs and followed by custodial or compounding personnel.

All cleaning materials, such as wipers, sponges, and mops, shall be nonshedding, preferably composed of synthetic micro fibers, and dedicated to use in the buffer or clean area, ante-area, and segregated compounding areas and shall not be removed from these areas except for disposal. Floor mops may be used in both the buffer or clean area and ante-area, but only in that order. Ideally, all cleaning tools are discarded after one use by collection in suitable plastic bags and removed with minimal agitation. If cleaning materials (e.g., mops) are reused, procedures shall be developed (based on manufacturers' recommendations) that ensure that the effectiveness of the cleaning device is maintained and that repeated use does not add to the bioburden of the area being cleaned.

Supplies and equipment removed from shipping cartons shall be wiped with a suitable disinfecting agent (e.g., sterile 70% IPA) delivered from a spray bottle or other suitable delivery method. After the disinfectant is sprayed or wiped on a surface to be disinfected, the disinfectant shall be allowed to dry, during which time the item shall not be used for compounding purposes.

Wiping with small sterile 70% IPA swabs that are commercially available in individual foil-sealed packages (or a comparable method) is preferred for disinfecting entry points on bags and vials, allowing the IPA to dry before piercing stoppers with sterile needles and breaking necks of ampuls. The surface of the sterile 70% IPA swabs used for disinfecting entry points of sterile packages and devices shall not contact any other object before contacting the surface of the entry point. Sterile 70% IPA wetted gauze pads or other particle-generating material shall not be used to disinfect the sterile entry points of packages and devices.

When sterile supplies are received in sealed pouches designed to keep them sterile until opening, the sterile supplies may be removed from the covering pouches as the supplies are introduced into the ISO Class 5 (see [Table 1](#)) PEC (LAFW, BSC, CAI, CACI) without the need to disinfect the individual sterile supply items. No shipping or other external cartons may be taken into the buffer or clean area or segregated compounding area.

#### Personnel Cleansing and Garbing

The careful cleansing of hands and arms and the correct donning of PPE by compounding personnel constitute the first major step in preventing microbial contamination in CSPs. Personnel shall also be thoroughly competent and highly motivated to perform flawless aseptic manipulations with ingredients, devices, and components of CSPs. Squamous cells are normally shed from the human body at a rate of 106 or more per hour, and those skin particles are laden with microorganisms.<sup>10,11</sup> When individuals are experiencing rashes, sunburn, weeping sores, conjunctivitis, active respiratory infection, as well as when they wear cosmetics, they shed these particles at even higher rates. Particles shed from compounding personnel pose an increased risk of microbial contamination of critical sites of CSPs. Therefore, compounding personnel with such conditions as mentioned above shall be excluded from working in ISO Class 5 (see [Table 1](#)) and ISO Class 7 (see [Table 1](#)) compounding areas until their conditions are remedied.

Before entering the buffer area or segregated compounding area (see Low-Risk Level CSPs with 12-Hour or Less BUD), compounding personnel shall remove personal outer garments (e.g., bandannas, coats, hats, jackets, scarves, sweaters, vests); all cosmetics, because they shed flakes and particles; and all hand, wrist, and other visible jewelry or piercings (e.g., earrings, lip or eyebrow piercings) that can interfere with the effectiveness of PPE (e.g., fit of gloves and cuffs of sleeves). The wearing of artificial nails or extenders is prohibited while working in the sterile compounding environment. Natural nails shall be kept neat and trimmed.

Personnel shall don the following PPE in an order that proceeds from those activities considered the dirtiest to those considered the cleanest. Garbing activities considered the dirtiest include donning of dedicated shoes or shoe covers, head and facial hair covers (e.g., beard covers in addition to face masks), and face masks/eye shields. Eye shields are optional unless working with irritants such as germicidal disinfecting agents or when preparing hazardous drugs.

After donning dedicated shoes or shoe covers, head and facial hair covers, and face masks, a hand cleansing procedure shall be performed by removing debris from underneath fingernails using a nail cleaner under running warm water followed by vigorous hand washing. Hands and forearms shall be washed to the elbows for at least 30 seconds with soap (either nonantimicrobial or antimicrobial) and water while in the ante-area. The use of antimicrobial scrub brushes is not recommended because they can cause skin irritation and skin damage. Hands and forearms to the elbows will be completely dried using either lint-free disposable towels or an electronic hand dryer. After completion of hand washing, a nonshedding gown with sleeves that fit snugly around the wrists and enclosed at the neck is donned. Gowns designated for buffer area use shall be worn, and preferably they should be disposable. If reusable gowns are worn, they should be laundered appropriately for buffer area use.

Once inside the buffer area or segregated compounding area (see Low-Risk Level CSPs with 12-Hour or Less BUD), and prior to donning sterile powder-free gloves, antiseptic hand cleansing shall be performed using a waterless alcohol-based surgical hand scrub with persistent activity<sup>12</sup> following manufacturers' recommendations. Hands are allowed to dry thoroughly before donning sterile gloves.

Sterile gloves shall be the last item donned before compounding begins. Gloves become contaminated when they contact nonsterile surfaces during compounding activities. Disinfection of contaminated gloved hands may be accomplished by wiping or rubbing sterile 70% IPA to all contact surface areas of the gloves and letting the gloved hands dry thoroughly. Only use gloves that have been tested for compatibility with alcohol disinfection by the manufacturer. Routine application of sterile 70% IPA shall occur throughout the compounding process and whenever nonsterile surfaces (e.g., vials, counter tops, chairs, carts) are touched. Gloves on hands shall also be routinely inspected for holes, punctures, or tears and replaced immediately if such are detected. Antiseptic hand cleansing shall be performed as indicated above. Compounding personnel shall be trained and evaluated in the avoidance of touching critical sites.

When compounding personnel exit the compounding area during a work shift, the exterior gown may be removed and retained in the compounding area if not visibly soiled, to be re-donned during that same work shift only. However, shoe covers, hair and facial hair covers, face masks/eye shields, and gloves shall be replaced with new ones before re-entering the compounding area, and proper hand hygiene shall be performed.

During high-risk compounding activities that precede terminal sterilization, such as weighing and mixing of nonsterile ingredients, compounding personnel shall be garbed and gloved the same as when performing compounding in an ISO Class 5 (see [Table 1](#)) environment. Properly garbed and gloved compounding personnel who are exposed to air quality that is either known or suspected to be worse than ISO Class 7 (see [Table 1](#)) shall re-garb PPE along with washing their hands properly, performing antiseptic hand cleansing with a waterless alcohol-based surgical hand scrub, and donning sterile gloves upon re-entering the ISO Class 7 (see [Table 1](#)) buffer area. When CAIs and CACIs are the source of the ISO Class 5 (see [Table 1](#)) environment, the garbing and gloving requirements for compounding personnel should be as described above, unless the isolator manufacturer can provide written documentation based on validated environmental testing that any component(s) of PPE or personnel cleansing are not required.

#### Personnel Training and Competency Evaluation of Garbing, Aseptic Work Practices, and Cleaning/Disinfection Procedures

Personnel who prepare CSPs shall be trained conscientiously and skillfully by expert personnel and through multimedia instructional sources and professional publications in the theoretical principles and practical skills of garbing procedures, aseptic work practices, achieving and maintaining ISO Class 5 (see [Table 1](#)) environmental conditions, and cleaning and disinfection procedures. This training shall be completed and documented before any compounding personnel begin to prepare CSPs. Compounding personnel shall complete didactic training, pass written competence assessments, undergo skill assessment using observational audit tools, and media-fill testing (see Appendices III–V).

Media-fill testing of aseptic work skills shall be performed initially before beginning to prepare CSPs and at least annually thereafter for low- and medium-risk level compounding and semiannually for high-risk level compounding.

Compounding personnel who fail written tests or observational audits or whose media-fill test vials have one or more units showing visible microbial contamination shall be re-instructed and re-evaluated by expert compounding personnel to ensure correction of all aseptic work practice deficiencies. Compounding personnel shall pass all evaluations prior to resuming compounding of sterile preparations. In addition to didactic evaluation and aseptic media fill, compounding personnel must demonstrate proficiency of proper hand hygiene, garbing, and consistent cleaning procedures.

In the event that cleaning and disinfecting procedures are also performed by other support personnel (e.g., institutional environmental services, housekeeping), thorough training of proper hand hygiene, garbing, and cleaning and disinfection procedures shall be done by a qualified aseptic compounding expert. After completion of training, support personnel shall routinely undergo performance evaluation of proper hand hygiene, garbing, and all applicable cleaning and disinfecting procedures conducted by a qualified aseptic compounding expert.

competency evaluation of garbing and aseptic work practice



The risk of contaminating a CSP prepared under low-risk level and medium-risk level conditions is highly dependent on proper hand hygiene and garbing practices, compounding personnel aseptic technique, and the presence of surface contamination, assuming that all work is performed in a certified and properly functioning ISO Class 5 (see [Table 1](#)) PEC and secondary engineering controls, ISO Class 7 (see [Table 1](#)) buffer area, and ISO Class 8 (see [Table 1](#)) ante-area. High-risk level CSPs pose the greatest threat to patients because compounding personnel are tasked with the requirement of processing nonsterile components and devices in order to achieve sterility. Compounding personnel shall be evaluated initially prior to beginning compounding CSPs and whenever an aseptic media fill is performed using a form such as the Sample Form for Assessing Hand Hygiene and Garbing

Related Practices of Compounding Personnel (see Appendix III) and the personnel glove fingertip sampling procedures indicated below.

**Aseptic Work Practice Assessment and Evaluation via Personnel Glove Fingertip Sampling**— Sampling of compounding personnel glove fingertips shall be performed for all CSP risk level compounding because direct touch contamination is the most likely source of introducing microorganisms into CSPs prepared by humans. Glove fingertip sampling shall be used to evaluate the competency of personnel in performing hand hygiene and garbing procedures in addition to educating compounding personnel on proper work practices, which include frequent and repeated glove disinfection using sterile 70% IPA during actual compounding of CSPs. All personnel shall demonstrate competency in proper hand hygiene and garbing procedures and in aseptic work practices (e.g., disinfection of component surfaces, routine disinfection of gloved hands).

Sterile contact agar plates shall be used to sample the gloved fingertips of compounding personnel after garbing in order to assess garbing competency and after completing the media-fill preparation (without applying sterile 70% IPA) in order to assess the adequacy of aseptic work practices prior to being initially allowed to prepare CSPs for human use and for more experienced personnel to maintain their qualifications to prepare CSPs for human use.

**Garbing And Gloving Competency Evaluation**— Compounding personnel shall be visually observed during the process of performing hand hygiene and garbing procedures (see Personnel Cleansing and Garbing under Personnel Training and Evaluation in Aseptic Manipulation Skills above). The visual observation shall be documented on a form such as the Sample Form for Assessing Hand Hygiene and Garbing Related Practices of Compounding Personnel (see Appendix III) and maintained to provide a permanent record and long-term assessment of personnel competency.

**Gloved Fingertip Sampling**— All compounding personnel shall successfully complete an initial competency evaluation and gloved fingertip/thumb sampling procedure (zero cfu) no less than three times before initially being allowed to compound CSPs for human use. Immediately after the compounding employee completes the hand hygiene and garbing procedure (e.g., donning of sterile gloves prior to any disinfection with sterile 70% IPA), the evaluator will collect a gloved fingertip and thumb sample from both hands of the compounding employee onto appropriate agar plates by lightly pressing each fingertip into the agar. The plates will be incubated for the appropriate incubation period and at the appropriate temperature (see Incubation Period). After completing the initial gowning and gloving competency evaluation, re-evaluation of all compounding personnel for this competency shall occur at least annually for personnel who compound low- and medium-risk level CSPs and semi-annually for personnel who compound high-risk level CSPs using one or more sample collections during any media-fill test procedure before they are allowed to continue compounding CSPs for human use.

Immediately prior to sampling, gloves shall not be disinfected with sterile 70% IPA. Disinfecting gloves immediately before sampling will provide false negative results. Plates filled with nutrient agar with neutralizing agents such as lecithin and polysorbate 80 added shall be used when sampling personnel fingertips. Personnel shall “touch” the agar with the fingertips of both hands in separate plates in a manner to create a slight impression in the agar. The sampled gloves shall be immediately discarded and proper hand hygiene performed after sampling. The nutrient agar plates shall be incubated as stated below (see Incubation Period). Results should be reported separately as number of cfu per employee per hand (left hand, right hand). The cfu action level for gloved hands will be based on the total number of cfu on both gloves, not per hand.

**Incubation Period**— At the end of the designated sampling period for compounding personnel competency assessment activities (surface or personnel), the agar plates are recovered and covers secured and they are inverted and incubated at a temperature and for a time period conducive to multiplication of microorganisms. TSA with lecithin and polysorbate 80 shall be incubated at 30° to 35° for 48 to 72 hours.

**Aseptic Manipulation Competency Evaluation**— After successful completion of an initial Hand Hygiene and Garbing Competency Evaluation, all compounding personnel shall have their aseptic technique and related practice competency evaluated initially during the Media-Fill Test Procedure and subsequent annual or semi-annual Media-Fill Test Procedures. Records of these evaluations will be maintained using a form such as the Sample Form for Assessing Aseptic Technique and Related Practices of Compounding Personnel (see Appendix IV) and maintained to provide a permanent record of and long-term assessment of personnel competency.

**Media-Fill Test Procedure**— The skill of personnel to aseptically prepare CSPs shall be evaluated using sterile fluid bacterial culture media-fill verification, (i.e., sterile bacterial culture medium transfer via a sterile syringe and needle). Media-fill testing is used to assess the quality of the aseptic skill of compounding personnel. Media-fill tests shall represent the most challenging or stressful conditions actually encountered by the personnel being evaluated when they prepare low- and medium-risk level CSPs and when sterilizing high-risk level CSPs. Media-fill challenge tests are also used to verify the capability of the compounding environment and processes to produce sterile preparations.

A commercially available sterile fluid culture media, such as Soybean-Casein Digest Medium (see [Sterility Tests \(71\)](#)), that is able to promote exponential colonization of bacteria that are most likely to be transmitted to CSPs from the compounding personnel and environment is commonly used. For high-risk level CSPs nonsterile commercially available Soybean-Casein Digest Medium may be used to make a 3% solution. Normal processing steps, including filter sterilization, shall be mimicked. Media-filled vials shall be incubated at 20° to 25° or at 30° to 35° for a minimum of 14 days. If two temperatures are used for incubation of media-filled samples, then these filled containers should be incubated for at least 7 days at each temperature (see [Microbiological Evaluation of Clean Rooms and Other Controlled Environments \(1116\)](#)). Failure is indicated by visible turbidity in any one of the media-fill units on or before 14 days. Other methodologies recommended by a competent microbiologist to enhance recovery time and sensitivity to detect microbial contamination may be considered (see CSP Microbial Contamination Risk Levels for examples of media-fill procedures).

#### surface cleaning and disinfection sampling and assessment

Surface sampling is an important component of the maintenance of a suitable microbially controlled environment for compounding CSPs, especially since transfer of microbial contamination from improperly disinfected work surfaces via inadvertent touch contact by compounding personnel can be a potential source of contamination into CSPs. It is useful for evaluating facility and work surface cleaning and disinfecting procedures and employee competency in work practices such as disinfection of component/vial surface cleaning. Surface sampling shall be performed in all ISO classified areas on a periodic basis. Sampling can be accomplished using contact plates or swabs, and it shall be done at the conclusion of compounding. Locations to be sampled shall be defined in a sample plan or on a form. The size of the plate to be used for each sampled location usually ranges from 24 to 30 cm<sup>2</sup>. Contact plates are filled with general solid agar growth medium and neutralizing agents above the rim of the plate, and they are used for sampling regular or flat surfaces. Swabs may be used for sampling irregular surfaces, especially for equipment (see [Microbiological Evaluation of Clean Rooms and Other Controlled Environments \(1116\)](#)).

**Cleaning and Disinfecting Competency Evaluation**— Compounding personnel and other personnel responsible for cleaning shall be visually observed during the process of performing cleaning and disinfecting procedures, during initial personnel training on cleaning procedures, during changes in cleaning staff, and at the completion of any media-fill test procedure (see Cleaning and Disinfecting of Compounding Areas).

The visual observation shall be documented using a form such as the Sample Form for Assessing Cleaning and Disinfection Procedures (see Appendix V) and maintained to provide a permanent record and long-term assessment of personnel competency.

**Surface Collection Methods**— To sample surfaces using a contact plate, gently touch the sample area with the agar surface and roll the plate across the surface to be sampled. The contact plate will leave a growth media residue behind; therefore, immediately after sampling with the contact plate, the sampled area shall be thoroughly wiped with a nonshedding wipe soaked in sterile 70% IPA.

If an area is sampled via the swab method, collection of the sample is processed by using appropriate procedures that will result in the surface location equivalent to that of a contact plate. After swabbing the surface to be sampled, swabs are placed in an appropriate diluent; an aliquot is plated on or in the specified nutrient agar. Results should be reported as cfu per unit of surface area.

#### Action Levels, Documentation, and Data Evaluation

The value of viable microbial monitoring of gloved fingertips and surfaces of components and the compounding environment are realized when the data are used to identify and correct an unacceptable work practice. Sampling data shall be collected and reviewed on a routine basis as a means of evaluating the overall control of the compounding environment. If an activity consistently shows elevated levels of microbial growth, competent microbiology personnel shall be consulted.



Any cfu count that exceeds its respective action level (see [Table 4](#)) should prompt a re-evaluation of the adequacy of personnel work practices, cleaning procedures, operational procedures, and air filtration efficiency within the aseptic compounding location. An investigation into the source of the contamination shall be conducted. Sources could include HVAC systems, damaged HEPA filters, and changes in personnel garbing or working practices. The source of the problem shall be eliminated, the affected area cleaned, and resampling performed.

When gloved fingertip sample results exceed action levels after proper incubation, a review of hand hygiene and garbing procedures as well as glove and surface disinfection procedures and work practices shall be performed and documented. Employee training may be required to correct the source of the problem.

Counts of cfu are to be used as an approximate measure of the environmental microbial bioburden. Action levels are determined on the basis of cfu data gathered at each sampling location and trended over time. The numbers in [Table 4](#) should be used only as guidelines. Regardless of the number of cfu identified in the compounding facility, further corrective actions will be dictated by the identification of microorganisms recovered (at least the genus level) by an appropriate credentialed laboratory of any microbial bioburden captured as a cfu using an impaction air sampler. Highly pathogenic microorganisms (e.g., Gram-negative rods, coagulase positive staphylococcus, molds and yeasts) can be potentially fatal to patients receiving CSPs and shall be immediately remedied, regardless of cfu count, with the assistance of a competent microbiologist, infection control professional, or industrial hygienist.

Table 4. Recommended Action Levels for Microbial Contamination\*

Classification	Fingertip Sample	Surface Sample (Contact Plate) (cfu per plate)
ISO Class 5	> 3	> 3
ISO Class 7	N/A	> 5
ISO Class 8 or worse	N/A	> 100

\* Pharmaceutical Inspection Co-operation Scheme (PIC/S) Guide to Good Manufacturing Practice for Medicinal Products Annexes PE 009-6, 5 April 2007.

#### SUGGESTED STANDARD OPERATING PROCEDURES (SOPs)

The compounding facility shall have written, properly approved SOPs designed to ensure the quality of the environment in which a CSP is prepared. The following procedures are recommended:

1. Access to the buffer area is restricted to qualified personnel with specific responsibilities or assigned tasks in the compounding area.
2. All cartoned supplies are decontaminated in the area by removing them from shipping cartons and wiping or spraying them with a nonresidue-generating disinfecting agent while they are being transferred to a clean and properly disinfected cart or other conveyance for introduction into the buffer area. Manufacturers' directions or published data for minimum contact time will be followed. Individual pouched sterile supplies need not be wiped because the pouches can be removed as these sterile supplies are introduced into the buffer area.
3. Supplies that are required frequently or otherwise needed close at hand but not necessarily needed for the scheduled operations of the shift are decontaminated and stored on shelving in the ante-area.
4. Carts used to bring supplies from the storeroom cannot be rolled beyond the demarcation line in the ante-area, and carts used in the buffer area cannot be rolled outward beyond the demarcation line unless cleaned and disinfected before returning.
5. Generally, supplies required for the scheduled operations of the shift are wiped down with an appropriate disinfecting agent and brought into the buffer area, preferably on one or more movable carts. Supplies that are required for back-up or general support of operations may be stored on the designated shelving in the buffer area, but excessive amounts of supplies are to be avoided.
6. Nonessential objects that shed particles shall not be brought into the buffer area, including pencils, cardboard cartons, paper towels, and cotton items (e.g., gauze pads).
7. Essential paper-related products (e.g., paper syringe overwraps, work records contained in a protective sleeve) shall be wiped down with an appropriate disinfecting agent prior to being brought into the buffer area.
8. Traffic flow in and out of the buffer area shall be minimized.
9. Personnel preparing to enter the buffer area shall remove all personal outer garments, cosmetics (because they shed flakes and particles), and all hand, wrist, and other visible jewelry or piercings that can interfere with the effectiveness of PPE.
10. Personnel entering the ante-area shall don attire as described in Personnel Cleansing and Garbing and Personnel Training and Competency Evaluation of Garbing, Aseptic Work Practices and Cleaning/Disinfection Procedures.
11. Personnel shall then thoroughly wash hands and forearms to the elbow with soap and water for at least 30 seconds. An air dryer or disposable nonshedding towels are used to dry hands and forearms after washing.
12. Personnel entering the buffer area shall perform antiseptic hand cleansing prior to donning sterile gloves using a waterless alcohol-based surgical hand scrub with persistent activity.
13. Chewing gum, drinks, candy, or food items shall not be brought into the buffer area or ante-area. Materials exposed in patient care and treatment areas shall never be introduced into areas where components and ingredients for CSPs are present.
14. At the beginning of each compounding activity session, and whenever liquids are spilled, the surfaces of the direct compounding environment are first cleaned with USP Purified Water to remove water-soluble residues. Immediately thereafter, the same surfaces are disinfected with a nonresidue-generating agent using a nonlinting wipe.
15. Primary engineering controls shall be operated continuously during compounding activity. When the blower is turned off and before other personnel enter to perform compounding activities, only one person shall enter the buffer area for the purposes of turning on the blower (for at least 30 minutes) and disinfecting the work surfaces.
16. Traffic in the area of the DCA is minimized and controlled.
17. Supplies used in the DCA for the planned procedures are accumulated and then decontaminated by wiping or spraying the outer surface with sterile 70% IPA or removing the outer wrap at the edge of the DCA as the item is introduced into the aseptic work area.
18. All supply items are arranged in the DCA so as to reduce clutter and provide maximum efficiency and order for the flow of work.
19. After proper introduction into the DCA of supply items required for and limited to the assigned operations, they are so arranged that a clear, uninterrupted path of HEPA-filtered air will bathe all critical sites at all times during the planned procedures. That is, no objects may be placed between the first air from HEPA filters and an exposed critical site.
20. All procedures are performed in a manner designed to minimize the risk of touch contamination. Gloves are disinfected with adequate frequency with an approved disinfectant such as sterile 70% IPA.
21. All rubber stoppers of vials and bottles and the necks of ampuls are disinfected by wiping with sterile 70% IPA and waiting for at least 10 seconds before they are used to prepare CSPs.
22. After the preparation of every CSP, the contents of the container are thoroughly mixed and then inspected for the presence of particulate matter, evidence of incompatibility, or other defects.
23. After procedures are completed, used syringes, bottles, vials, and other supplies are removed, but with a minimum of exit and re-entry into the DCA so as to minimize the risk of introducing contamination into the aseptic workspace.

#### ELEMENTS OF QUALITY CONTROL

A written description of specific training and performance evaluation program for individuals involved in the use of aseptic techniques for the preparation of sterile products shall be developed for each site. This program equips personnel with the appropriate knowledge and trains them in the required skills necessary to perform the assigned tasks. Each person assigned to the aseptic area in the preparation of sterile products shall successfully complete specialized training in aseptic techniques and aseptic area practices prior to preparing CSPs (see Personnel Training and Evaluation in Aseptic Manipulation Skills and Personnel Training and Competency Evaluation of Garbing, Aseptic Work Practices and



Cleaning/Disinfection Procedures).

#### Ingredients and Devices

Compounding personnel ascertain that ingredients for CSPs are of the correct identity and appropriate quality using the following information: vendor labels, labeling, certificates of analysis, direct chemical analysis, and knowledge of compounding facility storage conditions.

##### sterile ingredients and devices

Commercially available sterile drug products, sterile ready-to-use containers, and devices are examples of sterile components. A written procedure for unit-by-unit physical inspection preparatory to use is followed to ensure that these components are sterile, free from defects, and otherwise suitable for their intended use.

##### nonsterile ingredients and devices

If any nonsterile components, including containers and ingredients, are used to make a CSP, such CSPs must be high risk. Nonsterile active ingredients and added substances or excipients for CSPs should preferably be official USP or NF articles. When nonofficial ingredients are used, they shall be accompanied by certificates of analysis from their suppliers to aid compounding personnel in judging the identity, quality, and purity in relation to the intended use in a particular CSP. Physical inspection of a package of ingredients is necessary in order to detect breaks in the container, looseness in the cap or closure, and deviation from the expected appearance, aroma, and texture of the contents.

Bulk or unformulated drug substances and added substances or excipients shall be stored in tightly closed containers under temperature, humidity, and lighting conditions that are either indicated in official monographs or approved by suppliers. The date of receipt by the compounding facility shall be clearly and indelibly marked on each package of ingredient.

After receipt by the compounding facility, packages of ingredients that lack a supplier's expiration date cannot be used after 1 year unless either appropriate inspection or testing indicates that the ingredient has retained its purity and quality for use in CSPs.

Careful consideration and evaluation of nonsterile ingredient sources is especially warranted when the CSP will be administered into the vascular system, central nervous system, or eyes.

Upon receipt of each lot of the bulk drug substance or excipient used for CSPs, the individual compounding the preparation performs a visual inspection of the lot for evidence of deterioration, other types of unacceptable quality, and wrong identification. For bulk drug substances or excipients, visual inspection is performed on a routine basis as described in the written protocol.

#### Equipment

It is necessary that equipment, apparatus, and devices used to compound a CSP be consistently capable of operating properly and within acceptable tolerance limits. Written procedures outlining required equipment calibration, annual maintenance, monitoring for proper function, and controlled procedures for use of the equipment and specified time frames for these activities are established and followed. Routine maintenance and frequencies shall be outlined in these SOPs. Results from the equipment calibration, annual maintenance reports, and routine maintenance are kept on file for the lifetime of the equipment. Personnel are prepared through an appropriate combination of specific training and experience to operate or manipulate any piece of equipment, apparatus, or device they may use when preparing CSPs. Training includes gaining the ability to determine whether any item of equipment is operating properly or is malfunctioning.

#### VERIFICATION OF AUTOMATED COMPOUNDING DEVICES (ACDs) FOR PARENTERAL NUTRITION COMPOUNDING

ACDs for the preparation of parenteral nutrition admixtures are widely used by pharmacists in hospitals and other healthcare settings. They are designed to streamline the labor-intensive processes involved in the compounding of these multiple-component formulations by automatically delivering the individual nutritional components in a predetermined sequence under computerized control. Parenteral nutrition admixtures often contain 20 or more individual additives representing as many as 50 or more individual components (e.g., 15 to 20 crystalline amino acids, dextrose monohydrate, and lipids; 10 to 12 electrolyte salts; 5 to 7 trace minerals; and 12 vitamins). Thus, ACDs can provide improved accuracy and precision of the compounding process over the traditional manual compounding methods.

#### Accuracy

The accuracy of an ACD can be determined in various ways to ensure that the correct quantities of nutrients, electrolytes, or other nutritional components are delivered to the final infusion container. Initially, the ACD is tested for its volume and weight accuracy. For volume accuracy, a suitable volume of Sterile Water for Injection, USP, which represents a typical additive volume (e.g., 40 mL for small-volume range of 1 to 100 mL, 300 mL for large-volume range of 100 to 1000 mL), is programmed into the ACD and delivered to the appropriate volumetric container. The compounding personnel should then consult [Volumetric Apparatus](#) (31) for appropriate parameters to assess the volumetric performance of the ACD. For gravimetric accuracy, the balance used in conjunction with the ACD is tested using various weight sizes that represent the amounts typically used to deliver the various additives.

Compounding personnel should consult [Weights and Balances](#) (41) for acceptable tolerances of the weights used. In addition, the same volume of [Sterile Water for Injection](#) used to assess volumetric accuracy is then weighed on the balance used in conjunction with the ACD. For example, if 40 mL of water was used in the volumetric assessment, its corresponding weight should be about 40 g (assuming the relative density of water is 1.0). In addition, during the use of the ACD, certain additives, such as potassium chloride (corrected for density differences), can also be tested in the same manner as with an in-process test.

Finally, additional tests of accuracy may be employed that determine the content of certain ingredients in the final volume of the parenteral nutrition admixture. Generally, pharmacy departments do not have the capability to routinely perform chemical analyses such as analyses of dextrose or electrolyte concentrations. Consequently, hospital or institutional laboratories may be called upon to perform these quality assurance tests. However, the methods in such laboratories are often designed for biological, not pharmaceutical, systems.

Thus, their testing procedures shall be verified to meet the USP requirements stated in the individual monograph for the component being tested. For example, under [Dextrose Injection](#), the following is stated: It contains not less than 95.0% and not more than 105.0% of the labeled amount of C6H12O6·H2O. The hospital or institutional chemistry laboratories must validate their methods to apply to this range and correct for their typical measurement of anhydrous dextrose versus dextrose monohydrate. Similar ranges and issues exist, for example, for injections of calcium gluconate, magnesium sulfate, and potassium chloride. The critical point is the use of USP references and possible laboratory procedural differences.

#### Precision

The intermediate precision of the ACD can be determined on the basis of the day-to-day variations in performance of the accuracy measures. Thus, compounding personnel shall keep a daily record of the above-described accuracy assessments and review the results over time. This review shall occur at least at weekly intervals to avoid potentially clinically significant cumulative errors over time. This is especially true for additives with a narrow therapeutic index, such as potassium chloride.

#### FINISHED PREPARATION RELEASE CHECKS AND TESTS

The following quality metrics shall be performed for all CSPs before they are dispensed or administered.

##### Inspection of Solution Dosage Forms and Review of Compounding Procedures

All CSPs that are intended to be solutions shall be visually examined for the presence of particulate matter and not administered or dispensed when such matter is observed. The prescription orders, written compounding procedure, preparation records, and expended materials used to make CSPs at all contamination risk levels are inspected for accuracy of correct identities and amounts of ingredients, aseptic mixing and sterilization, packaging, labeling, and expected physical appearance before they are administered or dispensed.

##### physical inspection

Finished CSPs are individually inspected in accordance with written procedures after compounding. If not distributed promptly, these CSPs are individually inspected just prior to leaving the storage area. Those CSPs that are not immediately distributed are stored in an appropriate location as described in the written procedures. Immediately after compounding, and as a condition of release, each CSP unit, where possible, should be inspected against lighted white or black background or both for evidence of visible particulates or other foreign matter. Prerelase inspection also includes container-closure integrity and any other apparent visual defect. CSPs with observed defects should be immediately discarded or marked and segregated from acceptable products in a manner that prevents their administration. When CSPs are not distributed promptly after preparation, a predistribution inspection is



conducted to ensure that a CSP with defects, such as precipitation, cloudiness, and leakage, which may develop between the time of release and the time of distribution, is not released.

#### Compounding Accuracy Checks

Written procedures for double-checking compounding accuracy shall be followed for every CSP during preparation and immediately prior to release. The double-check system should meet state regulations and include label accuracy and accuracy of the addition of all drug products or ingredients used to prepare the finished product and their volumes or quantities. The used additive containers and, for those additives for which the entire container was not expended, the syringes used to measure the additive should be quarantined with the final products until the final product check is completed. Compounding personnel shall visually confirm that ingredients measured in syringes match the written order being compounded. Preferably, a person other than the compounder can verify that correct volumes of correct ingredients were measured to make each CSP. For example, compounding personnel would pull the syringe plunger back to the volume measured.

When practical, the accuracy of measurements is confirmed by weighing a volume of the measured fluid, then calculating that volume by dividing the weight by the accurate value of the density, or specific gravity, of the measured fluid. Correct density or specific gravity values programmed in ACDs, which measure by weight using the quotient of the programmed volume divided by the density or specific gravity, shall be confirmed to be accurate before and after delivering volumes of the liquids assigned to each channel or port. These volume accuracy checks and the following additional safety and accuracy checks in this section shall be included in the SOP manual of the CSP facility.

#### Sterility Testing

All high-risk level CSPs that are prepared in groups of more than 25 identical individual single-dose packages (e.g., ampuls, bags, syringes, vials) or in multiple-dose vials (MDVs) for administration to multiple patients or that are exposed longer than 12 hours at 2° to 8° and longer than 6 hours at warmer than 8° before they are sterilized shall meet the sterility test (see [Sterility Tests \(71\)](#)) before they are dispensed or administered. The Membrane Filtration method is the method of choice where feasible (e.g., components are compatible with the membrane). A method not described in the USP may be used if verification results demonstrate that the alternative is at least as effective and reliable as the USP Membrane

Filtration method or the USP Direct Inoculation of the Culture Medium method where the Membrane Filtration method is not feasible.

When high-risk level CSPs are dispensed before receiving the results of their sterility tests, there shall be a written procedure requiring daily observation of the incubating test specimens and immediate recall of the dispensed CSPs when there is any evidence of microbial growth in the test specimens. In addition, the patient and the physician of the patient to whom a potentially contaminated CSP was administered are notified of the potential risk. Positive sterility test results should prompt a rapid and systematic investigation of aseptic technique, environmental control, and other sterility assurance controls to identify sources of contamination and correct problems in the methods or processes.

#### Bacterial Endotoxin (Pyrogen) Testing

All high-risk level CSPs, except those for inhalation and ophthalmic administration, that are prepared in groups of more than 25 identical individual single-dose packages (e.g., ampuls, bags, syringes, vials) or in MDVs for administration to multiple patients or that are exposed longer than 12 hours at 2° to 8° and longer than 6 hours at warmer than 8° before they are sterilized shall be tested to ensure that they do not contain excessive bacterial endotoxins (see [Bacterial Endotoxins Test \(85\)](#) and [Pyrogen Test \(151\)](#)). In the absence of a bacterial endotoxins limit in the official monograph or other CSP formula source, the CSP shall not exceed the amount of USP Endotoxin Units (per hour per kilogram of body weight or square meters of body surface area) specified in [Bacterial Endotoxins Test \(85\)](#) referenced above for the appropriate route of administration.

#### Identity and Strength Verification of Ingredients

Compounding facilities shall have at least the following written procedures for verifying the correct identity and quality of CSPs before they are dispensed and administered:

1. That labels of CSPs bear correct names and amounts or concentrations of ingredients, the total volume, the BUD, the appropriate route(s) of administration, the storage conditions, and other information for safe use.
2. That there are correct identities, purities, and amounts of ingredients by comparing the original written order with the written compounding record for the CSP.
3. That correct fill volumes in CSPs and correct quantities of filled units of the CSPs were obtained. When the strength of finished CSPs cannot be confirmed to be accurate, based on the above three inspections, the CSPs shall be assayed by methods that are specific for the active ingredients.

#### STORAGE AND BEYOND-USE DATING

BUDs for compounded preparations are usually assigned on the basis of professional experience, which should include careful interpretation of appropriate information sources for the same or similar formulations (see Stability Criteria and Beyond-Use Dating under [Pharmaceutical Compounding—Nonsterile Preparations \(795\)](#)). BUDs for CSPs are rarely based on preparation-specific chemical assay results, which are used with the Arrhenius equation to determine expiration dates (see General Notices and Requirements) for manufactured products. The majority of CSPs are aqueous solutions in which hydrolysis of dissolved ingredients is the most common chemical degradation reaction. The extent of hydrolysis and other heat-catalyzed degradation reactions at any particular time point in the life of a CSP represents the thermodynamic sum of exposure temperatures and durations. Such lifetime stability exposure is represented in the mean kinetic temperature calculation (see [Pharmaceutical Calculations in Prescription Compounding \(1160\)](#)). Drug hydrolysis rates increase exponentially with arithmetic temperature increase; thus, exposure of a beta-lactam antibiotic solution for 1 day at controlled room temperature (see General Notices and Requirements) will have an equivalent effect on the extent of hydrolysis of approximately 3 to 5 days in cold temperatures (see General Notices and Requirements).

Personnel who prepare, dispense, and administer CSPs shall store them strictly in accordance with the conditions stated on the label of ingredient products and finished CSPs. When CSPs are known to have been exposed to temperatures warmer than the warmest labeled limit or to temperatures exceeding 40° (see General Notices and Requirements) for more than 4 hours, such CSPs should be discarded unless direct assay data or appropriate documentation confirms their continued stability.

#### Determining Beyond-Use Dates

BUDs and expiration dates are not the same (see General Notices and Requirements). Expiration dates for the chemical and physical stability of manufactured sterile products are determined from results of rigorous analytical and performance testing, and they are specific for a particular formulation in its container and at stated exposure conditions of illumination and temperature. When CSPs deviate from conditions in the approved labeling of manufactured products contained in CSPs, compounding personnel may consult the manufacturer of particular products for advice on assigning BUDs based on chemical and physical stability parameters. BUDs for CSPs that are prepared strictly in accordance with manufacturers' product labeling shall be those specified in that labeling or from appropriate literature sources or direct testing. BUDs for CSPs that lack justification from either appropriate literature sources or by direct testing evidence shall be assigned as described in Stability Criteria and Beyond-Use Dating under [Pharmaceutical Compounding—Nonsterile Preparations \(795\)](#).

In addition, compounding personnel may refer to applicable publications to obtain relevant stability, compatibility, and degradation information regarding the drug or its congeners. When assigning a beyond-use date, compounding personnel should consult and apply drug-specific and general stability documentation and literature where available, and they should consider the nature of the drug and its degradation mechanism, the container in which it is packaged, the expected storage conditions, and the intended duration of therapy (see [Expiration Date and Beyond-Use Date under Labeling in the General Notices and Requirements](#)). Stability information must be carefully interpreted in relation to the actual compounded formulation and conditions for storage and use. Predictions based on other evidence, such as publications, charts, and tables, would result in theoretical BUDs. Theoretically predicted beyond-use dating introduces varying degrees of assumptions and, hence, a likelihood of error or at least inaccuracy. The degree of error or inaccuracy would be dependent on the extent of differences between the CSPs' characteristics (e.g., composition, concentration of ingredients, fill volume, container type and material) and the characteristics of the products from which stability data or information is to be extrapolated. The greater the doubt of the accuracy of theoretically predicted beyond-use dating, the greater the need to determine dating periods experimentally. Theoretically predicted beyond-use dating periods should be carefully considered for CSPs prepared from nonsterile bulk active ingredients having therapeutic activity, especially where these CSPs are expected to be compounded routinely. When CSPs will be distributed to and administered in residential locations other than healthcare facilities, the effect of potentially uncontrolled and unmonitored temperature conditions shall be considered when assigning BUDs. It must be ascertained that CSPs will not be exposed to warm temperatures (see General Notices and Requirements) unless the compounding facility has evidence to justify stability of CSPs during such exposure.



It should be recognized that the truly valid evidence of stability for predicting beyond-use dating can be obtained only through product-specific experimental studies. Semiquantitative procedures such as thin-layer chromatography (TLC) may be acceptable for many CSPs. However, quantitative stability-indicating assays such as high-performance liquid chromatographic (HPLC) assays would be more appropriate for certain CSPs. Examples include CSPs with a narrow therapeutic index, where close monitoring or dose titration is required to ensure therapeutic effectiveness and to avoid toxicity; where a theoretically established beyond-use dating period is supported by only marginal evidence; or where a significant margin of safety cannot be verified for the proposed beyond-use dating period. In short, because beyond-use dating periods established from product-specific data acquired from the appropriate instrumental analyses are clearly more reliable than those predicted theoretically, the former approach is strongly urged to support dating periods exceeding 30 days.

To ensure consistent practices in determining and assigning BUDs, the compounding facility should have written policies and procedures governing the determination of the BUDs for all compounded products. When attempting to predict a theoretical BUD, a compounded or an admixed preparation should be considered as a unique system that has physical and chemical properties and stability characteristics that differ from its components. For example, antioxidant, buffering, or antimicrobial properties of a sterile vial for injection (SVI) might be lost upon its dilution, with the potential of seriously compromising the chemical stability of the SVI's active ingredient or the physical or microbiological stability of the SVI formulation in general. Thus, the properties stabilized in the SVI formulation usually cannot be expected to be carried over to the compounded or admixed preparation. Preparation-specific, experimentally determined stability data evaluation protocols are preferable to published stability information. Compounding personnel should consult general information chapter [Pharmaceutical Stability](#) (1150) for the appropriate stability parameters to be considered when initiating or evaluating a preparation-specific stability study.

Compounding personnel who assign BUDs to CSPs when lacking direct chemical assay results must critically interpret and evaluate the most appropriate available information sources to determine a conservative and safe BUD. The SOP manual of the compounding facility and each specific CSP formula record shall describe the general basis used to assign the BUD and storage conditions.

When manufactured MDVs (see Multiple-Dose Container under Preservation, Packaging, Storage, and Labeling in the General Notices and Requirements) of sterile ingredients are used in CSPs, the stoppers of the MDVs are inspected for physical integrity and disinfected by wiping with a sterile 70% IPA swab before each penetration with a sterile withdrawal device. When contaminants or abnormal properties are suspected or observed in MDVs, such MDVs shall be discarded. The BUD after initially entering or opening (e.g., needle puncturing) multiple-dose containers is 28 days (see [Antimicrobial Effectiveness Testing](#) (51)) unless otherwise specified by the manufacturer.

#### Proprietary Bag and Vial Systems

The sterility storage and stability beyond-use times for attached and activated (where activated is defined as allowing contact of the previously separate diluent and drug contents) container pairs of drug products for intravascular administration (e.g., ADD-Vantage®, Mini Bag Plus®) shall be applied as indicated by the manufacturer. In other words, follow manufacturers' instructions for handling and storing ADD-Vantage®, Mini Bag Plus®, Add A Vial®, Add-Ease® products, and any others.

#### Monitoring Controlled Storage Areas

To ensure that product potency is retained through the manufacturer's labeled expiration date, compounding personnel shall monitor the drug storage areas within the compounding facility. Controlled temperature areas in compounding facilities include controlled room temperature, 20° to 25° with mean kinetic temperature 25°; controlled cold temperature, 2° to 8° with mean kinetic temperature 8°; cold temperature, 2° to 8°; freezing temperature, -25° and -10° (see General Notices and Requirements) if needed to achieve freezing, and the media-specific temperature range for microbial culture media. A controlled temperature area shall be monitored at least once daily and the results documented on a temperature log.

Additionally, compounding personnel shall note the storage temperature when placing the product into or removing the product from the storage unit in order to monitor any temperature aberrations. Suitable temperature recording devices may include a calibrated continuous recording device or a National Institute of Standards and Technology (NIST) calibrated thermometer that has adequate accuracy and sensitivity for the intended purpose, and it shall be properly calibrated at suitable intervals. If the compounding facility uses a continuous temperature recording device, compounding personnel shall verify at least once daily that the recording device itself is functioning properly.

The temperature-sensing mechanisms shall be suitably placed in the controlled temperature storage space to reflect accurately its true temperature. In addition, the compounding facility shall adhere to appropriate procedures of all controlled storage spaces to ensure that such spaces are not subject to significantly prolonged temperature fluctuations as may occur, for example, by leaving a refrigerator door open too long.

#### MAINTAINING STERILITY, PURITY, AND STABILITY OF DISPENSED AND DISTRIBUTED CSPs

This section summarizes the responsibilities of compounding facilities for maintaining quality and control of CSPs that are dispensed and administered within their parent healthcare organizations.

Compounding personnel shall ensure proper storage and security of CSPs prepared by or dispensed from the compounding facility until either their BUDs are reached or they are administered to patients. In fulfilling this general responsibility, the compounding facility is responsible for the proper packaging, handling, transport, and storage of CSPs prepared by or dispensed from it, including the appropriate education, training, and supervision of compounding personnel assigned to these functions. The compounding facility should assist in the education and training of noncompounding personnel responsible for carrying out any aspect of these functions.

Establishing, maintaining, and ensuring compliance with comprehensive written policies and procedures encompassing these responsibilities is a further responsibility of the compounding facility. Where noncompounding personnel are assigned tasks involving any of these responsibilities, the policies and procedures encompassing those tasks should be developed by compounding supervisors. The quality and control activities related to distribution of CSPs are summarized in the following five subsections. Activities or concerns that should be addressed as the compounding facility fulfills these responsibilities are as follows.

#### Packaging, Handling, and Transport

Inappropriate processes or techniques involved with packaging, handling, and transport can adversely affect quality and package integrity of CSPs. Although compounding personnel routinely perform many of the tasks associated with these functions, some tasks, such as transport, handling, and placement into storage, may be fulfilled by noncompounding personnel who are not under the direct administrative control of the compounding facility. Under these circumstances, appropriate SOPs shall be established by the compounding facility with the involvement of other departments or services whose personnel are responsible for carrying out those CSP-related functions for which the compounding facility has a direct interest. The performance of the noncompounding personnel is monitored for compliance to established policies and procedures.

The critical requirements that are unique to CSPs and that are necessary to ensure CSP quality and packaging integrity shall be addressed in SOPs. For example, techniques should be specified to prevent the depression of syringe plungers or dislodging of syringe tips during handling and transport. Additionally, disconnection of system components (e.g., where CSPs are dispensed with administration sets attached to them) shall be prevented through the BUD of the CSP. Foam padding or inserts are particularly useful where CSPs are transported by pneumatic tube systems. Regardless of the methods used, the compounding facility must evaluate their effectiveness and the reliability of the intended protection.

Evaluation should be continuous—for example, through a surveillance system, including a system of problem reporting to the compounding facility.

Inappropriate transport and handling can adversely affect the quality of certain CSPs having unique stability concerns. For example, the physical shaking that might occur during pneumatic tube transport or undue exposure to heat or light must be addressed on a preparation-specific basis. Alternative transport modes or special packaging measures might be needed for the proper assurance of quality of these CSPs. The use of tamper-evident closures and seals on CSP ports can add an additional measure of security to ensure product integrity regardless of the transport method used.

Chemotoxic and other hazardous CSPs require safeguards to maintain the integrity of the CSP and to minimize the exposure potential of these products to the environment and to personnel who may come in contact with them. Transportation by pneumatic tube should be discouraged because of potential breakage and contamination. Special requirements associated with the packaging, transport, and handling of these agents include the prevention of accidental exposures or spills and the training of personnel in the event of an exposure or spill. Examples of special requirements of these agents also include exposure-reducing strategies such as the use of Luer lock syringes and connections, syringe caps, the capping of container ports, sealed plastic bags, impact-resistant containers, and cautionary labeling.

#### Use and Storage



The compounding facility is responsible for ensuring that CSPs in the patient-care setting maintain their quality until administered. The immediate labeling of the CSP container will display prominently and understandably the requirements for proper storage and expiration dating. Delivery and patient-care-setting personnel shall be properly trained to deliver the CSP to the appropriate storage location. Outdated and unused CSPs shall be returned to the compounding facility for disposition.

SOPs must exist to ensure that storage conditions in the patient-care setting are suitable for the CSP-specific storage requirements. Procedures include daily monitoring and documentation of drug storage refrigerators to ensure temperatures between 2° and 8° and the monthly inspection of all drug storage locations by compounding personnel. Inspections shall confirm compliance with appropriate storage conditions, separation of drugs and food, proper use of MDVs, and the avoidance of using single-dose products as MDVs. CSPs, as well as all other drug products, shall be stored in the patient-care area in such a way as to secure them from unauthorized personnel, visitors, and patients.

#### Readyng for Administration

Procedures essential for generally ensuring quality, especially sterility assurance, when readyng a CSP for its subsequent administration include proper hand washing, aseptic technique, site care, and change of administration sets. Additional procedures may also be essential for certain CSPs, devices, or techniques. Examples where such special procedures are needed include in-line filtration, the operation of automated infusion control devices, and the replenishment of CSPs into the reservoirs of implantable or portable infusion pumps. When CSPs are likely to be exposed to warmer than 30° for more than 1 hour during their administration to patients, the maintenance of their sterility and stability should be confirmed from either relevant and reliable sources or direct testing.

#### Redispsensed CSPs

The compounding facility shall have the sole authority to determine when unopened, returned CSPs may be redispsensed. Returned CSPs may be redispsensed only when personnel responsible for sterile compounding can ensure that such CSPs are sterile, pure, and stable (contain labeled strength of ingredients). The following may provide such assurance: the CSPs were maintained under continuous refrigeration and protected from light, if required, and no evidence of tampering or any readyng for use outside the compounding facility exists. Assignment of new storage times and BUDs that exceed the original dates for returned CSPs is permitted only when there is supporting evidence from sterility testing and quantitative assay of ingredients. Thus, initial preparation and thaw times should be documented and reliable measures should have been taken to prevent and detect tampering. Compliance with all procedures associated with maintaining product quality is essential. The CSPs shall not be redispsensed if there is not adequate assurance that preparation quality and packaging integrity (including the connections of devices, where applicable) were continuously maintained between the time the CSPs left and the time they were returned.

Additionally, CSPs shall not be redispsensed if redispsensing cannot be supported by the originally assigned BUD.

#### Education and Training

The assurance of CSPs' quality and packaging integrity is highly dependent on the proper adherence of all personnel to the pertinent SOPs. Compounding personnel shall design, implement, and maintain a formal education, training, and competency assessment program that encompasses all the functions and tasks addressed in the foregoing sections and all personnel to whom such functions and tasks are assigned. This program includes the assessment and documentation of procedural breaches, administration mishaps, side effects, allergic reactions, and complications associated with dosage or administration, such as extravasation. This program should be coordinated with the institution's adverse-events and incident reporting programs.

#### Packing and Transporting CSPs

The following sections describe how to maintain sterility and stability of CSPs until they are delivered to patient care locations for administration.

##### packing cpps for transit

When CSPs are distributed to locations outside the premises in which they are compounded, compounding personnel select packing containers and materials that are expected to maintain physical integrity, sterility, and stability of CSPs during transit. Packing is selected that simultaneously protects CSPs from damage, leakage, contamination, and degradation, and protects personnel who transport packed CSPs from harm. The SOP manual of the compounding facility specifically describes appropriate packing containers and insulating and stuffing materials, based on information from product specifications, vendors, and experience of compounding personnel. Written instructions that clearly explain how to safely open containers of packed CSPs are provided to patients and other recipients.

##### transit of cpps

Compounding facilities that ship CSPs to locations outside their own premises shall select modes of transport that are expected to deliver properly packed CSPs in undamaged, sterile, and stable condition to recipients.

Compounding personnel should ascertain that temperatures of CSPs during transit by the selected mode will not exceed the warmest temperature specified on the storage temperature range on CSP labels. It is recommended that compounding personnel communicate directly with the couriers to learn shipping durations and exposure conditions that CSPs may encounter.

Compounding personnel shall include specific handling and exposure instructions on the exteriors of containers packed with CSPs to be transported and obtain reasonable assurance of compliance therewith from transporters. Compounding personnel shall periodically review the delivery performance of couriers to ascertain that CSPs are being efficiently and properly transported.

#### Storage in Locations Outside Compounding Facilities

Compounding facilities that ship CSPs to patients and other recipients outside their own premises shall ascertain or provide, whichever is appropriate, the following assurances:

1. Labels and accessory labeling for CSPs include clearly readable BUDs, storage instructions, and disposal instructions for out-of-date units.
2. Each patient or other recipient is able to store the CSPs properly, including the use of a properly functioning refrigerator and freezer if CSPs are labeled for such storage.

#### PATIENT OR CAREGIVER TRAINING

A formal training program is provided as a means to ensure understanding and compliance with the many special and complex responsibilities placed on the patient or caregiver for the storage, handling, and administration of CSPs. The instructional objectives for the training program include all home care responsibilities expected of the patient or caregiver and is specified in terms of patient or caregiver competencies.

Upon the conclusion of the training program, the patient or caregiver should, correctly and consistently, be able to do the following:

1. Describe the therapy involved, including the disease or condition for which the CSPs are prescribed, goals of therapy, expected therapeutic outcome, and potential side effects of the CSPs.
2. Inspect all drug products, CSPs, devices, equipment, and supplies on receipt to ensure that proper temperatures were maintained during transport and that goods received show no evidence of deterioration or defects.
3. Handle, store, and monitor all drug products, CSPs, and related supplies and equipment in the home, including all special requirements related to same.
4. Visually inspect all drug products, CSPs, devices, and other items the patient or caregiver is required to use immediately prior to administration in a manner to ensure that all items are acceptable for use. For example, CSPs must be free from leakage, container cracks, particulates, precipitate, haziness, discoloration, or other deviations from the normal expected appearance, and the immediate packages of sterile devices must be completely sealed, with no evidence of loss of package integrity.
5. Check labels immediately prior to administration to ensure the right drug, dose, patient, and time of administration.
6. Clean the in-home preparation area, scrub hands, use proper aseptic technique, and manipulate all containers, equipment, apparatus, devices, and supplies used in conjunction with administration.
7. Employ all techniques and precautions associated with CSP administration; for example, preparing supplies and equipment, handling of devices, priming the tubing, and discontinuing an infusion.



8. Care for catheters, change dressings, and maintain site patency as indicated.
9. Monitor for and detect occurrences of therapeutic complications such as infection, phlebitis, electrolyte imbalance, and catheter misplacement.
10. Respond immediately to emergency or critical situations such as catheter breakage or displacement, tubing disconnection, clot formation, flow blockage, and equipment malfunction.
11. Know when to seek and how to obtain professional emergency services or professional advice.
12. Handle, contain, and dispose of wastes, such as needles, syringes, devices, biohazardous spills or residuals, and infectious substances.

Training programs include a hands-on demonstration and practice with actual items that the patient or caregiver is expected to use, such as CSP containers, devices, and equipment. The patient or caregiver practices aseptic and injection technique under the direct observation of a health professional.

The compounding facility, in conjunction with nursing or medical personnel, is responsible for ensuring initially and on an ongoing basis that the patient or caregiver understands, has mastered, and is capable of and willing to comply with all of these home care responsibilities. This is achieved through a formal, written assessment program. All specified competencies in the patient or caregiver training program are formally assessed. The patient or caregiver is expected to demonstrate to appropriate healthcare personnel mastery of assigned activities before being allowed to administer CSPs unsupervised by a health professional.

Printed material such as checklists or instructions provided during training may serve as continuing post-training reinforcement of learning or as reminders of specific patient or caregiver responsibilities. Post-training verbal counseling can also be used periodically, as appropriate, to reinforce training and to ensure continuing correct and complete fulfillment of responsibilities.

#### PATIENT MONITORING AND ADVERSE EVENTS REPORTING

Compounding facilities shall clinically monitor patients treated with CSPs according to the regulations and guidelines of their respective state healthcare practitioner licensure boards or of accepted standards of practice. Compounding facilities shall provide patients and other recipients of CSPs with a way to address their questions and report any concerns that they may have with CSPs and their administration devices.

The SOP manuals of compounding facilities shall describe specific instructions for receiving, acknowledging, and dating receipts, and for recording, or filing, and evaluating reports of adverse events and of the quality of preparation claimed to be associated with CSPs. Reports of adverse events with CSPs shall be reviewed promptly and thoroughly by compounding supervisors to correct and prevent future occurrences. Compounding personnel are encouraged to participate in adverse event reporting and product defects programs of the FDA and USP.

#### QUALITY ASSURANCE (QA) PROGRAM

A provider of CSPs shall have in place a formal QA program intended to provide a mechanism for monitoring, evaluating, correcting, and improving the activities and processes described in this chapter. Emphasis in the QA program is placed on maintaining and improving the quality of systems and the provision of patient care. In addition, the QA program ensures that any plan aimed at correcting identified problems also includes appropriate follow-up to make certain that effective corrective actions were performed.<sup>13</sup>

Characteristics of a QA program include the following:

1. Formalization in writing;
2. Consideration of all aspects of the preparations and dispensing of products as described in this chapter, including environmental testing and verification results;
3. Description of specific monitoring and evaluation activities;
4. Specification of how results are to be reported and evaluated;
5. Identification of appropriate follow-up mechanisms when action limits or thresholds are exceeded; and
6. Delineation of the individuals responsible for each aspect of the QA program.

In developing a specific plan, focus is on establishing objective, measurable indicators for monitoring activities and processes that are deemed high risk, high volume, or problem prone. In general, the selection of indicators and the effectiveness of the overall QA program is reassessed on an annual basis.

#### ABBREVIATIONS AND ACRONYMS

ACD	automated compounding device
ACPH	air changes per hour
ALARA	as low as reasonably achievable
ASHRAE	American Society of Heating, Refrigerating and Air-Conditioning Engineers
BI	biological indicator
BSC	biological safety cabinet
BUD	beyond-use date
CACI	compounding aseptic containment isolator
CAI	compounding aseptic isolator
CDC	Centers for Disease Control and Prevention
CETA	Controlled Environment Testing Association
cfu	colony-forming unit(s)
CSP	compounded sterile preparation
CSTD	closed-system vial-transfer device
DCA	direct compounding area
ECV	endotoxin challenge vial
EU	Endotoxin Unit
FDA	Food and Drug Administration
HEPA	high efficiency particulate air
HICPAC	Healthcare Infection Control Practices Advisory Committee
HVAC	heating, ventilation, and air conditioning
IPA	isopropyl alcohol
ISO	International Organization for Standardization
LAFW	laminar airflow workbench
MDVs	multiple-dose vials
MMWR	Morbidity and Mortality Weekly Report
NIOSH	National Institute for Occupational Safety and Health
NIST	National Institute of Standards and Technology



PEC	primary engineering control
PET	positron emission tomography
PPE	personnel protective equipment
psi	pounds per square inch
QA	quality assurance
SOP	standard operating procedure
SVI	sterile vial for injection
TSA	trypticase soy agar
USP	United States Pharmacopeia

## APPENDICES

## Appendix I. Principal Competencies, Conditions, Practices, and Quality Assurances That Are Required († "shall") and Recommended (‡ "should") in USP Chapter 797

note—This tabular appendix selectively abstracts and condenses the full text of 797 for rapid reference only. Compounding personnel are responsible for reading, understanding and complying with the full text and all official USP terminology, content, and conditions therein.

## INTRODUCTION

‡ Chapter purpose is to prevent harm and death to patients treated with CSPs.

† Chapter pertains to preparation, storage, and transportation, but not administration, of CSPs.

† Personnel and facilities to which 797 applies; therefore, for whom and which it may be enforced by regulatory and accreditation authorities.

† Types of preparations designated to be CSPs according to their physical forms, and their sites and routes of administration to patients.

† Compounding personnel must be meticulously conscientious to preclude contact contamination of CSPs both within and outside ISO Class 5 areas.

## ORGANIZATION

† All compounding personnel shall be responsible for understanding fundamental practices and precautions within USP 797, for developing and implementing appropriate procedures, and for continually evaluating these procedures and the quality of final CSPs to prevent harm.

## DEFINITIONS

† Twenty-eight terms are defined and integral to complying with USP 797.

## RESPONSIBILITY OF COMPOUNDING PERSONNEL

† Practices and quality assurances required to prepare, store, and transport CSPs that are sterile, and acceptably accurate, pure, and stable.

## CSP MICROBIAL CONTAMINATION RISK LEVELS

† Proper training and evaluation of personnel, proper cleansing and garbing of personnel, proper cleaning and disinfecting of compounding work environments, and proper maintenance and monitoring of controlled environmental locations (all of which are detailed in their respective sections).

## Low-Risk Level CSPs

† Aseptic manipulations within an ISO Class 5 environment using three or fewer sterile products and entries into any container.

† In absence of passing sterility test, store not more than 48 hours at controlled room temperature, 14 days at cold temperature, and 45 days in solid frozen state at  $-25^{\circ}$  to  $-10^{\circ}$  or colder.

† Media-fill test at least annually by compounding personnel.

## Low-Risk Level CSPs with 12-Hour or Less BUD

† Fully comply with all four specific criteria.

‡ Sinks should not be located adjacent to the ISO Class 5 primary engineering control.

‡ Sinks should be separated from the immediate area of the ISO Class 5 primary engineering control device.

## Medium-Risk Level CSPs

† Aseptic manipulations within an ISO Class 5 environment using prolonged and complex mixing and transfer, more than three sterile products and entries into any container, and pooling ingredients from multiple sterile products to prepare multiple CSPs.

† In absence of passing sterility test, store not more than 30 hours at controlled room temperature, 9 days at cold temperature, and 45 days in solid frozen state at  $-25^{\circ}$  to  $-10^{\circ}$  or colder.

† Media-fill test at least annually by compounding personnel.

## High-Risk Level CSPs

† Confirmed presence of nonsterile ingredients and devices, or confirmed or suspected exposure of sterile ingredients for more than one hour to air quality inferior to ISO Class 5 before final sterilization.

† Sterilization method verified to achieve sterility for the quantity and type of containers.

† Meet allowable limits for bacterial endotoxins.

† Maintain acceptable strength and purity of ingredients and integrity of containers after sterilization.

† In absence of passing sterility test, store not more than 24 hours at controlled room temperature, 3 days at cold temperature, and 45 days in solid frozen state at  $-25^{\circ}$  to  $-10^{\circ}$  or colder.

† Media-fill test at least semiannually by compounding personnel.

## PERSONNEL TRAINING AND EVALUATION IN ASEPTIC MANIPULATIONS SKILLS

† Pass didactic, practical skill assessment and media-fill testing initially, followed by an annual assessment for a low- and medium-risk level compounding and semi-annual assessment for high-risk level compounding.

† Compounding personnel who fail written tests, or whose media-fill test vials result in gross microbial colonization, shall be immediately restructured and re-evaluated by expert compounding personnel to ensure correction of all aseptic practice deficiencies.

## IMMEDIATE-USE CSPs

† Fully comply with all six specified criteria.

## SINGLE-DOSE AND MULTIPLE-DOSE CONTAINERS

† Beyond-use date 28 days, unless specified otherwise by the manufacturer, for closure sealed multiple-dose containers after initial opening or entry.

† Beyond-use time of 6 hours, unless specified otherwise by the manufacturer, for closure sealed single-dose containers in ISO Class 5 or cleaner air after initial opening or entry.

Beyond-use time of 1 hour for closure sealed single-dose containers after being opened or entered in worse than ISO Class 5 air.

† Storage of opened single-dose ampuls is not permitted.

#### HAZARDOUS DRUGS AS CSPs

† Appropriate personnel protective equipment.

† Appropriate primary engineering controls (BSCs and CACIs) are used for concurrent personnel protection and exposure of critical sites.

† Hazardous drugs shall be stored separately from other inventory in a manner to prevent contamination and personnel exposure.

† At least 0.01 inch water column negative pressure and 12 air changes per hour in non-cleanrooms in which CACIs are located.

† Hazardous drugs shall be handled with caution at all times using appropriate chemotherapy gloves during receiving, distribution, stocking, inventorying, preparing for administration, and disposal.

† Hazardous drugs shall be prepared in an ISO Class 5 environment with protective engineering controls in place, and following aseptic practices specified for the appropriate contamination risk levels.

† Access to drug preparation areas shall be limited to authorized personnel.

† A pressure indicator shall be installed that can readily monitor room pressurization, which is documented daily.

† Annual documentation of full training of personnel regarding storage, handling, and disposal of hazardous drugs.

† When used, a CSTD shall be used in an ISO Class 5 primary engineering control device.

† At least 0.01 inch water column negative pressure is required for compounding of hazardous drugs.

‡ Negative-pressure buffer area is not required for low-volume compounding operations when CSTD is used in BSC or CACI.

† Compounding personnel of reproductive capability shall confirm in writing that they understand the risks of handling hazardous drugs.

† Disposal of all hazardous drug wastes shall comply with all applicable federal and state regulations.

‡ Total external exhaust of primary engineering controls.

‡ Assay of surface wipe samples every 6 months.

#### RADIOPHARMACEUTICALS AS CSPs

† Positron Emission Tomography is according to USP chapter 823.

† Appropriate primary engineering controls and radioactivity containment and shielding.

† Radiopharmaceuticals compounded from sterile components, in closed sterile containers, with volume of 100 mL or less for a single-dose injection or not more than 30 mL taken from a multiple-dose container shall be designated as and conform to the standards for low-risk level CSPs.

† Radiopharmaceutical vials, designed for multi-use, compounded with technetium-99m, exposed to ISO Class 5 environment and punctured by needles with no direct contact contamination may be used up to the time indicated by manufacturers' recommendations.

† Location of primary engineering controls permitted in ISO Class 8 controlled environment.

† Technetium-99m/Molybdenum-99 generators used according to manufacturer, state, and federal requirements.

† Radiopharmaceuticals prepared as low-risk level CSPs with 12-hour or less BUD shall be prepared in a segregated compounding area.

† Materials and garb exposed in patient-care and treatment area shall not cross a line of demarcation into the segregated compounding area.

† Technetium-99m/Molybdenum-99 generators must be eluted in ISO Class 8 conditions.

† Segregated compounding area will be designated with a line of demarcation.

‡ Storage and transport of properly shielded vials of radiopharmaceutical CSPs may occur in a limited access ambient environment without a specific ISO class designation.

#### ALLERGEN EXTRACTS AS CSPs

† Allergen extracts as CSPs are not subject to the personnel, environmental, and storage requirements for all CSP Microbial Contamination Risk Levels when certain criteria are met.

#### VERIFICATION OF COMPOUNDING ACCURACY AND STERILITY

† Review labels and document correct measurements, aseptic manipulations, and sterilization procedures to confirm correct identity, purity, and strength of ingredients in, and sterility of, CSPs.

‡ Assay finished CSPs to confirm correct identity and, or, strength of ingredients.

‡ Sterility test finished CSPs.

#### Sterilization Methods

† Verify that methods achieve sterility while maintaining appropriate strength, purity, quality, and packaging integrity.

‡ Prove effectiveness by USP chapter 71, equivalent, or superior sterility testing.

#### Sterilization of High-Risk Level CSPs by Filtration

† Nominal 0.2-µm pore size sterile membranes that are chemically and physically compatible with the CSP.

† Complete rapidly without filter replacement.

† Subject filter to manufacturer's recommended integrity test (e.g., bubble point test) after filtering CSPs.

#### Sterilization of High-Risk Level CSPs by Steam

† Test to verify the mass of containers to be sterilized will be sterile after the selected exposure duration in the particular autoclave.

† Ensure live steam contacts all ingredients and surfaces to be sterilized.

† Pass solutions through a 1.2-µm or smaller nominal pore size filter into final containers to remove particulates before sterilization.

† Heated filtered air shall be evenly distributed throughout the chamber by a blower device.

† Dry heat shall only be used for those materials that cannot be sterilized by steam, when the moisture would either damage or be impermeable to the materials.

† Sufficient space shall be left between materials to allow for good circulation of the hot air.

† The description of dry heat sterilization conditions and duration for specific CSPs shall be included in written documentation in the compounding facility. The effectiveness of dry heat sterilization shall be verified using appropriate biological indicators and other confirmation.

‡ The oven should be equipped with a system for controlling temperature and exposure period.

#### Depyrogenation by Dry Heat

† Dry heat depyrogenation shall be used to render glassware or containers, such as vials free from pyrogens as well as viable microbes.

† The description of the dry heat depyrogenation cycle and duration for specific load items shall be included in written documentation in the compounding facility.

† The effectiveness of the dry heat depyrogenation cycle shall be verified using endotoxin challenge vials (ECVs).

The bacterial endotoxin test should be performed on the ECVs to verify the cycle is capable of achieving a 3 log reduction in endotoxin.

#### ENVIRONMENTAL QUALITY AND CONTROL

Exposure of Critical Sites

† ISO Class 5 or better air.

† Preclude direct contact (e.g., touch and secretions) contamination.

ISO Class 5 Air Sources, Buffer Areas, and Ante-Areas

† A buffer area is an area that provides at least ISO Class 7 air quality.

† New representations of facility layouts.

† Each compounding facility shall ensure that each source of ISO Class 5 environment for exposure of critical sites and sterilization by filtration is properly located, operated, maintained, monitored, and verified.

† Devices (e.g., computers and printers) and objects (e.g., carts and cabinets) can be placed in buffer areas and shall be verified by testing or monitoring.

Viable and Nonviable Environmental Sampling (ES) Testing

† Environmental sampling shall occur as part a comprehensive quality management program and shall occur minimally when several conditions exist.

‡ The ES program should provide information to staff and leadership to demonstrate that the engineering controls are maintaining an environment within the compounding area that consistently maintains acceptably low viable and nonviable particle levels.

Environmental Nonviable Particle Testing Program

† Certification and testing of primary (LAFWs, BSCs, CAIs and CACIs) and secondary engineering controls (buffer and ante areas) shall be performed by a qualified individual no less than every six months and whenever the device or room is relocated, altered, or major service to the facility is performed. Certification procedures such as those outlined in the CETA Certification Guide for Sterile Compounding Facilities (CAG-003-2006) shall be used.

Total Particle Counts

† Certification that each ISO classified area (e.g., ISO Class 5, 7 and 8) is within established guidelines shall be performed no less than every 6 months and whenever the LAFW, BSC, CAI, or CACI is relocated or the physical structure of the buffer room or ante-area has been altered.

† Testing shall be performed by qualified operators using current, state-of-the-art electronic equipment with results meeting ISO Class 5, 7, or 8 depending on the requirements of the area.

† All certification records shall be maintained and reviewed by supervising personnel or other designated employee to ensure that the controlled environments comply with the proper air cleanliness, room pressures, and air changes per hour.

Pressure Differential Monitoring

† A pressure gauge or velocity meter shall be installed to monitor the pressure differential or airflow between the buffer area and ante-area, and the ante-area and the general environment outside the compounding area.

† The results shall be reviewed and documented on a log at least every work shift (minimum frequency shall be at least daily) or by a continuous recording device.

† The pressure between the ISO Class 7 and general pharmacy area shall not be less than 5 Pa (0.02 inch water column (w.c.)).

† In facilities where low- and medium-risk level CSPs are prepared, differential airflow shall maintain a minimum velocity of 0.2 meter/second (40 fpm) between buffer area and ante-area.

Environmental Viable Airborne Particle Testing Program—Sampling Plan

† An appropriate environmental sampling plan shall be developed for airborne viable particles based on a risk assessment of compounding activities performed.

† Selected sampling sites shall include locations within each ISO Class 5 environment and in the ISO Class 7 and 8 areas, and the segregated compounding areas at greatest risk of contamination (e.g., work areas near the ISO Class 5 environment, counters near doors, pass-through boxes).

† The plan shall include sample location, method of collection, frequency of sampling, volume of air sampled, and time of day as related to activity in the compounding area and action levels.

‡ It is recommended that compounding personnel refer to USP Chapter [Microbiological Evaluation of Clean Rooms and Other Controlled Environments](#) 1116 and the CDC Guidelines for Environmental Infection Control in Healthcare Facilities-2003 for more information.

Growth Media

† A general microbiological growth medium such as Soybean-Casein Digest Medium (also known as trypticase soy broth (TSB) or agar (TSA)) shall be used to support the growth of bacteria.

† Malt extract agar (MEA) or some other media that supports the growth of fungi shall be used in high-risk level compounding environments.

† Media used for surface sampling shall be supplemented with additives to neutralize the effects of disinfecting agents (e.g., TSA with lecithin and polysorbate 80).

Viable Air Sampling

† Evaluation of airborne microorganisms using volumetric collection methods in the controlled air environments shall be performed by properly trained individuals for all compounding risk levels.

† Impaction shall be the preferred method of volumetric air sampling.

† For low-, medium-, and high-risk level compounding, air sampling shall be performed at locations that are prone to contamination during compounding activities and during other activities like staging, labeling, gowning, and cleaning.

† Locations shall include zones of air backwash turbulence within laminar airflow workbench and other areas where air backwash turbulence may enter the compounding area.

† For low-risk level CSPs with 12-hour or less BUD, air sampling shall be performed at locations inside the ISO Class 5 environment and other areas that are in close proximity to the ISO class 5 environment, during the certification of the primary engineering control.

‡ Consideration should be given to the overall effect the chosen sampling method will have on the unidirectional airflow within a compounding environment.

Air Sampling Devices

† The instructions in the manufacturer's user manual for verification and use of electric air samplers that actively collect volumes of air for evaluation shall be followed.

† A sufficient volume of air (400–1000 liters) shall be tested at each location in order to maximize sensitivity.

‡ It is recommended that compounding personnel also refer to USP Chapter 1116, which can provide more information on the use of volumetric air samplers and volume of air that should be sampled to detect environmental bioburden excursions.

Air Sampling Frequency and Process

† Air sampling shall be performed at least semiannually (i.e. every 6 months), as part of the re-certification of facilities and equipment for area where primary engineering controls are located.

† A sufficient volume of air shall be sampled and the manufacturer's guidelines for use of the electronic air sampling equipment followed.

‡ Any facility construction or equipment servicing may require the need to perform air sampling during these events.



## Incubation Period

† The microbial growth media plates used to collect environmental sampling are recovered, covers secured (e.g., taped), inverted, and incubated at a temperature and for a time period conducive to multiplication of microorganisms.

† The number of discrete colonies of microorganisms shall be counted and reported as colony-forming units (cfu) and documented on an environmental monitoring form. Counts from air monitoring need to be transformed into cfu/cubic meter of air and evaluated for adverse trends.

‡ TSA should be incubated at  $35^{\circ}\pm 2^{\circ}$  for 2–3 days.

‡ MEA or other suitable fungal media should be incubated at  $28^{\circ}\pm 2^{\circ}$  for 5–7 days.

## Action Levels, Documentation and Data Evaluation

† Sampling data shall be collected and reviewed on a periodic basis as a means of evaluating the overall control of the compounding environment.

† Competent microbiology personnel shall be consulted if an environmental sampling consistently shows elevated levels of microbial growth.

† An investigation into the source of the environmental contamination shall be conducted.

‡ Any cfu count that exceeds its respective action level should prompt a re-evaluation of the adequacy of personnel work practices, cleaning procedures, operational procedures, and air filtration efficiency within the aseptic compounding location.

‡ Table titled, Recommended Action Levels for Microbial Contamination should only be used as a guideline

## Facility Design and Environmental Controls

† Compounding facilities are physically designed and environmentally controlled to minimize airborne contamination from contacting critical sites.

† Compounding facilities shall provide a comfortable and well-lighted working environment, which typically includes a temperature of  $20^{\circ}$  or cooler to maintain comfortable conditions for compounding personnel when attired in the required aseptic compounding garb.

† Primary engineering controls provide unidirectional (i.e., laminar) HEPA air at a velocity sufficient to prevent airborne particles from contacting critical sites.

† In situ air pattern analysis via smoke studies shall be conducted at the critical area to demonstrate unidirectional airflow and sweeping action over and away from the product under dynamic conditions.

† Policies and procedures for maintaining and working within the primary engineering control area shall be written and followed. The policies and procedures will be determined by the scope and risk levels of the aseptic compounding activities used during the preparation of the CSPs.

† The principles of HEPA-filtered unidirectional airflow in the work environment shall be understood and practiced in the compounding process in order to achieve the desired environmental conditions.

† Clean rooms for nonhazardous and nonradioactive CSPs are supplied with HEPA that enters from ceilings with return vents low on walls, and that provides not less than 30 air changes per hour.

† Buffer areas maintain 0.02- to 0.05-inch water column positive pressure, and do not contain sinks or drains.

† Air velocity from buffer rooms or zones to ante-areas is at least 40 feet/minute.

† The primary engineering controls shall be placed within a buffer area in such a manner as to avoid conditions that could adversely affect their operation.

† The primary engineering controls shall be placed out of the traffic flow and in a manner to avoid disruption from the HVAC system and room cross-drafts.

† HEPA-filtered supply air shall be introduced at the ceiling.

† All HEPA filters shall be efficiency tested using the most penetrating particle size and shall be leak tested at the factory and then leak tested again in situ after installation.

† Activities and tasks carried out within the buffer area shall be limited to only those necessary when working within a controlled environment.

† Only the furniture, equipment, supplies, and other material required for the compounding activities to be performed shall be brought into the room.

† Surfaces and essential furniture in buffer rooms or zones and clean rooms shall be nonporous, smooth, nonshedding, impermeable, cleanable, and resistant to disinfectants.

† The surfaces of ceilings, walls, floors, fixtures, shelving, counters, and cabinets in the buffer area shall be smooth, impervious, free from cracks and crevices, and nonshedding, thereby promoting cleanability, and minimizing spaces in which microorganisms and other contaminants may accumulate.

† The surfaces shall be resistant to damage by disinfectant agents.

† Junctures of ceilings to walls shall be coved or caulked to avoid cracks and crevices where dirt can accumulate.

† Ceiling tiles shall be caulked around each perimeter to seal them to the support frame.

† The exterior lens surface of ceiling lighting fixtures shall be smooth, mounted flush, and sealed.

† Any other penetrations through the ceiling or walls shall be sealed.

† The buffer area shall not contain sources of water (sinks) or floor drains. Work surfaces shall be constructed of smooth, impervious materials, such as stainless steel or molded plastic, so that they are easily cleaned and disinfected.

† Carts shall be of stainless steel wire, nonporous plastic, or sheet metal construction with good quality, cleanable casters to promote mobility.

† Storage shelving, counters, and cabinets shall be smooth, impervious, free from cracks and crevices, nonshedding, cleanable, and disinfectable.

† Their number, design, and manner of installation the items above shall promote effective cleaning and disinfection.

‡ If ceilings consist of inlaid panels, the panels should be impregnated with a polymer to render them impervious and hydrophobic.

‡ Dust-collecting overhangs, such as ceiling utility pipes, or ledges, such as windowsills, should be avoided.

‡ Air returns should be mounted low on the wall creating a general top-down dilution of room air with HEPA-filtered make-up air.

## Placement of Primary Engineering Controls Within ISO Class 7 Buffer Areas

† Primary engineering controls for nonhazardous and nonradioactive CSPs are located in buffer areas, except for CAIs that are proven to maintain ISO Class 5 air when particle counts are sampled 6 to 12 inches upstream of critical site exposure areas during performance of normal inward and outward transfer of materials, and compounding manipulations when such CAIs are located in air quality worse than ISO Class 7.

† Presterilization procedures for high-risk level CSPs, such as weighing and mixing, shall be completed in no worse than an ISO Class 8 environment.

† Primary engineering controls shall be located out of traffic patterns and away from room air currents that could disrupt the intended airflow patterns.

† When isolators are used for sterile compounding, the recovery time to achieve ISO Class 5 air quality shall be documented and internal procedures developed to ensure that adequate recovery time is allowed after material transfer before and during compounding operations.

† When compounding activities require the manipulation of a patient's blood-derived or other biological material (e.g., radiolabeling a patient's or a donor's white blood cells), the manipulations shall be clearly separated from routine material-handling procedures and equipment used in CSP preparation activities, and they shall be controlled by specific standard operating procedures in order to avoid any cross-contamination.

† Food, drinks, and items exposed in patient care areas, and unpacking of bulk supplies and personnel cleansing and garbing are prohibited from buffer areas or rooms.

† Demarcation designation between buffer areas or rooms and ante-areas.

† Antiseptic hand cleansing and sterile gloves in buffer areas or rooms.

‡ Packaged compounding supplies and components, such as needles, syringes, tubing sets, and small- and large-volume parenterals, should be uncartoned and wiped down with a



disinfectant that does not leave a residue (e.g., sterile 70% IPA) when possible in an ante-area, of ISO Class 8 air quality, before being passed into the buffer areas.

#### Cleaning and Disinfecting the Sterile Compounding Areas

† Trained personnel write detailed procedures including cleansers, disinfectants, and non-shedding wipe and mop materials.

† Cleaning and disinfecting surfaces in the LAFWs, BSCs, CAIs, and CACIs shall be cleaned and disinfected frequently, including at the beginning of each work shift, before each batch preparation is started, every 30 minutes during continuous compounding periods of individual CSPs, when there are spills, and when surface contamination is known or suspected from procedural breaches.

† Trained compounding personnel are responsible for developing, implementing, and practicing the procedures for cleaning and disinfecting the DCAs written in the SOPs.

† Cleaning and disinfecting shall occur before compounding is performed. Items shall be removed from all areas to be cleaned, and surfaces shall be cleaned by removing loose material and residue from spills, e.g., water-soluble solid residues are removed with Sterile Water (for Injection or Irrigation) and low-shedding wipes. This shall be followed by wiping with a residue-free disinfecting agent, such as sterile 70% IPA, which is allowed to dry before compounding begins.

† Work surfaces in ISO Class 7 and 8 areas and segregated compounding areas are cleaned at least daily.

† Dust and debris shall be removed when necessary from storage sites for compounding ingredients and supplies, using a method that does not degrade the ISO Class 7 or 8 air quality.

† Floors in ISO Class 7 and 8 areas are cleaned daily when no compounding occurs.

† IPA (70% isopropyl alcohol) remains on surfaces to be disinfected for at least 30 seconds before such surfaces are used to prepare CSPs.

† Emptied shelving, walls, and ceilings in ante-areas are cleaned and disinfected at least monthly.

† Mopping shall be performed by trained personnel using approved agents and procedures described in the written SOPs.

† Cleaning and disinfecting agents, their schedules of use and methods of application shall be in accordance with written SOPs and followed by custodial and/or compounding personnel.

† All cleaning materials, such as wipers, sponges, and mops, shall be nonshedding, preferably composed of synthetic micro fibers, and dedicated to use in the buffer area, or ante-area, and segregated compounding areas and shall not be removed from these areas except for disposal.

† If cleaning materials are reused (e.g., mops), procedures shall be developed (based on manufacturer recommendations) that ensure that the effectiveness of the cleaning device is maintained and repeated use does not add to the bioburden of the area being cleaned.

† Supplies and equipment removed from shipping cartons shall be wiped with a suitable disinfecting agent (e.g., sterile 70% IPA) delivered from a spray bottle or other suitable delivery method.

† After the disinfectant is sprayed or wiped on a surface to be disinfected, the disinfectant shall be allowed to dry, and during this time the item shall not be used for compounding purposes.

† Sterile 70% IPA wetted gauze pads or other particle-generating material shall not be used to disinfect the sterile entry points of packages and devices.

#### Personnel Cleansing and Garbing

† Personnel shall also be thoroughly competent and highly motivated to perform flawless aseptic manipulations with ingredients, devices, and components of CSPs.

† Personnel with rashes, sunburn, weeping sores, conjunctivitis, active respiratory infection, and cosmetics are prohibited from preparing CSPs.

† Compounding personnel shall remove personal outer garments; cosmetics; artificial nails; hand, wrist, and body jewelry that can interfere with the fit of gowns and gloves; and visible body piercing above the neck.

† Order of compounding garb and cleansing in ante-area: shoes or shoe covers, head and facial hair covers, face mask, fingernail cleansing, hand and forearm washing and drying; non-shedding gown.

† Order of cleansing and gloving in buffer room or area: hand cleansing with a persistently active alcohol-based product with persistent activity; allow hands to dry; don sterile gloves.

† Routinely disinfect gloves with sterile 70% IPA after contacting nonsterile objects.

† Inspect gloves for holes and replace when breaches are detected.

† Personnel repeat proper procedures after they are exposed to direct contact contamination or worse than ISO Class 8 air.

† These requirements are exempted only for immediate-use CSPs and CAIs for which manufacturers provide written documentation based on validated testing that such personnel practices are not required to maintain sterility in CSPs.

#### Personnel Training and Competency Evaluation of Garbing, Aseptic Work Practices and Cleaning/Disinfection Procedures

† Personnel who prepare CSPs shall be trained conscientiously and skillfully by expert personnel, multi-media instructional sources, and professional publications in the theoretical principles and practical skills of garbing procedures, aseptic work practices, achieving and maintaining ISO Class 5 environmental conditions, and cleaning and disinfection procedures.

† This training shall be completed and documented before any compounding personnel begin to prepare CSPs.

† Compounding personnel shall complete didactic training, pass written competence assessments, undergo skill assessment using observational audit tools, and media-fill testing.

† Media-fill testing of aseptic work skills shall be performed initially before beginning to prepare CSPs and at least annually thereafter for low- and medium-risk level compounding; and semiannually for high-risk level compounding.

† Compounding personnel who fail written tests, observational audits, or whose media-fill test vials have one or more units showing visible microbial contamination, shall be retrained and re-evaluated by expert compounding personnel to ensure correction of all aseptic work practice deficiencies.

† Compounding personnel shall pass all evaluations prior to resuming compounding of sterile preparations.

† Compounding personnel must demonstrate proficiency of proper hand hygiene, garbing, and consistent cleaning procedures in addition to didactic evaluation and aseptic media fill.

† Cleaning and disinfecting procedures performed by other support personnel shall be thoroughly trained in proper hand hygiene, and garbing, cleaning, and disinfection procedures by a qualified aseptic compounding expert.

† Support personnel shall routinely undergo performance evaluation of proper hand hygiene, garbing, and all applicable cleaning and disinfecting procedures conducted by a qualified aseptic compounding expert.

#### Competency Evaluation of Garbing and Aseptic Work Practices

† Compounding personnel shall be evaluated initially prior to beginning compounding CSPs and whenever an aseptic media fill is performed using a Sample Form for Assessing Hand Hygiene and Garbing Related Practices of Compounding Personnel and the personnel glove fingertip sampling procedures.

#### Aseptic Work Practice Assessment and Evaluation via Personnel Glove Fingertip Sampling

† Monitoring of compounding personnel glove fingertips shall be performed for all CSP risk level compounding.

† Glove fingertip sampling shall be used to evaluate the competency of personnel in performing hand hygiene and garbing procedures in addition to educating compounding personnel on proper work practices.

† All personnel shall demonstrate competency in proper hand hygiene and garbing procedures in addition to aseptic work practices.

† Sterile contact agar plates shall be used to sample the gloved fingertips of compounding personnel after garbing to assess garbing competency and after completing the media-fill preparation.



Gloves shall not be disinfected with sterile 70% IPA immediately prior to sampling.

#### Garbing and Gloving Competency Evaluation

† Compounding personnel shall be visually observed during the process of performing hand hygiene and garbing procedures.

† The visual observation shall be documented on a Sample Form for Assessing Hand Hygiene and Garbing Related Practices of Compounding Personnel and maintained to provide a permanent record of and long-term assessment of personnel competency.

#### Gloved Fingertip Sampling

† Immediately after the compounding personnel completes the hand hygiene and garbing procedure, the evaluator shall collect a gloved fingertip and thumb sample from both hands of the compounding personnel onto appropriate agar plates by lightly pressing each finger tip into the agar.

† The plates shall be incubated for the appropriate incubation period and at the appropriate temperature.

† All employees shall successfully complete an initial competency evaluation and gloved fingertip/thumb sampling procedure (0 cfu) no less than three times before initially being allowed to compound CSPs for human use.

† After completing the initial gowning and gloving competency evaluation, re-evaluation of all compounding personnel shall occur at least annually for low- and medium-risk level CSPs and semiannually for high-risk level CSPs before being allowed to continue compounding CSPs.

† Gloves shall not be disinfected with sterile 70% IPA prior to testing.

† The sampled gloves shall be immediately discarded and proper hand hygiene performed after sampling. The nutrient agar plates shall be incubated as stated below.

† The cfu action level for gloved hands shall be based on the total number of cfu on both gloves and not per hand.

‡ Results should be reported separately as number of cfu per employee per hand (left hand, right hand).

#### Incubation Period

† At the end of the designated sampling period, the agar plates are recovered, covers secured, inverted and incubated at a temperature and for a time period conducive to multiplication of microorganisms. Trypticase soy agar (TSA) with lecithin and polysorbate 80 shall be incubated at  $35^{\circ}\pm 2^{\circ}$  for 2–3 days.

#### Aseptic Manipulation Competency Evaluation

† All compounding personnel shall have their aseptic technique and related practice competency evaluated initially during the media-fill test procedure and subsequent annual or semiannual media-fill test procedures on the Sample Form for Assessing Aseptic Technique and Related Practices of Compounding Personnel.

#### Media-Fill Test Procedure

† The skill of personnel to aseptically prepare CSPs shall be evaluated using sterile fluid bacterial culture media-fill verification.

† Media-filled vials shall be incubated within a range of  $35^{\circ}\pm 2^{\circ}$  for 14 days.

#### Surface Cleaning and Disinfection Sampling and Assessment

† Surface sampling shall be performed in all ISO classified areas on a periodic basis and can be accomplished using contact plates and/or swabs and shall be done at the conclusion of compounding.

† Locations to be sampled shall be defined in a sample plan or on a form.

#### Cleaning and Disinfecting Competency Evaluation

† Compounding personnel and other personnel responsible for cleaning shall be visually observed during the process of performing cleaning and disinfecting procedures during initial personnel training on cleaning procedures, changes in cleaning staff and at the completion of any Media-Fill Test Procedure.

† Visual observation shall be documented on a Sample Form for Assessing Cleaning and Disinfection Procedures and maintained to provide a permanent record of, and long-term assessment of, personnel competency.

#### Surface Collection Methods

† Immediately after sampling a surface with the contact plate, the sampled area shall be thoroughly wiped with a non-shedding wipe soaked in sterile 70% IPA.

‡ Results should be reported as cfu per unit of surface area.

#### Action Levels, Documentation, and Data Evaluation

† Environmental sampling data shall be collected and reviewed on a routine basis as a means of evaluating the overall control of the compounding environment.

† If an activity consistently shows elevated levels of microbial growth, competent microbiology personnel shall be consulted.

† An investigation into the source of the contamination shall be conducted.

† When gloved fingertip sample results exceed action levels after proper incubation, a review of hand hygiene and garbing procedures as well as glove and surface disinfection procedures and work practices shall be performed and documented.

‡ Any cfu count that exceeds its respective action level should prompt a re-evaluation of the adequacy of personnel work practices, cleaning procedures, operational procedures, and air filtration efficiency within the aseptic compounding location.

#### SUGGESTED STANDARD OPERATING PROCEDURES

† All facilities are required to have these, and they must include at least the items enumerated in this section.

#### FINISHED PREPARATION RELEASE CHECKS AND TESTS

##### Inspection of Solution Dosage Forms and Review of Compounding Procedures

† Review procedures and documents to ensure sterility, purity, correct identities and amounts of ingredients, and stability.

† Visually inspect for abnormal particulate matter and color, and intact containers and seals.

##### Sterility Testing

† High-risk level CSPs prepared in batches of more than 25 identical containers, or exposed longer than 12 hours at  $2^{\circ}$  to  $8^{\circ}$ , and 6 hours at warmer than  $8^{\circ}$  before being sterilized.

##### Bacterial Endotoxin (Pyrogen) Testing

† High-risk level CSPs, excluding those for inhalation and ophthalmic administration, prepared in batches of more than 25 identical containers, or exposed longer than 12 hours at  $2^{\circ}$  to  $8^{\circ}$ , and 6 hours at warmer than  $8^{\circ}$ , before being sterilized.

##### Identity and Strength Verification of Ingredients

† Written procedures to verify correct identity, quality, amounts, and purities of ingredients used in CSPs.

† Written procedures to ensure labels of CSPs contain correct names and amounts or concentrations of ingredients, total volumes, beyond-use dates, storage conditions, and route(s) of administration.

#### STORAGE AND BEYOND-USE DATING

##### Determining Beyond-Use Dates

† Use the general criteria in USP 795 in the absence of direct stability-indicating assays or authoritative literature that supports longer durations.



## MAINTAINING STERILITY, PURITY, AND STABILITY OF DISPENSED AND DISTRIBUTED CSPs

† Written procedures for proper packaging, storage, and transportation conditions to maintain sterility, quality, purity, and strength of CSPs.

## Redispersed CSPs

† When sterility, and acceptable purity, strength, and quality can be ensured.

† Assignment of sterility storage times and stability beyond-use dates that occur later than those of originally dispensed CSPs must be based on results of sterility testing and quantitative assay of ingredients.

## Packaging and Transporting CSPs

† Packaging maintains physical integrity, sterility, stability, and purity of CSPs.

† Modes of transport that maintain appropriate temperatures and prevent damage to CSPs.

## PATIENT OR CAREGIVER TRAINING

† Multiple component formal training program to ensure patients and caregivers understand the proper storage, handling, use, and disposal of CSPs.

## PATIENT MONITORING AND ADVERSE EVENTS REPORTING

† Written standard procedures describe means for patients to ask questions and report concerns and adverse events with CSPs, and for compounding supervisors to correct and prevent future problems.

‡ Adverse events and defects with CSPs reported to FDA's MedWatch and USP's MEDMARX programs.

Appendix II. Common Disinfectants Used in Health Care for Inanimate Surfaces and Noncritical Devices, and Their Microbial Activity and Properties<sup>1</sup>

## Chemical Category of Disinfectant

		Isopropyl alcohol	Accelerated Hydrogen peroxide <sup>3</sup>	Quaternary Ammonium (e.g., dodecyl dimethyl ammonium chloride)	Phenolics	Chlorine (e.g., sodium hypochlorite)	Iodophors (e.g., povidone-iodine)
Concentration Used		60–95%	0.5%	0.4–1.6% aq	0.4–1.6% aq	100–5000 ppm	30–50 ppm
Microbial Inactivation	Bacteria	+	+	+	+	+	+
	Lipophilic viruses	+	+	+	+	+	+
	Hydrophilic viruses	±	+	±	±	+	±
	M.tuberculosis	+	+	±	+	+	±
	Mycotic agents (fungi)	+	+	+	+	+	±
	Bacterial Spores	—	—	—	—	+	—
Important Chemical & Physical Properties	Shelf life >1 week	+	+	+	+	+	+
	Corrosive or deleterious effects	±	—	—	—	±	±
	Non-evaporable residue	—	—	+	+	—	+
	Inactivated by organic matter	+	±	+	±	+	+
	Skin irritant	±	—	+	+	+	±
	Eye irritant	+	—	+	+	+	+
	Respiratory irritant	—	—	—	—	+	—
	Systemic toxicity	+	—	+	+	+	+

Key to abbreviation and symbols: aq = diluted with water; ppm = parts per million; + = yes; — = no; ± = variable results.

1 Modified from World Health Organization, Laboratory Bio Safety Manual 1983 and Rutala WA, "Antiseptics, disinfection and sterilization in the hospital and related institutions," Manual of Clinical Microbiology, American Society for Microbiology, Washington, DC, 1995, pages 227-245.

2 Inactivation of the most common microorganisms (i.e., bacteria) occurs with a contact time of ≤1 minute; inactivation of spores requires longer contact times (e.g., 5-10 minutes for 5,000 ppm chlorine solution against C. difficile spores). Reference: Perez J, Springthorpe VS, Sattar SA, "Activity of selected oxidizing microbicides against the spores of Clostridium difficile: Relevance to environmental control," American Journal of Infection Control, August 2005, pages 320-325.

3 Accelerated hydrogen peroxide is a new generation of hydrogen peroxide-based germicides in which the potency and performance of the active ingredient have been enhanced and accelerated through the use of appropriate acids and detergents.

## Appendix III. Sample Form for Assessing Hand Hygiene and Garbing Related Practices of Compounding Personnel

	Printed name and position/title of person assessed:
Name of facility or location:	
Hand Hygiene and Garbing Practices: The qualified evaluator will check each space for which the person being assessed has acceptably completed the described activity, prints N/A if the activity is not applicable to the assessment session or N/O if the activity was not observed.*	
Presents in a clean appropriate attire and manner.	
Wears no cosmetics or jewelry (watches, rings, earrings, etc. piercing jewelry included) upon entry into ante-areas.	
Brings no food or drinks into or stored in the ante-areas or buffer areas.	
Is aware of the line of demarcation separating clean and dirty sides and observes required activities.	
Dons shoe covers or designated clean-area shoes one at a time, placing the covered or designated shoe on clean side of the line of demarcation, as appropriate.	
Dons beard cover if necessary.	
Dons head cover assuring that all hair is covered.	
Dons face mask to cover bridge of nose down to include chin.	
Performs hand hygiene procedure by wetting hands and forearms and washing using soap and warm water for at least 30 seconds.	
Dries hands and forearms using lint-free towel or hand dryer.	
Selects the appropriate sized gown examining for any holes, tears, or other defects.	
Dons gown and ensures full closure.	

Disinfects hands again using a waterless alcohol-based surgical hand scrub with persistent activity and allows hands to dry thoroughly before donning sterile gloves.
Dons appropriate sized sterile gloves ensuring that there is a tight fit with no excess glove material at the fingertips.
Examines gloves ensuring that there are no defects, holes, or tears.
While engaging in sterile compounding activities, routinely disinfects gloves with sterile 70% IPA prior to work in the direct compounding area (DCA) and after touching items or surfaces that may contaminate gloves.
Removes PPE on the clean side of the ante-area.
Removes gloves and performs hand hygiene.
Removes gown and discards it, or hangs it on hook if it is to be reused within the same work day.
Removes and discards mask, head cover, and beard cover (if used).
Removes shoe covers or shoes one at a time, ensuring that uncovered foot is placed on the dirty side of the line of demarcation and performs hand hygiene again. (Removes and discards shoe covers every time the compounding area is exited).

\*The person assessed is immediately informed of all unacceptable activities (i.e., spaces lacking check marks, N/A, or N/O) and shown and informed of specific corrections.

Signature of Person Assessed	Printed Name	Date
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Appendix IV. Sample Form for Assessing Aseptic Technique and Related Practices of Compounding Personnel

Printed name and position/title of person assessed:

**Aseptic Technique, Safety, and Quality Assurance Practices:** The qualified evaluator checks each space for which the person being assessed has acceptably completed the described activity, prints N/A if the activity is not applicable to the assessment session or N/O if the activity was not observed.\*

Completes the Hand Hygiene and Garbing Competency Assessment Form.
Performs proper hand hygiene, garbing, and gloving procedures according to SOPs.
Disinfects ISO Class 5 device surfaces with an appropriate agent.
Disinfects components/vials with an appropriate agent prior to placing into ISO Class 5 work area.
Introduces only essential materials in a proper arrangement in the ISO Class 5 work area.
Does not interrupt, impede, or divert flow of first-air to critical sites.
Ensures syringes, needles, and tubing remain in their individual packaging and are only opened in ISO Class 5 work area.
Performs manipulations only in the appropriate DCA of the ISO Class 5 device.
Does not expose critical sites to contact contamination or worse than ISO Class 5 air.
Disinfects stoppers, injection ports, and ampul necks by wiping with sterile 70% IPA and allows sufficient time to dry.
Affixes needles to syringes without contact contamination.
Punctures vial stoppers and spikes infusion ports without contact contamination.
Labels preparation(s) correctly.
Disinfects sterile gloves routinely by wiping with sterile 70% IPA during prolonged compounding manipulations.
Cleans, sets up, and calibrates automated compounding device (e.g., "TPN compounder") according to manufacturer's instructions.
Disposes of sharps and waste according to institutional policy or recognized guidelines.

\*The person assessed is immediately informed of all unacceptable activities (i.e., spaces lacking check marks, N/A, or N/O) and shown and informed of specific corrections.

The person assessed is immediately informed of an unacceptable activity (i.e., spousal/parenting behaviour, HIV, or STD) and informed of specific consequences.

## Appendix V. Sample Form for Assessing Cleaning and Disinfection Procedures

Printed name and position/title of person assessed:

Cleaning and Disinfection Practices: The qualified evaluator will check each space for which the person being assessed has acceptably completed the described activity, prints N/A if the activity is not applicable to the assessment session or N/O if the activity was not observed.\*

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### Daily Tasks:

Daily Tasks:	<p>Prepares correct concentration of disinfectant solution according to manufacturer's instructions.</p> <p>Uses appropriately labeled container for the type of surface to be cleaned (floor, wall, production bins, etc.).</p> <p>Documents disinfectant solution preparation.</p> <p>Follows garbing procedures when performing any cleaning activities.</p> <p>At the beginning of each shift, cleans all ISO Class 5 devices prior to compounding in the following order: walls, IV bar, automated compounders, and work surface.</p> <p>Uses a lint free wipe soaked with sterile 70% IPA or other approved disinfectant solution and allows to dry completely.</p> <p>Removes all compounding components and cleans all ISO Class 5 areas as stated above at the end of each shift.</p> <p>Cleans all counters and easily cleanable work surfaces.</p> <p>Mops floors, using the mop labeled "floors", starting at the wall opposite the room entry door; mops floor surface in even strokes toward the operator. Moves carts as needed to clean entire floor surface. Use of a microfiber cleaning system is an acceptable alternative to mops.</p> <p>In the ante-area, cleans sink and all contact surfaces; cleans floor with a disinfectant solution or uses microfiber cleaning system.</p>
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## Monthly Tasks:

Monthly Cleaning	Performs monthly cleaning on a designated day. Prepares a disinfectant solution as stated in daily tasks that is appropriate for the surfaces to be cleaned.
Ante-Area Cleaning	Cleans buffer area and ante-area ceiling, walls, and storage shelving with a disinfectant solution and a mop or uses a microfiber cleaning system.
ISO Class 5 Cleaning	Once ISO Class 5 area is clean, cleans compounding room ceiling, followed by walls and ending with the floor. Uses appropriate labeled mops or microfiber cleaning system.
Buffer Area Cleaning	Cleans all buffer area totes and storage shelves by removing contents and using a germicidal detergent soaked lint free wipe, cleans the inside surfaces of the tote and then the entire exterior surfaces of the tote. Allows totes to dry. Prior to replacing contents into tote, wipes tote with sterile 70% IPA to remove disinfectant residue. Uses new wipe as needed.
Storage Area Cleaning	

Cleans all buffer area carts by removing contents and using germicidal detergent soaked lint free wipe, cleans all carts starting with the top shelf and top of post, working down to wheels. Cleans the under side of shelves in a similar manner. Uses a new wipe for each cart. Allows to dry. Wipes carts with sterile 70% IPA wetted lint-free wipe to remove any disinfectant residue. Uses new wipe as needed.

Cleans buffer area chairs, the interior and exterior of trash bins, and storage bins using disinfectant solution soaked lint free wipe.

Documents all cleaning activities as to who performed such activities with date and time noted.

\*The person assessed is immediately informed of all unacceptable activities (i.e., spaces lacking check marks, N/A, or N/O) and shown and informed of specific corrections.

Signature of Person Assessed	Printed Name	Date
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1 See American Society of Heating, Refrigerating and Air-Conditioning Engineers, Inc. (ASHRAE), Laboratory Design Guide.

2 CETA Applications Guide for the Use of Compounding Isolators in Compounding Sterile Preparations in Healthcare Facilities, CAG-001-2005, Controlled Environment Testing Association (CETA), November 8, 2005.

3 U.S. Food and Drug Administration, Guidance for Industry, Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice, September 2004.

4 Guidelines for Environmental Infection Control in Health-Care Facilities, Recommendations of CDC and the Healthcare Infection Control Practices Advisory Committee (HICPAC), MMWR, vol. 52, no. RR-10, June 6, 2003, figure 3, pg. 12.

5 NSF/ANSI 49.

6 ISO 14644-4:2001 Cleanrooms and associated controlled environments—Design, construction, and start-up, Case Postale 56, CH-1211 Geneve 20, Switzerland, tel. +41 22 749 01 11.

7 By definition (IEST RP CC 001.4), HEPA filters are a minimum of 99.97% efficient when tested using 0.3- $\mu$ m thermally generated particles and a photometer or rated at their most penetrating particle size using a particle counter.

8 Sample procedures are detailed in CETA Applications Guide CAG-002-2006—section 2.09.

9 Controlled Environment Testing Association, 1500 Sunday Drive, Ste. 102, Raleigh, NC 27607; [www.CETAinternational.org](http://www.CETAinternational.org).

10 Agalloco J, Akers JE. Aseptic Processing: A Vision of the Future. Pharmaceutical Technology, 2005. Aseptic Processing supplement, s16.

11 Eaton T. Microbial Risk Assessment for Aseptically Prepared Products. Am Pharm Rev. 2005; 8 (5, Sep/Oct): 46–51.

12 Guideline for Hand Hygiene in Health care Settings, MMWR, October 25, 2002, vol. 51, No. RR-16 available on the Internet at <http://www.cdc.gov/handhygiene/>.

13 The use of additional resources, such as the Accreditation Manual for Home Care from the Joint Commission on Accreditation of Healthcare Organizations, may prove helpful in the development of a QA plan.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Claudia C. Okeke, Ph.D.</a> Scientific Fellow 1-301-816-8345	(SCC05) Sterile Compounding 05

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#### « 801 » POLAROGRAPHY

Polarography is an electrochemical method of analysis based on the measurement of the current flow resulting from the electrolysis of a solution at a polarizable microelectrode, as a function of an applied voltage. The polarogram (see [Figure 1](#)) obtained by this measurement provides qualitative and quantitative information on electro-reducible and electro-oxidizable substances. The normal concentration range for substances being analyzed is from 10–2 molar to 10–5 molar.

In direct current (dc) polarography, the microelectrode is a dropping mercury electrode (DME) consisting of small reproducible drops of mercury flowing from the orifice of a capillary tube connected to a mercury reservoir. A saturated calomel electrode (SCE) with a large surface area is the most commonly employed reference electrode. As the voltage applied to the cell increases, only a very small residual current flows until the substance under assay undergoes reduction or oxidation. Then the current increases, at first gradually, then almost linearly with voltage, and it gradually reaches a limiting value as is shown in [Figure 1](#).

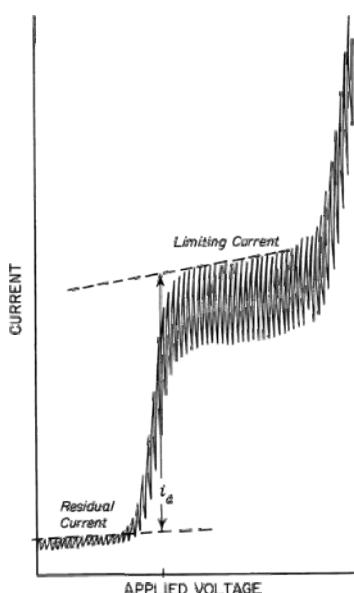


Fig. 1. Typical Polarogram Showing Change in Current Flow with Increasing Potential Applied to the Dropping Mercury Electrode.

On the initial rising portion of the polarographic wave, the increased flow of current results in a decrease in the concentration of the electro-active species at the electrode surface. As



voltage and current increase, the concentration of the reactive species decreases further to a minimal value at the electrode surface. The current is then limited by the rate at which the reacting species can diffuse from the bulk of the solution to the surface of the microelectrode. The final current rise is caused by the reaction of the supporting electrolyte. This large concentration of electrolyte is inert within the potential range used in the analysis, and it prevents the reactive species from reaching the electrode by electrical migration, thus assuring that the limiting current is diffusion-controlled.

Since, in the case of the DME, the electrode surface is being constantly renewed in a cyclic fashion, the current increases from a small value as the drop begins to form to a maximum value as the drop falls. By the use of a suitable recorder to measure the current, the characteristic saw-toothed record is obtained. The limiting current is the sum of the residual and the diffusion currents. The residual current is subtracted from the limiting current to give the wave height.

Ilkovic Equation— The linear relationship between the diffusion current (id) and the concentration of electro-active species is shown by the Ilkovic equation:

$$id = 708nD1 / 2Cm2 / 3t1 / 6$$

in which id is the maximum current in microamperes; n is the number of electrons required per molecule of electro-active substance; D is its diffusion coefficient, in square cm per second; C is the concentration, in millimoles per L; m is the rate of mercury flow from the DME, in mg per second; and t is the drop time, in seconds.

Modern polarographs are equipped with recorders capable of following the current during the latter portion of the drop life; consequently, the maximum of the oscillations is the measure of the current. When the current is measured only at the end of the drop life, the technique is termed sampled dc polarography. In this case, only the maximum currents are recorded and oscillations due to drop growth are not observed.

For instruments equipped with galvanometers to measure the current or recorders operated in a damped mode, the saw-toothed waves correspond to oscillations about the average current. In the latter case, the average of the oscillations is the measure of the current. For polarograms obtained in this manner, the id given by the Ilkovic equation is the average current in microamperes observed during the life of the drop, when the coefficient 708 is replaced by 607.

Control of the Diffusion Current— The Ilkovic equation identifies the variables that must be controlled to ensure that the diffusion current is directly proportional to the concentration of electro-active material. At 25° the diffusion coefficients for aqueous solutions of many ions and organic molecules increase 1% to 2% per degree rise in temperature. Thus the temperature of the polarographic cell must be controlled to within  $\pm 0.5^{\circ}$ . The quantities m and t depend upon the dimensions of the capillary and the height of the mercury column above the electrode. Although results obtained with different capillaries can be compared if the product  $m^2/3t1/6$  is known, it is advisable to use the same capillary with a constant head of mercury during a series of analyses. The diffusion current is proportional to the square root of the height of the mercury column. A mercury reservoir with a diameter greater than 4 cm prevents any significant drop in the mercury level during a series of runs.

The capillary for the DME has a bore of approximately 0.04 mm and a length of 6 cm to 15 cm. The height of the mercury column, measured from the tip of the capillary to the top of the mercury pool, ranges from 40 cm to 80 cm. The exact length of the capillary and the height of the mercury column are adjusted to give a drop-time of between 3 and 5 seconds at open circuit with the capillary immersed in the test solution.

Equipment is available that allows controlled drop-times of fractions of a second to several seconds. As detail within a polarogram is related to the number of drops delivered during a given potential change, such short drop-times allow more rapid recording of the polarogram.

The current flowing through the test solution during the recording of a polarogram is in the microampere range. Thus, the current flow produces negligible changes in the test solution and several polarograms can be run on the same test solution without significant differences.

Half-wave Potential— The half-wave potential ( $E_{1/2}$ ) occurs at the point on the polarogram one-half the distance between the residual current and the limiting current plateau. This potential is characteristic of the electro-active species and is largely independent of its concentration or the capillary used to obtain the wave. It is dependent upon the solution composition and may change with variations in the pH or in the solvent system or with the addition of complexing agents. The half-wave potential thus serves as a criterion for the qualitative identification of a substance.

The potential of the DME is equal to the applied voltage versus the reference electrode after correction for the iR drop (that potential need to pass the current, i, through the solution with a resistance R). It is especially important to make this correction for nonaqueous solutions, which ordinarily possess high resistance, if an accurate potential for the DME is needed. Correction of the half-wave potential is not required for quantitative analysis. Unless otherwise indicated, it is to be understood that potentials represent measurements made against the SCE.

Removal of Dissolved Oxygen— Inasmuch as oxygen is reduced at the DME in two steps, first to hydrogen peroxide and then to water, it interferes where polarograms are to be made at potentials more negative than about 0 volt versus SCE, and must be removed. This may be accomplished by bubbling oxygen-free nitrogen through the solution for 10 to 15 minutes immediately before recording the wave, the nitrogen first having been "conditioned" to minimize changes due to evaporation, by being passed through a separate portion of the solution.

It is necessary that the solution be quiet and vibration-free during the time the wave is recorded, to ensure that the current is diffusion-controlled. Therefore, the nitrogen aeration should be stopped and the gas be directed to flow over the surface of the solution before a polarogram is recorded.

In alkaline media, sodium bisulfite may be added to remove oxygen, provided the reagent does not react with other components of the system.

Measurement of Wave Height— To use a polarogram quantitatively, it is necessary to measure the height of the wave. Since this is a measure of the magnitude of the diffusion current, it is measured vertically. To compensate for the residual current, the segment of the curve preceding the wave is extrapolated beyond the rise in the wave. For a well-formed wave where this extrapolation parallels the limiting current plateau, the measurement is unambiguous. For less well-defined waves, the following procedure may be used unless otherwise directed in the individual monograph. Both the residual current and the limiting current are extrapolated with straight lines, as shown by the graph (Figure 1). The wave height is taken as the vertical distance between these lines measured at the half-wave potential.

Procedure— [Caution—Mercury vapor is poisonous, and metallic mercury has a significant vapor pressure at room temperature. The work area in which mercury is used should be constructed in such a way that any spilled or spattered droplets of mercury can be completely recovered with relative ease. Scrupulously clean up mercury after each use of the instrument. Work in a well-ventilated laboratory, taking care to clean up any spilled mercury. ] Transfer a portion of the final dilution of the substance being assayed to a suitable polarographic cell immersed in a water bath regulated to  $25 \pm 0.5^{\circ}$ . Pass a stream of nitrogen through the solution for 10 to 15 minutes to remove dissolved oxygen. Start the mercury dropping from the capillary, insert the capillary into the test solution, and adjust the height of the mercury reservoir. Switch the flow of nitrogen to pass over the surface of the solution, and record the polarogram over the potential range indicated in the individual monograph, using the appropriate recorder or galvanometer sensitivity to give a suitable wave. Measure the height of the wave, and unless otherwise directed in the monograph, compare this with the wave height obtained with the appropriate USP Reference Standard, measured under the same conditions.

Pulse Polarography— In conventional dc polarography, the current is measured continuously as potential is applied as a linear ramp (see Figure 2).

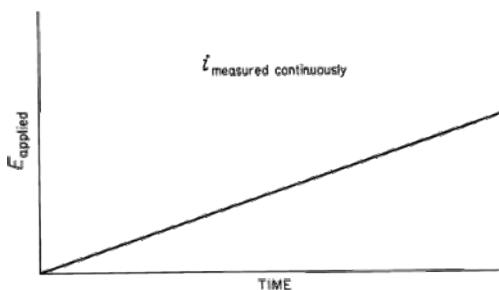


Fig. 2. Direct Current (dc) Polarography.

This current is composed of two elements. The first, the diffusion (faradaic) current, is produced by the substance undergoing reduction or oxidation at the working electrode, and is directly proportional to the concentration of this substance. The second element is the capacitative current (charging of the electrochemical double layer). The changes in these currents as the mercury drop varies in size produce the oscillations present in typical dc polarograms.

In normal pulse polarography, a potential pulse is applied to the mercury electrode near the end of the drop life, with the drop being held at the initial potential during growth period (see [Figure 3](#)).

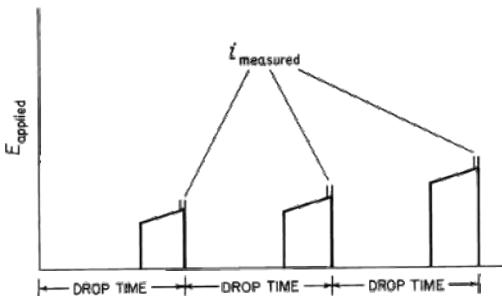


Fig. 3. Pulse Polarography.

Each succeeding drop has a slightly higher pulse applied to it, with the rate of increase being determined by the selected scan rate. The current is measured at the end of the pulse where the capacitative current is nearly zero, and thus primarily faradaic current is measured (see [Figure 4](#)).

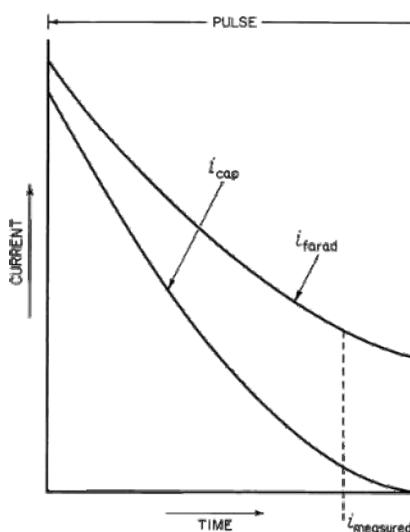


Fig. 4. Plot of Current Versus Time in Pulse Polarography.

In addition, since the pulse is applied for only a short duration, the diffusion layer is not depleted as extensively as in dc polarography and larger current levels are obtained for equivalent concentrations. Concentrations as low as  $10^{-6}$  M can be measured, providing approximately a ten-fold increase in sensitivity over that with dc polarography. Limiting current values are more easily measured, since the waves are free from oscillations.

Differential pulse polarography is a technique whereby a fixed-height pulse applied at the end of the life of each drop is superimposed on a linear increasing dc ramp (see [Figure 5](#)).

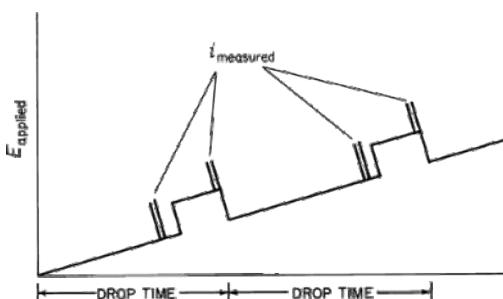


Fig. 5. Differential Pulse Polarography.

Current flow is measured just before application of the pulse and again at the end of the pulse. The difference between these two currents is measured and presented to the recorder.

Such a differential signal provides a curve approximating the derivative of the polarographic wave, and gives a peak presentation. The peak potential is equivalent to:

$$E_1 / 2 - \Delta E / 2$$

where  $\Delta E$  is the pulse height. The peak height is directly proportional to concentration at constant scan rates and constant pulse heights. This technique is especially sensitive (levels of  $10^{-7}M$  may be determined) and affords improved resolution between closely spaced waves.

**Anodic Stripping Voltammetry**— Anodic stripping voltammetry is an electrochemical technique whereby trace amounts of substances in solution are concentrated (reduced) onto an electrode and then stripped (oxidized) back into solution by scanning the applied voltage anodically. The measurement of the current flow as a function of this voltage and scanning rate provides qualitative and quantitative information on such substances. The concentration step permits analyses at  $10^{-7}M$  to  $10^{-9}M$  levels.

Basic instrumentation includes a voltage ramp generator; current-measuring circuitry; a cell with working, reference, and counter electrodes; and a recorder or other read-out device. Instruments having dc or pulse-polarographic capabilities are generally quite adequate for stripping application. The working electrode commonly used is the hanging mercury drop electrode (HMDE), although the mercury thin-film electrode (MTFE) has acquired acceptance. For analysis of metals such as silver, platinum, and gold, whose oxidation potentials are more positive than mercury, and mercury itself, the use of solid electrodes such as platinum, gold, or carbon is required. A saturated calomel electrode or a silver–silver chloride electrode serves as the reference except for the analysis of mercury or silver. A platinum wire is commonly employed as the counter electrode.

Test specimens containing suitable electrolyte are pipeted into the cell. Dissolved oxygen is removed by bubbling nitrogen through the cell for 5 to 10 minutes.

Generally, an electrolysis potential equivalent to 200 to 300 mV more negative than the half-wave potential of the material to be analyzed is applied (although this potential is to be determined experimentally), with stirring for 1 to 10 minutes. For reproducible results, maintain constant conditions (i.e., deposition time, stirring rate, temperature, specimen volume, and drop size if HMDE is used).

After deposition, the stirring is discontinued and the solution and electrode are allowed to equilibrate for a short period. The potential is then rapidly scanned anodically (10 mV/second or greater in dc polarography and 5 mV/second in differential pulse polarography). As in polarography, the limiting current is proportional to concentration of the species (wave height in dc and pulse; peak height in differential pulse), while the half-wave potential (dc, pulse) or peak potential (differential pulse) identifies the species. It is imperative that the choice of supporting electrolyte be made carefully in order to obtain satisfactory behavior. Quantitation is usually achieved by a standard addition or calibration method.

This technique is appropriate for trace-metal analysis, but has limited use in organic determinations, since many of these reactions are irreversible. In analyzing substances such as chloride, cathodic stripping voltammetry may be used. The technique is the same as anodic stripping voltammetry, except that the substance is deposited anodically and then stripped by a cathodic voltage scan.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Antonio Hernandez-Cardoso, B.S.</a> Scientist, Latin American Specialist 1-301-816-8308	(GC05) General Chapters 05

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#### 811 POWDER FINENESS

Test procedures for sieving powder materials are described under [Particle Size Distribution Estimation by Analytical Sieving](#) (786), and, where practical, the particle size distribution should be estimated by this procedure. The classification of powder fineness in this Pharmacopeia, expressed in descriptive terms, is provided in the table. For practical reasons, sieves are the preferred means of measuring powder fineness for most pharmaceutical purposes. Sieving is most suitable where a majority of the particles are larger than about 75  $\mu\text{m}$ , although it can be used for some powders having smaller particle sizes where the method can be validated. Avoid processing conditions that would alter the true particle size distribution of the powder being tested.

**Sieves for Pharmacopeial Testing**— Sieves for Pharmacopeial testing are described under [Particle Size Distribution Estimation by Analytical Sieving](#) (786).

**Powdered Vegetable and Animal Drugs**— In determining the powder fineness of a vegetable or animal drug, no portion of the drug may be rejected during milling or sifting unless specifically permitted in the individual monograph.

**Air Permeation Method for Determining Fineness of Sub-sieve Size Particles**— The average particle size measured is in the range of 0.2 to 50  $\mu\text{m}$ . The test specimen is loaded into a precision bore tube and is compacted between two paper disks and porous plugs by a rack-and-pinion packing plunger. The determination of the particle size of the specimen in the uniformly packed column is based on its resistance to the flow of a closely regulated current of dried air. The liquid level of a flowmeter-manometer corresponds directly to particle size. Special handling instructions and procedures are provided in the individual monographs.

**Classification of Powder Fineness**— Powder fineness may be classified by determining the smallest sieve opening through which a specified quantity of material passes. Results are typically reported as the following:

- d90 = smallest sieve opening through which 90% or more of the material passes
- d50 = smallest sieve opening through which 50% or more of the material passes
- d10 = smallest sieve opening through which 10% or more of the material passes.



The upper and lower limit of the sieve opening values may be reported when results of two or more test lots are combined, e.g., "Lot A has a d50 value of 1000  $\mu\text{m}$  with a range 850–1180  $\mu\text{m}$ ."

An alternative but less informative method of classifying powder fineness is by use of the terms in the following table.

Classification of Powders by Fineness	
Classification of Powder	d50 Sieve Opening ( $\mu\text{m}$ )
Very Coarse	> 1000
Coarse	355–1000
Moderately Fine	180–355
Fine	125–180
Very Fine	90–125

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Robert H. Lafaver, B.A.</a> Scientist 1-301-816-8335	(EGC05) Excipient General Chapters

USP32–NF27 Page 357  
Pharmacopeial Forum: Volume No. 28(2) Page 611

### 821 RADIOACTIVITY

Radioactive pharmaceuticals require specialized techniques in their handling and testing in order that correct results may be obtained and hazards to personnel be minimized. All operations should be carried out or supervised by personnel having had expert training in handling radioactive materials.

The facilities for the production, use, and storage of radioactive pharmaceuticals are generally subject to licensing by the federal Nuclear Regulatory Commission, although in certain cases this authority has been delegated to state agencies. The federal Department of Transportation regulates the conditions of shipment of radioactive materials. State and local agencies often have additional special regulations. Each producer or user must be thoroughly cognizant of the applicable regulations of the federal Food, Drug, and Cosmetic Act, and any additional requirements of the U. S. Public Health Service and of state and local agencies pertaining to the articles concerned.

Definitions, special considerations, and procedures with respect to the Pharmacopeial monographs on radioactive drugs are set forth in this chapter.

#### GENERAL CONSIDERATIONS

##### Fundamental Decay Law

The decay of a radioactive source is described by the equation:

$$N_t = N_0 e^{-\lambda t}$$

in which  $N_t$  is the number of atoms of a radioactive substance at elapsed time  $t$ ,  $N_0$  is the number of those atoms when  $t = 0$ , and  $\lambda$  is the transformation or decay constant, which has a characteristic value for each radionuclide. The half-life,  $T_{1/2}$ , is the time interval required for a given activity of a radionuclide to decay to one-half of its initial value, and is related to the decay constant by the equation:

$$T_{1/2} = \frac{0.69315}{\lambda}$$

The activity of a radioactive source ( $A$ ) is related to the number of radioactive atoms present by the equation:

$$A = \lambda N$$

from which the number of radioactive atoms at time  $t$  can be computed, and hence the mass of the radioactive material can be determined.

The activity of a pure radioactive substance as a function of time can be obtained from the exponential equation or from decay tables, or by graphical means based on the half-life (see [Normalized Decay Chart, Figure 1](#)).

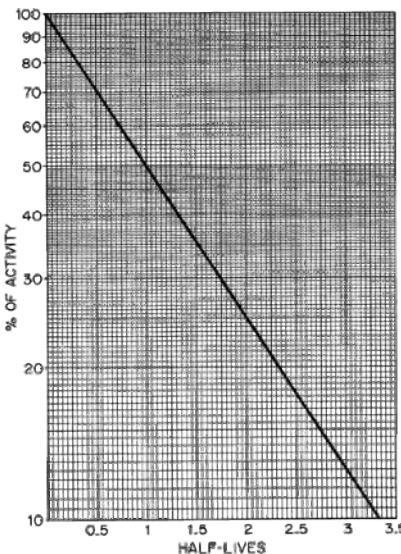


Fig. 1. Normalized Decay Chart.

The activity of a radioactive material is expressed as the number of nuclear transformations per unit time. The fundamental unit of radioactivity, the curie (Ci), is defined as  $3.700 \times 10^{10}$  nuclear transformations per second. The millicurie (mCi) and microcurie ( $\mu$ Ci) are commonly used subunits. The "number of nuclear transformations per unit time" is the sum of rates of decay from all competing modes of disintegration of the parent nuclide. Before the activity of any given radionuclide in a measured specimen can be expressed in curies, it is often necessary to know the abundance(s) of the emitted radiation(s) measured.

#### Geometry

The validity of relative calibration and measurement of radionuclides is dependent upon the reproducibility of the relationship of the source to the detector and its surroundings. Appropriate allowance must be made for source configuration.

#### Background

Cosmic rays, radioactivity present in the detector and shielding materials, and radiation from nearby radioactive sources not properly shielded from the measuring equipment, all contribute to the background count rate. All radioactivity measurements must be corrected by subtracting the background count rate from the gross count rate in the test specimen.

#### Statistics of Counting

Since the process of radioactive decay is a random phenomenon, the events being counted form a random sequence in time. Therefore, counting for any finite time can yield only an estimate of the true counting rate. The precision of this estimate, being subject to statistical fluctuations, is dependent upon the number of counts accumulated in a given measurement and can be expressed in terms of the standard deviation  $\sigma$ . An estimate for  $\sigma$  is

$$\sqrt{n}$$

where  $n$  is the number of counts accumulated in a given measurement. The probability of a single measurement falling within

$$\pm 100/\sqrt{n}\%$$

of the mean of a great many measurements is 0.68. That is, if many measurements of  $n$  counts each were to be made, approximately two-thirds of the observations would lie within

$$\pm 100/\sqrt{n}\%$$



of the mean, and the remainder outside.

Because of the statistical nature of radioactive decay, repeated counting of an undisturbed source in a counting assembly will yield count-rate values in accordance with the frequency of a normal distribution. Deviations in these values from the normal distribution conform to the  $\chi^2$  test. For this reason, the  $\chi^2$  test is frequently applied to determine the performance and correct operation of a counting assembly. In the selection of instruments and conditions for assay of radioactive sources, the figure of merit  $E^2/B$  should be maximized (where  $E$  = counter efficiency = observed count rate/sample disintegration rate, and  $B$  = background count rate).

#### Counting Losses

The minimum time interval that is required for the counter to resolve two consecutive signal pulses is known as the dead time. The dead time varies typically from the order of microseconds for proportional and scintillation counters, to hundreds of microseconds for Geiger-Müller counters. Nuclear events occurring within the dead time of the counter will not be registered. To obtain the corrected count rate,  $R$ , from the observed count rate,  $r$ , it is necessary to use the formula:

$$R = \frac{r}{1 - r\tau}$$

in which  $\tau$  is the dead time. The foregoing correction formula assumes a nonextendable dead time. Thus, for general validity, the value of  $r\tau$  should not exceed 0.1. The observed count rate,  $r$ , refers to the gross specimen count rate and is not to be corrected for background before use in the foregoing equation.

#### Calibration Standards

Perform all radioactivity assays using measurement systems calibrated with appropriately certified radioactivity standards. Such calibration standards may be purchased either direct from the National Institute of Standards and Technology or from other sources that have established traceability to the National Institute of Standards and Technology through participation in a program of inter-comparative measurements. Where such calibration standards are unavailable, the Pharmacopelia provides the nuclear decay data required for calibration. These data, as well as half-life values, are obtained from the Evaluated Nuclear Structure Data File of the Oak Ridge Nuclear Data Project, and reflect the most recent values at the time of publication.

#### Carrier

The total mass of radioactive atoms or molecules in any given radioactive source is directly proportional to the activity of the radionuclide for a given half-life, and the amount present in radiopharmaceuticals is usually too small to be measured by ordinary chemical or physical methods. For example, the mass of  $^{131}\text{I}$  having an activity of 100 mCi is  $8 \times 10^{-7}$  g. Since such small amounts of material behave chemically in an anomalous manner, carriers in the form of nonradioactive isotopes of the same radionuclide may be added during processing to permit ready handling. In many cases, adsorption can be prevented merely by increasing the hydrogen-ion concentration of the solution. Amounts of such material, however, must be sufficiently small that undesirable physiological effects are not produced. The term "carrier-free" refers only to radioactive preparations in which nonradioactive isotopes of the radionuclide are absent. This implies that radioactive pharmaceuticals produced by means of  $(n, \gamma)$  reactions cannot be considered carrier-free.

The activity per unit volume or weight of a medium or vehicle containing a radionuclide either in the carrier-free state or in the presence of carrier is referred to as the radioactive concentration, whereas the term specific activity is used to express the activity of a radionuclide per gram of its element.

#### Radiochemical Purity

Radiochemical purity of a radiopharmaceutical preparation refers to the fraction of the stated radionuclide present in the stated chemical form. Radiochemical impurities in radiopharmaceuticals may result from decomposition and from improper preparative procedures. Radiation causes decomposition of water, a main ingredient of most radiopharmaceuticals, leading to the production of reactive hydrogen atoms and hydroxyl radicals, hydrated electrons, hydrogen, hydrogen ions, and hydrogen peroxide. The last-mentioned is formed in the presence of oxygen radicals, originating from the radiolytic decomposition of dissolved oxygen. Many radiopharmaceuticals show improved stability if oxygen is excluded. Radiation may also affect the radiopharmaceutical itself, giving rise to ions, radicals, and excited states. These species may combine with one another and/or with the active species formed from water. Radiation decomposition may be minimized by the use of chemical agents that act as electron or radical scavengers. Electrons trapped in solids cause discoloration due to formation of F-centers and the darkening of glass containers for radiopharmaceuticals, a situation that typifies the case. The radiochemical purity of radiopharmaceuticals is determined by column, paper, and thin-layer chromatography or other suitable analytical separation techniques as specified in the individual monograph.

#### Radionuclidic Purity

Radionuclidic purity of a radiopharmaceutical preparation refers to the proportion of radioactivity due to the desired radionuclide in the total radioactivity measured. Radionuclidic purity is important in the estimation of the radiation dose received by the patient when the preparation is administered. Radionuclidic impurities may arise from impurities in the target materials, differences in the values of various competing production cross-sections, and excitation functions at the energy or energies of the bombarding particles during production.

#### Terms and Definitions

The date of manufacture is the date on which the manufacturing cycle for the finished product is completed.

The date of assay is the date (and time, if appropriate) when the actual assay for radioactivity is performed.

The date of calibration is an arbitrary assigned date and time to which the radioactivity of the product is calculated for the convenience of the user.

The expiration date is the date that establishes a limit for the use of the product. The expiration period (i.e., the period of time between the date of manufacture and the expiration date) is based on a knowledge of the radioactive properties of the product and the results of stability studies on the finished dosage form.

#### Labeling

Individual radiopharmaceutical monographs indicate the expiration date, the calibration date, and the statement, "Caution—Radioactive Material." The labeling indicates that in making dosage calculations, correction is to be made for radioactive decay, and also indicates the radioactive half-life of the radionuclide. Articles that are Injections comply with the requirements for Labeling under [Injections \(1\)](#), and those that are Biologics comply with the requirements for Labeling under [Biologics \(104\)](#).

#### IDENTIFICATION AND ASSAY OF RADIONUCLIDES

##### Instrumentation

##### ionization chambers

An ionization chamber is an instrument in which an electric field is applied across a volume of gas for the purpose of collecting ions produced by a radiation field. The positive ions and negative electrons drift along the lines of force of the electric field, and are collected on electrodes, producing an ionization current. In a properly designed well-type ionization chamber, the ionization current should not be too dependent on the position of the radioactive specimen, and the value of the current per unit activity, known as the calibration factor, is characteristic of each gamma-ray-emitting radionuclide.

The ionization current produced in an ionization chamber is related to the mean energy of the emitted radiation and is proportional to the intensity of the radiation. If standard sources of known disintegration rates are used for efficiency calibration, the ionization chamber may then be used for activity determinations between several microcuries and several hundred



...licures or more. The upper limit of activity that may be measured in an ionization chamber usually is not sharply defined and may be limited by saturation considerations, range of the amplifier, and design of the chamber itself. The data supplied with or obtained from a particular instrument should be reviewed to ascertain the useful ranges of energies and intensities of the device.

Reproducibility within approximately 5% or less can be readily obtained in about 10 seconds, with a deep re-entrant well-type chamber. The most commonly used form of ionization chamber for measurement of the activities of radiopharmaceuticals is known as a dose calibrator.

Although the calibration factor for a radionuclide may be interpolated from an ionization chamber energy-response curve, there are a number of sources of error possible in such a procedure. It is therefore recommended that all ionization chamber calibrations be performed with the use of authentic reference sources of the individual radionuclides, as described hereinafter.

The calibration of a dose calibrator should be maintained by relating the measured response of a standard to that of a long-lived performance standard, such as radium 226 in equilibrium with its daughters. The instrument must be checked daily with the 226Ra or other source to ascertain the stability over a long period of time. This check should include performance standard readings at all radionuclide settings employed. To obtain the activity ( $A_x$ ) of the radionuclide being measured, use the relationship:

$$A_x = \frac{R_x R}{R_n}$$

in which  $R_n$  is the new reading for the radium or other source,  $R_c$  is the reading for the same source obtained during the initial calibration procedure, and  $R$  is the observed reading for the radionuclide specimen. Obviously, any necessary corrections for radioactive decay of the reference source must first be applied. Use of this procedure should minimize any effects due to drift in the response of the instrument. The recommended activity of the 226Ra or other monitor used in the procedure described above is 75 to 150  $\mu\text{Ci}$ . It is recommended also that the reproducibility and/or stability of multirange instruments be checked for all ranges with the use of appropriate standards.

The size and shape of a radioactive source may affect the response of a dose calibrator, and it is often necessary to apply a small correction when measuring a bulky specimen.

#### scintillation and semiconductor detectors

When all or part of the energy of beta or gamma radiation is dissipated within scintillators, photons of intensity proportional to the amount of dissipated energy are produced. These pulses are detected by an electron multiplier phototube and converted to electrical pulses, which are subsequently analyzed with a pulse-height analyzer to yield a pulse-height spectrum related to the energy spectrum of the radiation emitted by the source. In general, a beta-particle scintillation pulse-height spectrum approximates the true beta-energy spectrum, provided that the beta-particle source is prepared in such a manner that self-absorption is minimized. Beta-ray spectra may be obtained by using calcium fluoride or anthracene as the scintillator, whereas gamma-ray spectra are usually obtained with a thallium-activated sodium iodide crystal or a large-volume lithium-drifted germanium semiconductor detector. The spectra of charged particles also may be obtained using silicon semiconductor detectors and/or gas proportional counters. Semiconductor detectors are in essence solid-state ionization chambers, but the energy required to create an electron-hole pair or to promote an electron from the valence band to the conduction band in the semiconductor is about one-tenth the energy required for creation of an ion-pair in a gas-filled ionization chamber or proportional counter and is far less than the energy needed to produce a photon in a NaI(Tl) scintillation crystal. In gamma-ray spectrometry, a Ge(Li) detector can yield an energy resolution of 0.33% for 1.33 MeV gamma-rays from  $^{60}\text{Co}$ , while a 3- x 3-inch NaI(Tl) crystal can give a value of 5.9% for the same gamma-ray energy. The energy resolution is a measure of the ability to distinguish the presence of two gamma rays closely spaced in energy and is defined by convention as the full width of the photopeak at its half maximum (FWHM), expressed in percentage of the photopeak energy.

Gamma-ray spectra exhibit one or more sharp, characteristic photopeaks, or full-energy peaks, as a result of total absorption in the detector of the full energy of gamma radiations from the source; these photopeaks are useful for identification purposes. Other secondary peaks are observed as a consequence of backscatter, annihilation radiation, coincidence summing, fluorescent X-rays, etc., accompanied by a broad band known as the Compton continuum arising from scattering of the photons in the detector and from surrounding materials. Since the photopeak response varies with gamma-ray energy, calibration of a gamma-ray spectrometer should be achieved with radionuclide standards having well-known gamma-ray energies and emission rates. The shape of the gamma-ray spectrum is dependent upon the shape and size of the detector and the types of shielding materials used.

When confirming the identity of a radionuclide by gamma-ray spectrometry, it is necessary to make a comparison of the specimen spectrum with that of a specimen of known purity of the same radionuclide obtained under identical instrument parameters and specimen geometry. Where the radionuclides emit coincident X- or gamma-radiations, the character of the pulse-height distribution often changes quite dramatically because of the summing effect of these coincident radiations in the detector as the efficiency of detection is increased (e.g., by bringing the source closer to the detector). Such an effect is particularly evident in the case of iodine 125. Among the more useful applications of gamma-ray spectrometry are those for the identification of radionuclides and the determination of radionuclidic impurities.

Where confirmation of the identity of a given radionuclide by means of a direct comparison with the spectrum of a specimen of the same radionuclide of known purity is not possible, the identity of the radionuclide in question must then be established by the following method. Two or more of the following nuclear decay scheme parameters of the radionuclide specimen to be identified shall be measured, and agreement shall be within  $\pm 10\%$ : (1) half-life, (2) energy of each gamma- or X-ray emitted, (3) the abundance of each emission, and (4)  $E_{\text{max}}$  for those radionuclides that decay with beta-particle emissions. Such measurements are to be performed as directed in the Identification and Assay sections of this chapter.

Agreement of two or more of the measured parameters with the corresponding published nuclear decay scheme data constitutes confirmation of the identity of the radionuclide.

#### liquid-scintillation counters

Alpha- and beta-emitting radionuclides may be assayed with the use of a liquid-scintillation detector system. In the liquid scintillator, the radiation energy is ultimately converted into light quanta that are usually detected by two multiplier phototubes so arranged as to count only coincidence radiation. The liquid scintillator is a solution consisting of a solvent, primary and secondary solutes, and additives. The charged particle dissipates its energy in the solvent, and a fraction of this energy is converted into fluorescence in the primary solute. The function of the secondary solute is to shift the fluorescence radiation to longer wavelengths that are more efficiently detected by the multiplier phototubes. Frequently used solvents are toluene and p-xylene; primary solutes are 2,5-diphenyloxazole (PPO) and 2-(4'-tert-butylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole (butyl-PBD); and secondary solutes are 2,2'-p-phenylenebis[4-methyl-5-phenyloxazole] (dimethyl-POPOP) and p-bis(o-methylstyryl)benzene (bis-MSB). As a means of attaining compatibility and miscibility with aqueous specimens to be assayed, many additives, such as surfactants and solubilizing agents, are also incorporated into the scintillator. For an accurate determination of radioactivity of the specimen, care must be exercised to prepare a specimen that is truly homogeneous. The presence of impurities or color in solution causes a decrease in photon output of the scintillator; such a decrease is known as quenching. Accurate radioactivity measurement requires correcting for count-rate loss due to quenching.

The disintegration rate of a beta-particle source may be determined by a procedure in which the integral count rate of the specimen is measured as a function of the pulse-height discriminator bias, and the emission rate is then obtained by extrapolation to zero bias. Energetic alpha-particle emitters may be similarly measured by this method.

#### Identification

A radionuclide can be identified by its mode of decay, its half-life, and the energies of its nuclear emissions.

The radioactive half-life is readily determined by successive counting of a given source of the radionuclide over a period of time that is long compared to its half-life. The response of the counting assembly when employed for the decay measurement of long-lived radionuclides should be monitored with an even longer-lived reference source to assess and

compensate for errors arising from electronic drift. In the case of short-lived radionuclides, when the counting period constitutes a significant fraction of the half-life of the radionuclide, the recorded count rate must be corrected to the time when the count is initiated, as follows:

$$R_t = \frac{r\lambda t}{1 - e^{-\lambda t}}$$

in which  $R_t$  is the count rate at the beginning of a counting period,  $r$  is the count rate observed over the entire counting period,  $t$  is the duration of the counting period,  $\lambda$  is the decay constant of the radionuclide, and  $e$  is the base of the natural logarithm. When  $t$  is small compared to the half-life of the radionuclide under study so that  $\lambda t < 0.05$ , then  $(1 - e^{-\lambda t})$  approaches  $\lambda t$ , and no such correction is necessary.

The energy of nuclear emissions is often determined by the maximum range of penetration of the radiation in matter (in the case of alpha- and beta-particles) and by the full-energy peak or photopeak in the gamma-ray spectrum (in the case of X- and gamma-rays). Since beta-particles are emitted with a continuous energy spectrum, the maximum beta-energy,  $E_{max}$ , is a unique index for each beta-emitting radionuclide. In addition to the maximum range and energy spectrum of the beta-particles, the absorption coefficient, when obtained under reproducible counting conditions, can serve as a reliable index for identification of a beta-emitter. Fortunately, beta-particles are absorbed in matter in an approximately exponential manner, and a plot of the logarithm of the beta-particle count rate as a function of the absorber thickness is known as the absorption curve. The initial portion of the absorption curve shows linearity from which the absorption coefficient can be obtained. The maximum range is determined by the use of absorbers of varying thickness, and the energy spectrum is measured by beta-ray scintillation spectrometry.

The absorption of gamma-rays in matter is strictly exponential, but the half-value layers of attenuation have not been very useful for the purpose of radionuclide characterization. Gamma-rays from each isomeric transition are mono-energetic; their energy can be directly measured by gamma-ray spectrometry. Because of their high energy resolution, solid-state detectors [Ge(Li)] are vastly superior to scintillation detectors [NaI(Tl)] in gamma-ray spectrometry.

The activities of radiopharmaceutical solutions are frequently in the range of millicuries per mL. Such solutions usually must be extensively diluted before they can be accurately assayed. The diluent should be compatible with the radiopharmaceutical with respect to factors such as pH and redox potentials, so that no hydrolysis or change in oxidation state occurs upon dilution, which could lead to adsorption and separation of the radionuclide from solution.

#### beta-emitting radionuclides

Mass Absorption Coefficient Procedure— Deposit and dry an aliquot of the radioactive phosphorus 32 solution on a thin plastic film to minimize backscattering, and place it under a suitable counter. Determine the counting rates successively, using not less than six different "thicknesses" of aluminum each between 20 and 50 mg/cm2 and a single absorber thicker than 800 mg/cm2, which is used to measure the background. (The absorbers are inserted between the test specimen and the counter but are placed nearer the counter window to minimize scattering.) Net beta-particle count rates are obtained after subtraction of the count rate found with the absorber having a thickness of 800 mg/cm2 or greater. Plot the logarithm of the net beta-particle count rate as a function of the total absorber "thickness." The total absorber "thickness" is the "thickness" of the aluminum absorbers plus the "thickness" of the counter window (as stated by the manufacturer) plus the air-equivalent "thickness" (the distance in centimeters of the specimen from the counter window multiplied by

1.205 mg/cm3 at 20° and 76 cm of mercury), all expressed in mg/cm2. An approximately straight line results.

Choose two total absorber "thicknesses" that differ by 20 mg/cm2 or more and that fall on the linear plot, and calculate the mass absorption coefficient,  $\mu$ , by the equation:

$$\mu = \frac{1}{t_2 - t_1} \cdot \ln \left( \frac{N_{t_1}}{N_{t_2}} \right) = \frac{2.303}{t_2 - t_1} (\log N_{t_1} - \log N_{t_2})$$

in which  $t_1$  and  $t_2$  represent the total absorber "thicknesses," in mg/cm2,  $t_2$  being the thicker absorber, and  $N_{t_1}$  and  $N_{t_2}$  being the net beta-particle rates with the  $t_1$  and  $t_2$  absorbers, respectively.

For characterization of the radionuclide, the mass absorption coefficient should be within  $\pm 5\%$  of the value found for a pure specimen of the same radionuclide when determined under identical counting conditions and geometry.

Other Methods of Identification— Other methods for determining the identity of a beta emitter also rely upon the determination of  $E_{max}$ . This may be accomplished in several ways. For example, (1) utilization of the range energy relationships of beta particles in an absorber, or (2) determination of  $E_{max}$  from a beta-particle spectrum obtained on an energy-calibrated beta-spectrometer using a thin source of the radionuclide (see Scintillation and Semiconductor Detectors in this chapter).

#### gamma-emitting radionuclides

The gamma-ray spectrum of a radionuclide is a valuable tool for the qualitative identification of gamma-ray emitting radionuclides. The full-energy peak, or the photopeak, is identified with the gamma-ray transition energy that is given in the decay scheme of the radionuclide.

In determining radionuclidic identity and purity, the gamma-ray spectrum of a radioactive substance is obtained with either a NaI(Tl) crystal or a semiconductor Ge(Li) detector. The latter has an energy resolution more than an order of magnitude better than the former and is highly preferred for analytical purposes. The spectrum obtained shall be identical in shape to that of a specimen of the pure radionuclide, measured with the same detection system and in the same geometry. The gamma-ray spectrum of the radiopharmaceutical shall contain only photopeaks identifiable with the gamma-ray transition energies found in the decay scheme of the same radionuclide. For low geometrical efficiencies, the areas under the photopeaks, after correction for the measured detector efficiency, shall be proportional to the abundances or emission rates of the respective gamma-rays in the radionuclide.

#### radionuclidic impurities

Because they are extremely toxic, alpha-emitting nuclides must be strictly limited in radiopharmaceutical preparations. Procedures for identifying beta- and gamma-active radionuclides as given in the foregoing text are applicable to the detection of gamma and usually beta contaminants.

The gross alpha-particle activity in radiopharmaceutical preparations can be measured by the use of a windowless proportional counter or a scintillation detector employing a silver-activated zinc-sulfide phosphor or by the techniques of liquid-scintillation counting.

The heavy ionization caused by alpha particles allows the measurement of alpha-emitting radionuclides in the presence of large quantities of beta- and gamma-active nuclides by the use of appropriate techniques for discriminating the amplitudes of signal pulses. In proportional counting, the operating voltage region for counting alpha particles, referred to as the "alpha plateau," is considerably lower than the "beta plateau" for counting beta and gamma radiations. Typical "alpha plateau" and "beta plateau" voltage settings with P-10 counting gas are 900 to 1300 and 1600 to 2000 volts, respectively.

When silver-activated zinc-sulfide phosphor is employed for alpha-particle detection, the alpha particles can be distinguished from other interfering radiation by pulse-height discrimination. Care must be exercised to minimize self-absorption at the source whenever specimens are prepared for alpha-particle counting.

#### Assay

#### beta-emitting radionuclides

Procedure— The disintegration rate (A) of a beta-particle-emitting specimen is obtained by counting a quantitatively deposited aliquot in a fixed geometry according to the formula:



$$A = \frac{R}{\epsilon \times f_r \times f_b \times f_s}$$

in which  $\epsilon$  is the counting efficiency of the counter;  $f_r$  is the correction factor for counter dead time;  $f_b$  is the correction factor for backscatter; and  $f_s$  is the correction factor for self-absorption. The count rate for zero absorber is obtained by extrapolation of the initial linear portion of the absorption curve to zero absorber "thickness," taking into consideration the  $\text{mg/cm}^2$  "thickness" of specimen coverings, counter window, and the intervening air space between specimen and the counter window. The counter efficiency,  $\epsilon$ , is determined by use of a long-lived secondary standard with similar spectral characteristics. RaD + E has frequently been used for efficiency calibration of counters for phosphorus 32. By the use of identical measurement conditions for the specimen and the standard (and extrapolation to zero absorber), the ratio of the values of  $f_r$ ,  $f_b$ , and  $f_s$  for the standard and the specimen approaches unity.

The previous relationship is valid also when the counter has been calibrated with a standard of the radionuclide to be assayed. In this case, however, the extrapolations to zero absorber "thickness" for the specimen and standard are not required, as the two absorption corrections cancel for a given geometry.

Another useful and frequently employed method for the determination of the disintegration rate of beta-emitting radionuclides is liquid-scintillation counting, which also utilizes an extrapolation of the specimen count rate to zero pulse-height discriminator bias.

#### gamma-emitting radionuclides

For the assay of gamma-emitting radionuclides, three methods are provided. The selection of the preferred method is dictated by the availability of a calibration standard of the radionuclide to be assayed and the radionuclitic purity of the article itself.

Direct comparison with a calibration standard is required if a calibration standard of the radionuclide to be assayed is available and if the upper limit of conceivable error in the activity determination arising from the presence of radionuclitic impurities has been determined to be less than 3%. If the required calibration standard is not routinely available, as would probably be the case for a short-lived radionuclide, but was available at some time prior to the performance of the assay for determination of efficiency of the counting system for the radionuclide to be assayed, use a calibrated counting system, provided the radionuclitic impurity content of the specimen meets the requirements stated for the direct comparison method. If the requirements for either of the first two methods cannot be met, use the method for determination of activity from a calibration curve.

With the exception of the first method, the counting systems used are monitored for stability. This requirement is met by daily checks with a long-lived performance check source and weekly checks with at least three sources covering a broad range of gamma-ray emission energies (e.g., 57Co, 137Cs, and 60Co). If a discrepancy for any of the aforementioned measurements is found, either completely recalibrate or repair and recalibrate the system prior to further use.

**Assay by Direct Comparison with a Calibration Standard**— An energy selective measurement system (e.g., pulse-height analyzer) is not required for this procedure. Use either an ionization chamber or an integral counting system with a NaI(Tl) detector. A consistently reproducible geometrical factor from specimen to specimen is essential for accurate results.

With proper precautions, the accuracy of this method approaches the accuracy with which the disintegration rate of the calibration standard is known.

Determine the counting rate of the detector system for a calibration standard of the radionuclide to be assayed (e.g., active enough to give good measurement statistics in a reasonable time, but not so active as to cause serious dead-time problems), selecting such a standard as to provide optimum accuracy with the particular assembly used. Place an accurately measured aliquot of the unknown assay specimen (diluted, if necessary) in a container identical to that used for the standard, and measure this specimen at approximately the same time and under the same geometrical conditions as for the standard. If the elapsed time between the measurements of the calibration standard and the specimen exceeds 12 hours, check the stability of the measurement system within 8 hours of the specimen measurement time with a long-lived performance check source. Record the system response with respect to the same check source at the time of calibration, and if subsequent checks exceed the original recorded response by more than  $\pm 3\%$ , recalibration is required. Correct both activity determinations for background, and calculate the activity, in  $\mu\text{Ci}$  per mL, by the formula:

$$SD(g/b)$$

in which  $S$  is the  $\mu\text{Ci}$  strength of the standard,  $D$  is the dilution factor, and  $g$  and  $b$  are the measured values of counting rate for the specimen and the standard, respectively.

**Assay with a Calibrated Integral Counting System**— The procedure and precautions given for the preceding direct-comparison method apply, except that the efficiency of the detector system is determined and recorded for each radionuclide to be assayed, rather than simply recording the counting rate of the standard. Thus, the efficiency for a given radionuclide,  $x$ , is determined by  $\epsilon_x = bx/sx$ , in which  $bx$  is the counting rate, corrected for background and dead-time, for the calibration standard of the radionuclide,  $x$ , and  $sx$  is the corresponding activity of the certified calibration standard in nuclear transformations per second. For subsequent specimen assays, the activity is given by the formula:

$$A_x = Dg_x/\epsilon_x$$

in which  $D$  is the dilution factor,  $g_x$  is the specimen counting rate (corrected for background and dead-time), and  $\epsilon_x$  is the corresponding efficiency for the radionuclide.

**Determination of Activity from a Calibration Curve**— Versatility in absolute gamma-ray intensity measurements can be achieved by employing multi-channel pulse-height analysis. The photopeak efficiency of a detector system can be determined as a function of gamma-ray energy by means of a series of gamma-ray emission rate standard specimens, and the gamma-ray emission rate of any radionuclide for which no standard is available can be determined by interpolation from this efficiency curve. However, exercise care to ensure that the efficiency curve for the detector system is adequately defined over the entire region of interest by using a sufficient number of calibration points along the photopeak-energy axis.

**Selection of a Counting Assembly**— A gamma-ray spectrometer is used for the identification of radionuclides that emit X-rays or gamma rays in their decay. Requirements for an assembly suitable for identification and assay of the radionuclides used in radiopharmaceuticals are that (a) the resolution of the detector based on the 662-keV photopeak of 137Cs-137mBa must be 8.0% or better, (b) the detector must be equipped with a specimen holder designed to facilitate exact duplication of counting geometry, and (c) the pulse-height analyzer must have enough channels to delineate clearly the photopeak being observed.

**Procedure**— Minimal requirements for the maintenance of instrument calibrations shall consist of weekly performance checks with a suitable reference source and a complete recalibration semi-annually. Should the weekly performance check deviate from the value determined at the time of calibration by more than 4.0%, a complete recalibration of the instrument is required at that time.

This method involves three basic steps, namely photopeak integration, determination of the photopeak efficiency curve, and calculation of the activity of the specimen.

**photopeak integration**— The method for the determination of the required photopeak area utilizes a Gaussian approximation for fitting the photopeak. A fixed fraction of the total

number of photopeak counts can be obtained by taking the peak width,  $a$ , at some fraction of the maximum, where the shape has been experimentally found to be very close to Gaussian, and multiplying by the counting rate of the peak channel,  $P$ , after correction for any Compton and background contributions to the peak channel count rate. This background usually can be adequately determined by linear interpolation. This is illustrated in [Figure 2](#).

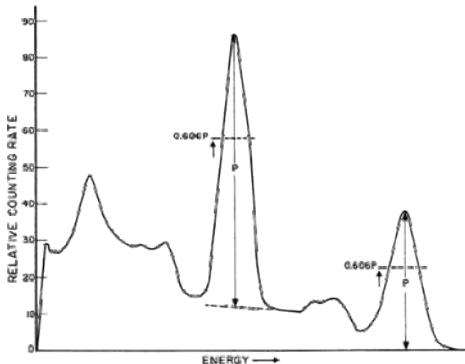


Fig. 2 Typical Gamma-ray Spectrum Showing the Selection of the Peak Channel Counting Rate,  $P$ , after the Correction for Compton and Background Contributions.

The photopeak-curve shape is closest to a straight line at  $0.606P$ , and the contribution of the fractional channels to  $a$  can be accurately estimated by interpolation. Calculate  $a$  by the equation:

$$a = D' - D + \frac{d - 0.606P}{d - c} + \frac{d' - 0.606P}{d' - c'}$$

in which  $c$  and  $d$  and also  $c'$  and  $d'$  are the single channel counting rates on either side of  $0.606P$ , and  $D$  and  $D'$  are the channel numbers (locations) of  $d$  and  $d'$ , respectively. The location of the required variables on the photopeak is illustrated in [Figure 3](#).

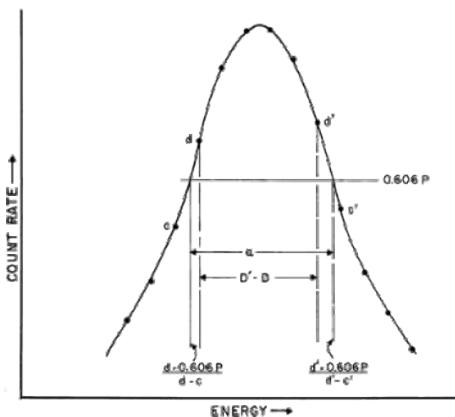


Fig. 3. Location of the Variables Required for the Determination of the Peak Width,  $a$ , at  $0.606P$ .

From the known values for the counting rate in the peak channel of the photopeak,  $P$ , and the width of the peak at  $0.606P$ ,  $a$ , a calibrated fraction of the photopeak area is then obtained from the product,  $(aP)$ .

To summarize the procedures involved in obtaining a calibrated fraction of a photopeak area using this method, the necessary steps or calculations are presented below in a stepwise manner:

(1) Subtract any Compton and background contributions from the photopeak to be measured.

(2) Determine the counting rate of the peak channel (maximum channel counting rate after subtracting Compton and background),  $P$ .

(3) Multiply  $P$  by 0.606, and locate the horizontal line corresponding to the peak width,  $a$ .

(4) Obtain the peak width,  $a$ , by inserting the values of variables (obtained as shown in the preceding figure) into the equation defining  $a$ .

(5) The desired calibrated fraction of the peak area is then equal to the product of  $a$  times  $P$  or  $F = aP$ , where  $F$  is a fractional area of the peak proportional to the emission rate of the source.

This method provides a quick and accurate means of determining the gamma-ray emission rate of sources while avoiding, to a large extent, subjective estimates of the detailed shape of the tails of the peaks. The error due to using the maximum channel counting rate, rather than the theoretical maximum or peak channel rate, is of the order of 1.0% if  $a$  is 6 or greater.

photopeak efficiency calibration— Radionuclides such as those listed in the accompanying table together with some of their nuclear decay data are available as certified reference standards.<sup>2</sup> A sufficient number of radioactive standard reference sources should be selected in order to obtain the calibration curve over the desired range. Where possible, standard sources of those radionuclides that are to be assayed should be included.

Nuclear Properties of Selected Calibration Standards<sup>(1,2)</sup>

Principal Photon Emissions	Energy (keV)	Photons per 100 Disintegrations
133Ba (T1/2 = 10.5 years)		
K <sup>41</sup>	30.97	63.4
K <sup>42</sup>	30.62	34.2
K <sup>40</sup>	35.0	22.8



$\gamma_1$	53.15	2.14
$\gamma_2$	79.62	2.55
$\gamma_3$	80.99	33.0
$\gamma_6$	276.39	6.9
$\gamma_7$	302.83	17.8
$\gamma_8$	356.0	60.0
$\gamma_9$	383.85	8.7
137Cs = 137mBa (T1/2 = 30.17 years)		
K $\alpha_1$	32.19	3.82
K $\alpha_2$	31.82	2.07
K $\beta$	36.4	1.39
Weighted Mean <sup>(4)</sup>	(32.9)	(7.28)
$\gamma_1$	661.6	89.98
22Na (T1/2 = 2.60 years)		
h $\nu$	511	179.80 <sup>(5)</sup>
$\gamma_1$	1274.54	99.94
60Co (T1/2 = 5.27 years)		
$\gamma_1$	1173.2 <sup>(6)</sup>	100.0
$\gamma_2$	1332.5 <sup>(6)</sup>	100.0
57Co (T1/2 = 270.9 days)		
$\Sigma X$	7.0	56.0
$\gamma_1$	14.4	9.5
$\gamma_2$	122.06	85.51
$\gamma_3$	136.47	10.60
Weighted Mean	(125.0)	(96.11)
( $\gamma_2 + \gamma_3$ ) <sup>(4)</sup>		
54Mn (T1/2 = 312.7 days)		
$\Sigma X$	6.0	25.0
$\gamma_1$	834.83	99.98
109Cd = 109Ag (T1/2 = 464 days)		
K $\alpha_1$	22.16	35.3
K $\alpha_2$	21.99	18.6
K $\beta$	24.9	11.4
Weighted Mean <sup>(4)</sup>		63.5
$\gamma_1$	88.0	3.72
129I (T1/2 = 1.57 $\times$ 107 years)		
K $\alpha_1$ <sup>(3)</sup>	29.78	37.0
K $\alpha_2$	29.46	20.0
K $\beta$	13.2	37.0
$\gamma_1$	39.58	7.52
Weighted Mean <sup>(4)</sup>	(31.3)	(77.80)

(1) In measurements for gamma- (or X-ray) assay purposes, fluorescent radiation from lead shielding (specifically, lead K X-rays  $\sim$ 76 ke V) may interfere with quantitative results. Allowance must be made for these effects, or the radiation suppressed; a satisfactory means of absorbing this radiation is covering the exposed lead with cadmium sheet 0.06 to 0.08 inch thick, and then covering the cadmium with copper 0.02 to 0.04 inch thick.

(2) Only those photon emissions having an abundance  $\geq 1\%$  are normally included.

(3) The K notation refers to X-ray emissions.

(4) The weighted mean energies and total intensities are given for groups of photons that would not be resolved by a NaI(Tl) detector.

(5) For this photon intensity to be usable, all emitted positrons must be annihilated in the source material.

(6) Cascade.

Calculate the gamma-ray emission rate from the equation:

$$\Gamma = Asb$$

in which As is the activity, in disintegrations per second, of the standard used, and b is the number of gamma rays per disintegration at that energy. Accurately measure quantities of standard solutions of each radionuclide into identical containers, and determine the fractional photopeak area (F) for each of the standards.

Using the equation  $F_p = F/\Gamma$ , calculate the photopeak efficiency,  $F_p$ , and construct a log-log plot of  $F_p$  versus the gamma-ray energy as shown in Figure 4.

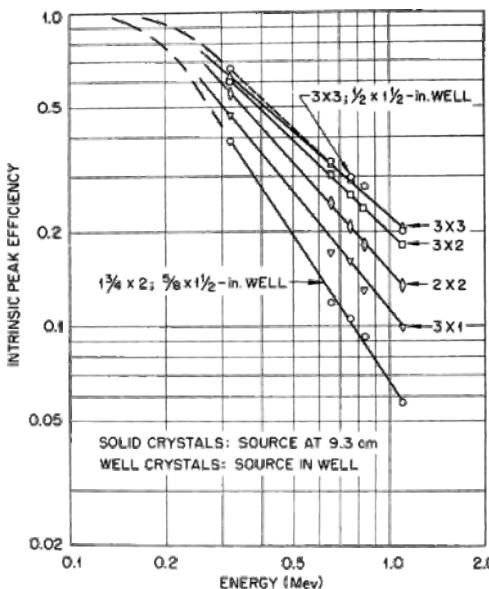


Fig. 4. Typical Photopeak Efficiency Calibration Curves for Various NaI(Tl) Detectors.

determination of specimen activity— In the same manner as in the preparation of the calibration curve, determine the fractional area (F) of the principal photopeak of the specimen under assay or an accurately measured aliquot adjusted to the same volume in an identical container as used for the standards. From the calibration curve, find the value of  $\mathcal{E}p$  for this radionuclide. Using the equation  $\Gamma = F/\mathcal{E}p$ , calculate the gamma-ray emission rate ( $\Gamma$ ). Calculate the activity (A), in disintegrations per second, of the specimen using the equation  $A = (\Gamma/b)(D)$ , in which b is the number of gamma rays per disintegration and D is the dilution factor. To obtain the activity, in  $\mu\text{Ci}$  or  $\text{mCi}$ , divide A by  $3.7 \times 10^4$  or  $3.7 \times 10^7$ , respectively. The above relationship is equally valid for obtaining the activity of an undiluted specimen or capsule; in this case, the dilution factor, D, is unity.

\* These certified reference standards are obtainable from the National Institute of Standards and Technology, Washington, DC 20234.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Ian DeVeau, Ph.D.</a> Director, Veterinary Drugs and Radiopharmaceuticals 1-301-816-8178	(RMI05) Radiopharmaceuticals and Medical Imaging Agents 05

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823 RADIOPHARMACEUTICALS  
FOR POSITRON EMISSION  
TOMOGRAPHY—COMPOUNDING

Physicians frequently prescribe special formulations of noncommercially available drugs for patient care. Upon receipt of a prescription for such a preparation, pharmacists (or other qualified individuals working under the authority and supervision of a physician) compound the drug formulation and dispense it to the patient. For convenience, a limited bulk quantity of the special formulation may be compounded in anticipation of future dispensing requirements. Such medical and pharmacy practices are regulated by state boards of medicine and pharmacy. Physicians who prescribe a drug that must be compounded extemporaneously bear the professional responsibility to base its use on sound scientific and medical evidence.

Pharmacists and physicians who compound (or oversee the compounding of) drug preparations on prescription orders, bear the professional responsibility to ensure that the preparation meets prescribed and appropriate standards of strength, quality, and purity.

Radiopharmaceuticals administered for positron emission tomography (PET) procedures typically incorporate radionuclides that possess very short physical half-lives,  $T_{1/2}$  (e.g.,  $T_{1/2}$  of  $^{18}\text{F} = 109.7$  minutes, of  $^{11}\text{C} = 20.4$  minutes, of  $^{13}\text{N} = 9.96$  minutes, and of  $^{15}\text{O} = 2.03$  minutes). As a result, these radionuclides are usually produced using particle acceleration techniques (e.g., cyclotron) at or within close proximity to the site where the PET procedure will be conducted. The radionuclides may then be synthetically incorporated into the final PET radiopharmaceutical for subsequent patient administration.

The following requirements address the compounding of PET radiopharmaceuticals for human use (see also [Automated Radiochemical Synthesis Apparatus](#) (1015)).

Control of Components, Materials, and Supplies

The following activities are to be established and performed. A designated person shall be responsible for ensuring that these activities are carried out and completed properly.

(1) Establish written specifications for

- the identity, purity, and quality of components (including ingredients, reagents, target solutions, and gases); the identity and quality of containers and closures, and other materials (e.g., transfer lines, purification devices, membrane filters) that come into contact with the final PET radiopharmaceutical; and the identity, purity, and quality of analytical supplies (e.g., solvents, chromatography columns, and reference materials), sterility test media, endotoxin test reagents, and other supplies intended for use in PET radiopharmaceutical quality control procedures; and
- the appropriate storage (i.e., based on heat, light, and humidity considerations) of components, containers and closures, materials and supplies used for the compounding of PET radiopharmaceuticals.

(2) Log-in each lot of shipments of components, containers and closures, materials, and supplies used for the compounding of PET radiopharmaceuticals, and record the date of receipt, quantity received, manufacturer, lot number, and expiration date. If no expiration date is designated by the manufacturer, an expiration date is to be assigned to the component, material, or supply based on knowledge of its physical and chemical properties and prior experience with its use. For organic substrates, reactants, and reagent materials that are potentially susceptible to degradation or to a change in composition, the expiration date is based on the component's documented evidence of stability.

(3) Determine that each batch of components, containers and closures, materials, and supplies used for the compounding of PET radiopharmaceuticals are in compliance with



Established written specifications. A reliable manufacturer is routinely used as the source of a given product. Certification of compliance with the specifications for containers, closures, and materials marketed commercially for the intended purpose(s) may be accomplished by inspection of the product labeling and/or inspection of the certificate of analysis provided by the manufacturer. Certification of compliance with the specifications for other components and materials used in the compounding of PET radiopharmaceuticals may be accomplished by inspection of the certificate of analysis provided by the manufacturer. The identities of each lot of components, containers and closures, and materials used in the compounding of PET radiopharmaceuticals are to be verified by defined procedures, tests, and/or documented certificates of analysis, as appropriate.

(4) Store components, containers and closures, materials, and supplies used for the compounding of PET radiopharmaceuticals in a controlled access area according to established storage conditions.

#### Compounding Procedure Verification

The following activities are to be established or performed. A designated, qualified, and trained person shall be responsible for ensuring that these activities are carried out and properly completed by qualified and trained personnel.

(1) Written acceptance criteria for the identity, purity, and quality of each PET radiopharmaceutical being compounded. If a USP monograph exists for a particular PET, then these standards are the minimum acceptance criteria (see Official and Official Articles under the General Notices and Requirements).

(2) Written and verified procedures for the compounding of each PET radiopharmaceutical that

- incorporate, for each PET radiopharmaceutical intended for parenteral administration, sterile membrane filtration (0.22 µm);
- incorporate, for each PET radiopharmaceutical intended for inhalation, particulate filtration (0.45 µm); and
- are routinely updated and verified as changes in the compounding procedures are implemented or are reviewed and verified at a minimum of once a year to ensure that they are current. A master file of written compounding procedures currently used for each PET radiopharmaceutical is to be maintained within the PET facility. Copies of outdated compounding procedures shall also be retained, separate from the master file, for review purposes.

(3) Appropriate controls over computer and related automated equipment to ensure that changes in compounding software are instituted only by authorized personnel, that such changes are documented and verified, and that only current versions of the software are available and used in PET radiopharmaceutical compounding procedures. A diskette copy and printout of current computer software programs used in the compounding of each PET radiopharmaceutical is to be maintained within a master file located in the PET facility. Copies of outdated computer software programs shall also be retained, separate from the master file, for review purposes.

(4) Verification studies to ensure that the written compounding procedures, computer software program, equipment, and facilities result in a PET radiopharmaceutical that meets established acceptance criteria. Such verification studies must

- include documented evaluations of the radiochemical identity and purity, radionuclidic identity and purity, specific activity, sterility (for parenteral agents), bacterial endotoxins (for parenteral agents), pH, osmolarity (for parenteral agents), if appropriate, appearance, stereochemical purity (for applicable compounds), potential organic volatile impurities, other toxic chemicals that may have been used during the synthesis or purification procedure, effective concentration of a stabilizer (if any), chemical purity of the PET radiopharmaceutical [note—Evaluations for chemical purity must include analyses for the presence of starting materials, known intermediates, by-products, and known degradation products], and equivalency of initial and final sub-batches (for PET radiopharmaceuticals with radionuclides having a  $T\frac{1}{2} < 20.0$  minutes). For purposes of this chapter, "sub-batch" is defined as a quantity of PET drug product having uniform character and quality, within specified limits, that is produced during one succession of multiple irradiations, using a given synthesis and/or purification operation; and
- be signed, and dated, and retained as an indication that the compounding procedures, equipment, and facilities have resulted in a PET radiopharmaceutical that meets established acceptance criteria.

Whenever there is a change in the compounding procedures, computer software program, or component specifications that has the potential to alter the identity, quality, or purity of the drug product, verification procedures and studies must be conducted. Verification studies on a minimum of three consecutive batches, which show that the product meets acceptance criteria, are to be performed prior to the approval, for human use, of new or revised compounding procedures for a given PET radiopharmaceutical. For routine verified processes that are being used with consistent success, a minimum of one verification study that shows the product meets acceptance criteria must be conducted on an annual basis.

#### Stability Testing and Expiration Dating

Written specifications for the expiration dating and storage conditions of each PET radiopharmaceutical are to be established based on the results of stability testing and specific activity considerations. The stability test specimen must be taken from the product stored in the container and closure system specified for storing the product. The PET radiopharmaceutical must meet all acceptance criteria at expiry. Whenever there is a change in the compounding procedures, computer software program, or component specifications that has the potential to affect the stability of the drug product, stability testing must be conducted.

#### PET Radiopharmaceutical Compounding for Human Use

The following are to be performed according to established written procedures and documented. A designated, qualified, and trained person shall be responsible for ensuring that these activities are carried out and completed properly by qualified and trained personnel.

(1) Inspect the compounding and dispensing area and all equipment for cleanliness and suitability immediately before use. Before initiating compounding and dispensing activities, extraneous materials and labels must be removed from involved areas and equipment. For PET radiopharmaceuticals intended for parenteral administration, all manipulations of components, containers and closures, and materials distal to sterile membrane filtration must be performed using an appropriate aseptic technique in an appropriately controlled environment.

(2) Ensure the correct identity, quantity, and suitability of components, containers and closures, and other materials used in compounding the PET radiopharmaceutical.

(3) Label all subdivided components used in the compounding procedure for identity and traceability.

(4) Label the final PET radiopharmaceutical container or dispensing-administration assembly prior to initiating the compounding procedure. The following information must appear on the label or labeling attached to the final container or dispensing-administration assembly: the identity of the PET radiopharmaceutical, and added substances (e.g., stabilizers and preservatives), an assigned batch or lot number, and the required warning (e.g., radioactive) statements or symbols. The final PET radiopharmaceutical shall also be labeled to include the total radioactivity and radioactive concentration at the stated time of calibration, the expiration time and date, and any required or applicable warning statements (e.g., "Caution- Radioactive Material", "Do not use if cloudy or contains particulate matter") and/or the radioactivity symbol.

(5) Compound the PET radiopharmaceutical according to current, verified procedures. A written record must be maintained for each batch (i.e., the material produced during a single synthesis and purification) of the compounded PET radiopharmaceutical. This written record includes

- lot numbers, manufacturer identities, expiration dates, and quantities of all components, containers and closures, and materials used in the compounding procedure;
- a description of the individual compounding procedures to be followed;
- the initials of the responsible individual indicating that the compounding procedure for the batch is an accurate reproduction of the current, verified compounding procedure;
- the initials of the responsible individual indicating that critical steps and processes in the compounding procedure were completed [note—Critical steps in automated compounding processes shall be monitored through direct observation (if possible, considering visual or radiation exposure constraints) or via computer or other feedback mechanisms];
- documentation of the investigation of any unplanned deviations in, or unexpected results of, verified compounding procedures or processes, including documentation of the outcome of the investigation;
- the percent yield calculated on the basis of the known or expected decay-corrected amount of the starting radionuclide that is synthetically incorporated into the final radiopharmaceutical;



- raw analytical data on each batch of compounded PET radiopharmaceutical; and
- the date and the signature of the individual assuming overall responsibility for, and adherence to, the verified compounding procedure.

#### Quality Control

The following are to be performed according to established, written procedures and documented. A designated, qualified, and trained person shall be responsible for ensuring that these activities are carried out and completed properly by qualified and trained personnel.

(1) Establish, in writing, the quality control tests to be performed on individual batches of the PET radiopharmaceutical, the analytical procedures, and the corresponding acceptance criteria.

- For PET radiopharmaceuticals labeled with a nuclide having a  $T_{1/2} \geq 20.0$  minutes, the following quality control procedures are to be performed on each batch (i.e., the material produced during a single synthesis and purification operation) prior to release: measurement of the pH of parenteral and oral dosage forms; visual inspection of parenteral and oral dosage forms; determination of the radiochemical purity and identity of all dosage forms; determination of the radionuclidic identity of all dosage forms; and assessment of the specific activity of PET radiopharmaceuticals with mass-dependent localization or toxicity concerns; and evidence of compliance with the established acceptance criteria for residual solvents and other toxic chemicals used during the synthesis or purification procedures.
- For PET radiopharmaceuticals labeled with a nuclide having a  $T_{1/2} < 20.0$  minutes, a batch is defined as all related sub-batches of the PET radiopharmaceutical compounded during a given day. The following quality control procedures are to be performed on an initial quality control sub-batch of each such PET radiopharmaceutical prior to release for human use of subsequent sub-batches: measurement of the pH of parenteral and oral dosage forms; visual inspection of parenteral and oral dosage forms; determination of the radiochemical purity and identity of all dosage forms; determination of radionuclidic identity of all dosage forms; and assessment of the specific activity of PET radiopharmaceuticals with mass-dependent localization or toxicity concerns; and evidence of compliance with the established acceptance criteria for residual solvents and other toxic chemicals used or produced during the synthesis or purification procedures.
- For each batch of PET radiopharmaceutical intended for parenteral administration, perform a membrane filter integrity test immediately after completion of product filtration. This post-filtration integrity test is to be completed prior to release of the batch for human use, except in the case of 15O water, where it may be necessary to release the batch prior to completion of the post-filtration integrity test. In this case, the test is completed as soon as possible after release of the batch.
- For PET radiopharmaceuticals intended for parenteral administration, perform an in-process 20-minute endotoxin "limit test" (i.e., incorporating positive controls in the range of 5 EU per mL to 175 EUV, where V is the maximum volume of injection) on each batch ( $T_{1/2} \geq 20.0$  minutes) or quality control sub-batch ( $T_{1/2} < 20.0$  minutes) of the radiopharmaceutical prior to release, for human use, of the batch or subsequent sub-batches.
- For PET radiopharmaceuticals intended for parenteral administration, a standard 60-minute bacterial endotoxin test must be performed on each batch ( $T_{1/2} \geq 20.0$  minutes) or quality control sub-batch ( $T_{1/2} < 20.0$  minutes) of the radiopharmaceutical. Endotoxin testing may also be performed using other recognized procedures (see [Bacterial Endotoxins Test](#) (85)). Regardless of which test is utilized, an assessment of the bacterial endotoxins should be performed prior to release of each batch ( $T_{1/2} \geq 20.0$  minutes) or quality control sub-batch ( $T_{1/2} < 20.0$  minutes) of the radiopharmaceutical before release for human use of the batch or subsequent sub-batches.
- Sterility tests for each PET radiopharmaceutical intended for parenteral administration are performed on each batch ( $T_{1/2} \geq 20.0$  minutes) or quality control sub-batch ( $T_{1/2} < 20.0$  minutes). Sterility tests are also performed following the replacement of system components. Sterility tests are initiated within 24 hours of sterile filtration. Product samples are tested individually and are not pooled.

(2) Establish written procedures for the performance of quality control tests on batches of PET radiopharmaceuticals intended for human use.

(3) Conduct verification testing of equipment and procedures used for the quality control testing of PET radiopharmaceuticals. Using internal or external standards, the correct operation of analytical equipment, such as gas chromatography or high-performance liquid chromatography (see System Suitability under [Chromatography](#) (621)) must be confirmed upon initial installation or upon major repair. Correct operation of analytical equipment must also be checked (i.e., a system suitability test must be performed) on a scheduled basis, and maintenance must be performed according to appropriate, written, scheduled procedures. Dose calibrators used in measuring the bulk radioactivity and the radioactivity of dispensed dosages of PET radiopharmaceuticals should be tested in accordance with applicable state regulations governing the medical use of radioactive materials.

(4) Perform quality control tests on batches of PET radiopharmaceuticals according to written procedures, and initial the results of such testing.

(5) Accept or reject the individual batch of the PET radiopharmaceutical based on the conformity of quality control test results with established acceptance criteria. If the individual batch of the PET radiopharmaceutical is acceptable, sign and date the batch.

(6) Investigate unacceptable quality control test results and document the outcome of such investigations.

#### Sterilization and Sterility Assurance

A complete system of process controls is required to assure sterility of PET radiopharmaceuticals. Sterilization activities for the following elements of the process are to be established, documented, and performed.

**Compounding Equipment and Components**— Equipment used to prepare PET radiopharmaceuticals must be properly cleaned and kept in sanitary condition. Equipment in contact with a PET drug solution may be processed to remove endotoxin and may be sterilized to eliminate bioburden. Prepared equipment is stored and protected to maintain cleanliness and, if necessary, sterility. It is recommended that components for PET products be obtained from qualified suppliers after verifying that the components meet specifications for sterile drug products. It is further recommended that sterile vials, syringes, transfer sets, and filters be obtained from commercial sources. If components are sterilized by the PET facility, the sterilization processes and asepsis of assembly components must be verified. Verification of sterilizer performance must be repeated periodically. Solutions for parenteral administration must be filter-sterilized and aseptically transferred to a sterile, nonpyrogenic, multiple-dose vial. Certain finished dosage forms of PET products may not be transferred to a vial and require special consideration.

**Environmental Controls**— The work area used for compounding the finished dosage form must be clean. The aseptic hood is protected from sources of microbial contamination and is located in an area where surrounding personnel traffic is controlled and limited. Appropriate clean laboratory clothing shall be worn when performing functions in the aseptic hood. Components, materials, and equipment are transferred to the aseptic area in protective wrapping or containers. Aseptic techniques are used whenever a sterile solution dosage form is handled. The containers, filter assembly, vent filters, and needles for the final dosage form must be sterile, disposable, and for single use only. After the filter and the product container are assembled, the sterility of this assembly must be preserved. If the sterility of any component is compromised, the component or set must be replaced. Before penetrating the finished product container, the septum of the product vial must be thoroughly swabbed with a disinfectant solution (i.e., freshly filtered or certified sterile 70% alcohol) and allowed to air-dry in the aseptic hood.

**Aseptic Hood**— Assemble the product filter and the container and closure system for the finished product in an aseptic hood. Sterile (aseptic) operations should be conducted within an aseptic workstation with an air cleanliness rating of class 100 (e.g., Laminar flow hood or isolator). The aseptic hood surfaces and equipment surfaces allow easy cleaning and disinfecting. Disinfectants are filtered or certified sterile with a manufacturer's certificate of analysis, and the hood's internal surfaces are cleaned and disinfected daily before use and after new equipment is brought in. Microbiological testing of the aseptic hood is performed periodically (e.g., weekly). This may be done by swab or contact plate for surfaces and settle plate or dynamic air sampler. Airborne, nonviable particle counting may be performed less frequently.

**Aseptic Technique**— All aseptic operations, including the assembly of sterile components, compounding, filtration, and manipulations of sterile solutions must be performed by operators qualified to work with aseptic techniques. Aseptic manipulations shall be performed using sterile items sealed in protective covering and opened within the aseptic hood. Any sterile equipment or component that is compromised by contact with a nonsterile surface must be replaced. Sterile components shall be transferred from the hood with closures in place. Aseptic operations are performed by operators wearing laboratory clothing appropriate for pharmaceutical compounding. Gloved hands are disinfected immediately before reaching into the aseptic hood.

Aseptic area operators are trained and evaluated periodically through observation as well as through microbiological tests. Aseptic techniques used to make sterile products are evaluated by simulations, in which a microbiological growth medium is substituted for the PET radiopharmaceutical solution. Process simulations include manipulations such as

connecting vents and filtration. Verification of the medium's growth promotion capability in the PET drug container is an essential control for process simulations. After completing the simulation process, the final product container is gently shaken to permit the medium to contact all surfaces, and the container and the medium are incubated (at 30° to 35°, 20° to 25°, or another suitable temperature) for 14 days with periodic examination for evidence of growth: the absence of growth in the containers is necessary for an acceptable test result. Simulations are performed in triplicate to qualify a new operator. Each operator repeats one simulation about once a year or any time procedures are changed.

**Qualification of the Filtration Process—** Sterilizing filtration is the final safeguard in removing microorganisms from solutions of PET radiopharmaceuticals. This critical procedure requires that microbial retention by membrane filters be demonstrated under specified conditions. Filters must not release particles or soluble compounds, bind product ingredients, or lose integrity during use. When the filters are prepared and sterilized by a commercial filter manufacturer, the filter manufacturer generally provides filtration conditions (i.e., pressure and flow rate); these conditions are not to be exceeded when preparing a PET drug product. For most aqueous solutions of near neutral pH, certification regarding microbial retention challenges to the selected filter may be obtained from the filter manufacturer. Certification of conformance to specifications must be examined and maintained for each filter lot.

Before using filters from a particular lot, a sample is tested for integrity to demonstrate that the membrane and housing have not lost the ability to retain microorganisms. The manufacturer's recommended method or an alternative method, if demonstrated to be acceptable, may be used.

The sterilizing membrane filter must also be tested for integrity after filtering the compounded PET radiopharmaceutical but before the product is released. An example of a simple test is the "Bubble Point" test, which uses a pressure gauge and a source of air pressure connected to the transfer set attached to the filter. The filter disk is placed in a beaker of water with the filter outlet below the water surface, and air pressure is applied gently to the nonsterile side of the filter assembly. The air pressure is increased until the validated bubble point is reached, at which point the pressure is maintained briefly to allow equilibration. Filter integrity is demonstrated to be acceptable in the absence of a steady stream of bubbles.

**Microbiological Testing of Finished Products—** PET radiopharmaceuticals for parenteral administration must be sterile and free of endotoxins, as demonstrated by sterility and endotoxin tests. Endotoxin tests are initiated promptly after compounding, and sterility tests are started no later than 24 hours after compounding. Each lot shall be assayed individually and not pooled with other lots. If a microbiological test fails, an investigation shall be undertaken to identify the cause, and corrections shall be undertaken. After a record of successful sterility tests is established for a particular PET drug, only the first lot prepared each day shall be subject to a sterility test using cultivation methods. However, when a different PET drug is made at the facility or a new lot of sterile components (for example, filter or final product container) is substituted, then the first daily lot of that PET drug is tested for sterility.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Ian DeVeau, Ph.D.</a> Director, Veterinary Drugs and Radiopharmaceuticals 1-301-816-8178	(RMI05) Radiopharmaceuticals and Medical Imaging Agents 05

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#### 831 REFRACTIVE INDEX

The refractive index (*n*) of a substance is the ratio of the velocity of light in air to the velocity of light in the substance. It is valuable in the identification of substances and the detection of impurities.

Although the standard temperature for Pharmacopeial measurements is 25°, many of the refractive index specifications in the individual monographs call for determining this value at 20°. The temperature should be carefully adjusted and maintained, since the refractive index varies significantly with temperature.

The values for refractive index given in this Pharmacopeia are for the D line of sodium (doublet at 589.0 nm and 589.6 nm). Most instruments available are designed for use with white light but are calibrated to give the refractive index in terms of the D line of sodium light.

The Abbé refractometer measures the range of refractive index for those Pharmacopeial materials for which such values are given. Other refractometers of equal or greater accuracy may be employed.

To achieve the theoretical accuracy of ±0.0001, it is necessary to calibrate the instrument against a standard provided by the manufacturer and to check frequently the temperature control and cleanliness of the instrument by determining the refractive index of distilled water, which is 1.3330 at 20° and 1.3325 at 25°.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Gary E. Ritchie, M.Sc.</a> Scientific Fellow 1-301-816-8353	(GC05) General Chapters 05

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#### 841 SPECIFIC GRAVITY

Unless otherwise stated in the individual monograph, the specific gravity determination is applicable only to liquids, and, unless otherwise stated, is based on the ratio of the weight of a liquid in air at 25° to that of an equal volume of water at the same temperature. Where a temperature is specified in the individual monograph, the specific gravity is the ratio of the weight of the liquid in air at the specified temperature to that of an equal volume of water at the same temperature. When the substance is a solid at 25°, determine the specific gravity of the melted material at the temperature directed in the individual monograph, and refer to water at 25°.

Unless otherwise stated in the individual monograph, the density is defined as the mass of a unit volume of the substance at 25°, expressed in kilograms per cubic meter or grams per cubic centimeter (1 kg/m<sup>3</sup> = 10–3 g/cm<sup>3</sup>).

Unless otherwise directed in the individual monograph, use Method I.

#### METHOD I

**Procedure—** Select a scrupulously clean, dry pycnometer that previously has been calibrated by determining its weight and the weight of recently boiled water contained in it at 25°. Adjust the temperature of the liquid to about 20°, and fill the pycnometer with it. Adjust the temperature of the filled pycnometer to 25°, remove any excess liquid, and weigh. When the monograph specifies a temperature different from 25°, filled pycnometers must be brought to the temperature of the balance before they are weighed. Subtract the tare weight of the pycnometer from the filled weight.

The specific gravity of the liquid is the quotient obtained by dividing the weight of the liquid contained in the pycnometer by the weight of water contained in it, both determined at 25°, unless otherwise directed in the individual monograph.

#### METHOD II

The procedure includes the use of the Oscillating transducer density meter. The apparatus consists of the following:

- a U-shaped tube, usually of borosilicate glass, which contains the liquid to be examined;
- a magneto-electrical or piezo-electrical excitation system that causes the tube to oscillate as a cantilever oscillator at a characteristic frequency depending on the density of the

liquid to be examined;

- a means of measuring the oscillation period (T), which may be converted by the apparatus to give a direct reading of density or used to calculate density by using the constants A and B described below; and
- a means to measure and/or control the temperature of the oscillating transducer containing the liquid to be tested.

The oscillation period is a function of the spring constant (c) and the mass of the system:

$$T^2 = \left( \frac{M}{c} + \frac{\rho \times V}{c} \right) \times 4\pi^2$$

where  $\rho$  is the density of the liquid to be tested, M is the mass of the tube, and V is the volume of the filled tube.

Introduction of two constants A = c / (4π² × V) and B = M / V, leads to the classical equation for the oscillating transducer:

$$\rho = A \times T^2 - B$$

The specific gravity of the liquid is given by the formula:

$$\rho(L) / \rho(W)$$

where  $\rho(L)$  and  $\rho(W)$  are the densities of the liquid and water, respectively, both determined at 25°, unless otherwise directed in the individual monograph.

Calibration— The constants A and B are determined by operating the instrument with the U-tube filled with two different samples of known density (e.g., degassed water and air). Perform the control measurements daily, using degassed water: the results displayed for the control measurement using degassed water do not deviate from the reference value ( $\rho$  = 0.997043 g/cm³) by more than its specified error. Precision is a function of the repeatability and stability of the oscillator frequency. Density meters are able to achieve measurements with an error on the order of 1×10-3g/cm-3 to 1×10-5g/cm-3 and a repeatability of 1×10-4g/cm-3 to 1×10-6g/cm-3. For example, an instrument specified to ±1×10-4g/cm-3 must display 0.9970 ± 0.0001 g/cm-3 in order to be suitable for further measurement, otherwise a readjustment is necessary. Calibration with certified reference materials should be carried out regularly.

Procedure— Using the manufacturer's instructions, perform the measurements using the same procedure as for Calibration. If necessary, equilibrate the liquid to be examined at 25° before introduction into the tube to avoid the formation of bubbles and to reduce the time required for measurement. Factors affecting accuracy include the following:

- temperature uniformity throughout the tube,
- nonlinearity over a range of density,
- parasitic resonant effects, and
- viscosity, if the oscillating transducer density meters used do not provide automatic compensation of sample viscosity influence.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Gary E. Ritchie, M.Sc.</a> Scientific Fellow 1-301-816-8353	(GC05) General Chapters 05

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846 SPECIFIC SURFACE AREA

## INTRODUCTION

The specific surface area of a powder is determined by physical adsorption of a gas on the surface of the solid and by calculating the amount of adsorbate gas corresponding to a monomolecular layer on the surface. Physical adsorption results from relatively weak forces (van der Waals forces) between the adsorbate gas molecules and the adsorbent surface of the test powder. The determination is usually carried out at the temperature of liquid nitrogen. The amount of gas adsorbed can be measured by a volumetric or continuous flow procedure.

### BRUNAUER, EMMETT AND TELLER (BET) THEORY AND SPECIFIC SURFACE AREA DETERMINATION

#### Multipoint Measurement

The data are treated according to the Brunauer, Emmett and Teller (BET) adsorption isotherm equation:

$$\left[ \frac{1}{V_a \left( \frac{P_o}{P} - 1 \right)} \right] = \frac{C-1}{V_m C} \times \frac{P}{P_o} + \frac{1}{V_m C} \quad (1)$$

P = partial vapor pressure of adsorbate gas in equilibrium with the surface at 77.4 K (b.p. of liquid nitrogen), in Pa,

P<sub>o</sub> = saturated pressure of adsorbate gas, in Pa,

V<sub>a</sub> = volume of gas adsorbed at standard temperature and pressure (STP) [273.15 K and atmospheric pressure (1.013 × 10<sup>5</sup> Pa)], in mL,

V<sub>m</sub> = volume of gas adsorbed at STP to produce an apparent monolayer on the sample surface, in mL,

C = dimensionless constant that is related to the enthalpy of adsorption of the adsorbate gas on the powder sample.

A value of V<sub>a</sub> is measured at each of not less than three values of P/P<sub>o</sub>.

Then the BET value

1

$$\frac{1}{V_a \left( \frac{P_o}{P} - 1 \right)}$$

is plotted against  $P/P_o$ , according to equation (1). This plot should yield a straight line usually in the approximate relative pressure range 0.05 to 0.3. The data are considered acceptable if the correlation coefficient,  $r$ , of the linear regression is not less than 0.9975; that is,  $r^2$  is not less than 0.995. From the resulting linear plot, the slope, which is equal to  $(C - 1)/VmC$ , and the intercept, which is equal to  $1/VmC$ , are evaluated by linear regression analysis. From these values,  $Vm$  is calculated as  $1/(slope + intercept)$ , while  $C$  is calculated as  $(slope/intercept) + 1$ . From the value of  $Vm$  so determined, the specific surface area,  $S$ , in  $m^2 \cdot g^{-1}$ , is calculated by the equation:

$$S = \frac{Vm Na}{m \times 22400} \quad (2)$$

$N$  = Avogadro constant ( $6.023 \times 1023 \text{ mol}^{-1}$ ),

$a$  = effective cross-sectional area of one adsorbate molecule, in square meters (0.162 nm $^2$  for nitrogen and 0.195 nm $^2$  for krypton),

$m$  = mass of test powder, in g,

22400 = volume, in mL, occupied by one mole of the adsorbate gas at STP allowing for minor departures from the ideal.

A minimum of three data points is required. Additional measurements may be carried out especially when nonlinearity is obtained at a  $P/P_o$  value close to 0.3. Because nonlinearity is often obtained at a  $P/P_o$  value below 0.05, values in this region are not recommended. The test for linearity, the treatment of the data, and the calculation of the specific surface area of the sample are described above.

#### Single-Point Measurement

Normally, at least three measurements of  $Va$ , each at different values of  $P/P_o$ , are required for the determination of specific surface area by the dynamic flow gas adsorption technique (Method I) or by volumetric gas adsorption (Method II). However, under certain circumstances described below, it may be acceptable to determine the specific surface area of a powder from a single value of  $Va$  measured at a single value of  $P/P_o$  such as 0.300 (corresponding to 0.300 mole of nitrogen or 0.001038 mole fraction of krypton), using the following equation for calculating  $Vm$ :

$$Vm = V_a \left( 1 - \frac{P}{P_o} \right) \quad (3)$$

The specific surface area is then calculated from the value of  $Vm$  by equation (2) given above.

The single-point method may be employed directly for a series of powder samples of a given material for which the material constant  $C$  is much greater than unity. These circumstances may be verified by comparing values of specific surface area determined by the single-point method with that determined by the multipoint method for the series of powder samples. Close similarity between the single-point values and multipoint values suggests that  $1/C$  approaches zero.

The single-point method may be employed indirectly for a series of very similar powder samples of a given material for which the material constant  $C$  is not infinite but may be assumed to be invariant. Under these circumstances, the error associated with the single-point method can be reduced or eliminated by using the multipoint method to evaluate  $C$  for one of the samples of the series from the BET plot, from which  $C$  is calculated as  $(1 + \text{slope}/\text{intercept})$ . Then  $Vm$  is calculated from the single value of  $Va$  measured at a single value of  $P/P_o$ , by the equation:

$$Vm = V_a \left( \frac{P_o}{P} - 1 \right) \left[ \frac{1}{C} + \frac{C-1}{C} \times \left( \frac{P}{P_o} \right) \right] \quad (4)$$

The specific surface area is calculated from  $Vm$  by equation (2) given above.

#### EXPERIMENTAL TECHNIQUES

This section describes the methods to be used for the sample preparation, the dynamic flow gas adsorption technique (Method I) and the volumetric gas adsorption technique (Method II).

##### Sample Preparation

##### outgassing

Before the specific surface area of the sample can be determined, it is necessary to remove gases and vapors that may have become physically adsorbed onto the surface after manufacture and during treatment, handling, and storage. If outgassing is not achieved, the specific surface area may be reduced or may be variable because an intermediate area of the surface is covered with molecules of the previously adsorbed gases or vapors. The outgassing conditions are critical for obtaining the required precision and accuracy of specific surface area measurements on pharmaceuticals because of the sensitivity of the surface of the materials.

The outgassing conditions must be demonstrated to yield reproducible BET plots, a constant weight of test powder, and no detectable physical or chemical changes in the test powder.

The outgassing conditions defined by the temperature, pressure, and time are chosen so that the original surface of the solid is reproduced as closely as possible. Outgassing of many

Substances is often achieved by applying a vacuum by purging the sample in a flowing stream of a nonreactive, dry gas or by applying a desorption-adsorption cycling method. In either case, elevated temperatures are sometimes applied to increase the rate at which the contaminants leave the surface. Caution should be exercised when outgassing powder samples using elevated temperatures to avoid affecting the nature of the surface and the integrity of the sample.

If heating is employed, the recommended temperature and time of outgassing are as low as possible to achieve reproducible measurement of specific surface area in an acceptable time. For outgassing sensitive samples, other outgassing methods such as the desorption-adsorption cycling method may be employed.

#### adsorbate

The standard technique is the adsorption of nitrogen of analytical quality at liquid nitrogen temperature.

For powders of low specific surface area (< 0.2 m<sup>2</sup>g<sup>-1</sup>), the proportion adsorbed is low. In such cases, the use of krypton at the liquid nitrogen temperature is preferred because the low vapor pressure exerted by this gas greatly reduces error. The use of larger sample quantities, where feasible (equivalent to 1 m<sup>2</sup> or greater total surface area using nitrogen), may compensate for the errors in determining low surface areas.

All gases used must be free from moisture.

#### quantity of sample

A quantity of the test powder is accurately weighed such that the total surface of the sample is at least 1 m<sup>2</sup> when the adsorbate is nitrogen and 0.5 m<sup>2</sup> when the adsorbate is krypton.

Lower quantities of sample may be used after appropriate validation.

#### Measurements

Because the amount of gas adsorbed under a given pressure tends to increase when the temperature is decreased, adsorption measurements are usually made at a low temperature. Measurement is performed at 77.4 K, the boiling point of liquid nitrogen.

#### Method I: The Dynamic Flow Method

##### principle

In the dynamic flow method (see [Figure 1](#)), the recommended adsorbate gas is dry nitrogen or krypton, while helium is employed as a diluent gas, which is not adsorbed under the recommended conditions.

A minimum of three mixtures of the appropriate adsorbate gas with helium are required within the P/P<sub>0</sub> range 0.05 to 0.30.

The gas detector-integrator should provide a signal that is approximately proportional to the volume of the gas passing through it under defined conditions of temperature and pressure. For this purpose, a thermal conductivity detector with an electronic integrator is one among various suitable types. A minimum of three data points within the recommended range of 0.05 to 0.30 for P/P<sub>0</sub> is determined.

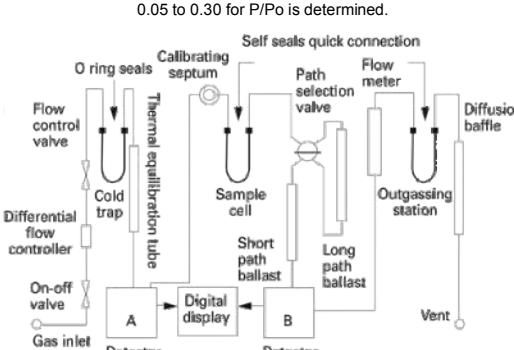


Fig. 1. Schematic diagram of the dynamic flow method apparatus.

#### procedure

A known mixture of the gases, usually nitrogen and helium, is passed through a thermal conductivity cell, through the sample again, through the thermal conductivity cell, and then to a recording potentiometer.

The sample cell is immersed in liquid nitrogen, and the sample adsorbs nitrogen from the mobile phase. This unbalances the thermal conductivity cell, and a pulse is generated on a recorder chart.

The sample is removed from the coolant; this gives a desorption peak equal in area and in the opposite direction to the adsorption peak. Because this is better defined than the adsorption peak, it is the one used for the determination.

To effect the calibration, a known quantity of adsorbate, sufficient to give a peak of similar magnitude to the desorption peak, is injected into the system, and the proportion of gas volume per unit peak area is obtained.

A mixture of nitrogen and helium is used for a single-point determination; and several such mixtures or premixing two streams of gas are used for a multipoint determination.

The calculation is the same as the volumetric method.

#### Method II: The Volumetric Method

##### principle

In the volumetric method (see [Figure 2](#)), the recommended adsorbate gas is nitrogen, which is admitted into the evacuated space above the previously outgassed powder sample to give a defined equilibrium pressure, P, of the gas. The use of a diluent gas, such as helium, is therefore unnecessary, although helium may be employed for other purposes, such as to measure the dead volume.

Because only pure adsorbate gas, instead of a gas mixture, is employed, interfering effects of thermal diffusion are avoided in this method.

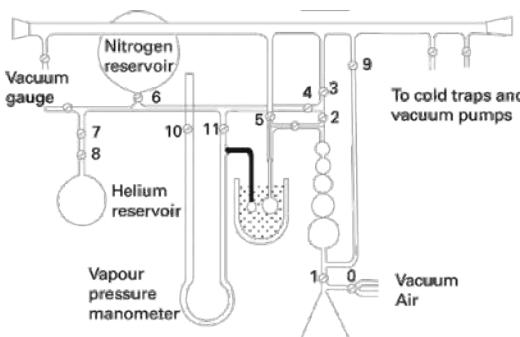


Fig. 2. Schematic diagram of the volumetric method apparatus.

## procedure

A small amount of dry nitrogen is admitted into the sample tube to prevent contamination of the clean surface, the sample tube is removed, a stopper is inserted, the tube is weighed, and the weight of the sample is calculated. Then the sample tube is attached to the volumetric apparatus. The sample is cautiously evacuated down to the specified pressure (e.g., between 2 Pa and 10 Pa). Alternately, some instruments are operated by evacuating to a defined rate of pressure change (e.g., less than 13 Pa/30 s) and by holding for a defined period of time before commencing the next step.

If the principle of operation of the instrument requires the determination of the dead volume in the sample tube, for example, by the admission of a nonadsorbed gas, such as helium, this procedure is carried out at this point, followed by evacuation of the sample. The determination of dead volume may be avoided using difference measurements: that is, by means of reference and sample tubes connected by a differential transducer. The adsorption of nitrogen gas is then measured as described below.

Raise a Dewar vessel containing liquid nitrogen at 77.4 K up to a defined point on the sample cell. Admit a sufficient volume of adsorbate gas to give the lowest desired relative pressure. Measure the volume adsorbed,  $V_a$ . For multipoint measurements, repeat the measurement of  $V_a$  at successively higher  $P/P_0$  values. When nitrogen is used as the adsorbate gas,  $P/P_0$  values of 0.10, 0.20, and 0.30 are often suitable.

## Reference Materials

Periodically verify the functioning of the apparatus using appropriate reference materials of known surface area that have a specific surface area similar to that of the sample to be examined.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Kahkashan Zaidi, Ph.D.</a> Senior Scientist 1-301-816-8269	(GC05) General Chapters 05

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## 851 SPECTROPHOTOMETRY AND LIGHT-SCATTERING

### ULTRAVIOLET, VISIBLE, INFRARED, ATOMIC ABSORPTION, FLUORESCENCE, TURBIDIMETRY, NEPHELOMETRY, AND RAMAN MEASUREMENT

Absorption spectrophotometry is the measurement of an interaction between electromagnetic radiation and the molecules, or atoms, of a chemical substance. Techniques frequently employed in pharmaceutical analysis include UV, visible, IR, and atomic absorption spectroscopy. Spectrophotometric measurement in the visible region was formerly referred to as colorimetry; however, it is more precise to use the term "colorimetry" only when considering human perception of color.

Fluorescence spectrophotometry is the measurement of the emission of light from a chemical substance while it is being exposed to UV, visible, or other electromagnetic radiation. In general, the light emitted by a fluorescent solution is of maximum intensity at a wavelength longer than that of the exciting radiation, usually by some 20 to 30 nm.

Light-Scattering involves measurement of the light scattered because of submicroscopic optical density inhomogeneities of solutions and is useful in the determination of weight-average molecular weights of polydisperse systems in the molecular weight range from 1000 to several hundred million. Two such techniques utilized in pharmaceutical analysis are turbidimetry and nephelometry.

Raman spectroscopy (inelastic light-scattering) is a light-scattering process in which the specimen under examination is irradiated with intense monochromatic light (usually laser light) and the light scattered from the specimen is analyzed for frequency shifts.

The wavelength range available for these measurements extends from the short wavelengths of the UV through the IR. For convenience of reference, this spectral range is roughly divided into the UV (190 to 380 nm), the visible (380 to 780 nm), the near-IR (780 to 3000 nm), and the IR (2.5 to 40  $\mu$ m or 4000 to 250  $\text{cm}^{-1}$ ).

### COMPARATIVE UTILITY OF SPECTRAL RANGES

For many pharmaceutical substances, measurements can be made in the UV and visible regions of the spectrum with greater accuracy and sensitivity than in the near-IR and IR. When solutions are observed in 1-cm cells, concentrations of about 10  $\mu$ g of the specimen per mL often will produce absorbances of 0.2 to 0.8 in the UV or the visible region. In the IR and near-IR, concentrations of 1 to 10 mg per mL and up to 100 mg per mL, respectively, may be needed to produce sufficient absorption; for these spectral ranges, cell lengths of from 0.01 mm to upwards of 3 mm are commonly used.

The UV and visible spectra of substances generally do not have a high degree of specificity. Nevertheless, they are highly suitable for quantitative assays, and for many substances they are useful as additional means of identification.

There has been increasing interest in the use of near-IR spectroscopy in pharmaceutical analysis, especially for rapid identification of large numbers of samples, and also for water determination.

The near-IR region is especially suitable for the determination of  $-\text{OH}$  and  $-\text{NH}$  groups, such as water in alcohol,  $-\text{OH}$  in the presence of amines, alcohols in hydrocarbons, and primary and secondary amines in the presence of tertiary amines.

The IR spectrum is unique for any given chemical compound with the exception of optical isomers, which have identical spectra. However, polymorphism may occasionally be responsible for a difference in the IR spectrum of a given compound in the solid state. Frequently, small differences in structure result in significant differences in the spectra. Because of the large number of maxima in an IR absorption spectrum, it is sometimes possible to quantitatively measure the individual components of a mixture of known qualitative composition without prior separation.

The Raman spectrum and the IR spectrum provide similar data, although the intensities of the spectra are governed by different molecular properties. Raman and IR spectroscopy



...hibit different relative sensitivities for different functional groups, e.g., Raman spectroscopy is particularly sensitive to C–S and C–C multiple bonds, and some aromatic compounds are more easily identified by means of their Raman spectra. Water has a highly intense IR absorption spectrum, but a particularly weak Raman spectrum. Therefore, water has only limited IR "windows" that can be used to examine aqueous solutes, while its Raman spectrum is almost completely transparent and useful for solute identification. The two major limitations of Raman spectroscopy are that the minimum detectable concentration of specimen is typically 10-1 M to 10-2 M and that the impurities in many substances fluoresce and interfere with the detection of the Raman scattered signal.

Optical reflectance measurements provide spectral information similar to that obtained by transmission measurements. Since reflectance measurements probe only the surface composition of the specimen, difficulties associated with the optical thickness and the light-scattering properties of the substance are eliminated. Thus, reflectance measurements are frequently more simple to perform on intensely absorbing materials. A particularly common technique used for IR reflectance measurements is termed attenuated total reflectance (ATR), also known as multiple internal reflectance (MIR). In the ATR technique, the beam of the IR spectrometer is passed through an appropriate IR window material (e.g., KRS-5, a TiBr-TlI eutectic mixture), which is cut at such an angle that the IR beam enters the first (front) surface of the window, but is totally reflected when it impinges on the second (back) surface (i.e., the angle of incidence of the radiation upon the second surface of the window exceeds the critical angle for that material). By appropriate window construction, it is possible to have many internal reflections of the IR beam before it is transmitted out of the window. If a specimen is placed in close contact with the window along the sides that totally reflect the IR beam, the intensity of reflected radiation is reduced at each wavelength (frequency) that the specimen absorbs. Thus, the ATR technique provides a reflectance spectrum that has been increased in intensity, when compared to a simple reflectance measurement, by the number of times that the IR beam is reflected within the window. The ATR technique provides excellent sensitivity, but it yields poor reproducibility, and is not a reliable quantitative technique unless an internal standard is intimately mixed with each test specimen.

Fluorescence spectrophotometry is often more sensitive than absorption spectrophotometry. In absorption measurements, the specimen transmittance is compared to that of a blank; and at low concentrations, both solutions give high signals. Conversely, in fluorescence spectrophotometry, the solvent blank has low rather than high output, so that the background radiation that may interfere with determinations at low concentrations is much less. Whereas few compounds can be determined conveniently at concentrations below 10-5 M by light absorption, it is not unusual to employ concentrations of 10-7 M to 10-8 M in fluorescence spectrophotometry.

#### THEORY AND TERMS

The power of a radiant beam decreases in relation to the distance that it travels through an absorbing medium. It also decreases in relation to the concentration of absorbing molecules or ions encountered in that medium. These two factors determine the proportion of the total incident energy that emerge. The decrease in power of monochromatic radiation passing through a homogeneous absorbing medium is stated quantitatively by Beer's law,  $\log(1/T) = A = abc$ , in which the terms are as defined below.

Absorbance [Symbol: A]—The logarithm, to the base 10, of the reciprocal of the transmittance (T). [note—Descriptive terms used formerly include optical density, absorbancy, and extinction.]

Absorptivity [Symbol: a]—The quotient of the absorbance (A) divided by the product of the concentration of the substance (c), expressed in g per L, and the absorption path length (b) in cm. [note—It is not to be confused with absorbancy index; specific extinction; or extinction coefficient.]

Molar Absorptivity [Symbol: ε]—The quotient of the absorbance (A) divided by the product of the concentration, expressed in moles per L, of the substance and the absorption path length in cm. It is also the product of the absorptivity (a) and the molecular weight of the substance. [note—Terms formerly used include molar absorbancy index; molar extinction coefficient; and molar absorption coefficient.]

For most systems used in absorption spectrophotometry, the absorptivity of a substance is a constant independent of the intensity of the incident radiation, the internal cell length, and the concentration, with the result that concentration may be determined photometrically.

Beer's law gives no indication of the effect of temperature, wavelength, or the type of solvent. For most analytical work the effects of normal variation in temperature are negligible.

Deviations from Beer's law may be caused by either chemical or instrumental variables. Apparent failure of Beer's law may result from a concentration change in solute molecules because of association between solute molecules or between solute and solvent molecules, or dissociation or ionization. Other deviations might be caused by instrumental effects such as polychromatic radiation, slit-width effects, or stray light.

Even at a fixed temperature in a given solvent, the absorptivity may not be truly constant. However, in the case of specimens having only one absorbing component, it is not necessary that the absorbing system conform to Beer's law for use in quantitative analysis. The concentration of an unknown may be found by comparison with an experimentally determined standard curve.

Although, in the strictest sense, Beer's law does not hold in atomic absorption spectrophotometry because of the lack of quantitative properties of the cell length and the concentration, the absorption processes taking place in the flame under conditions of reproducible aspiration do follow the Beer relationship in principle. Specifically, the negative log of the transmittance, or the absorbance, is directly proportional to the absorption coefficient, and, consequently, is proportional to the number of absorbing atoms. On this basis, calibration curves may be constructed to permit evaluation of unknown absorption values in terms of concentration of the element in solution.

Absorption Spectrum—A graphic representation of absorbance, or any function of absorbance, plotted against wavelength or function of wavelength.

Transmittance [Symbol: T]—The quotient of the radiant power transmitted by a specimen divided by the radiant power incident upon the specimen. [note—Terms formerly used include transmittancy and transmission.]

Fluorescence Intensity [Symbol: I]—An empirical expression of fluorescence activity, commonly given in terms of arbitrary units proportional to detector response. The fluorescence emission spectrum is a graphical presentation of the spectral distribution of radiation emitted by an activated substance, showing intensity of emitted radiation as ordinate, and wavelength as abscissa. The fluorescence excitation spectrum is a graphical presentation of the activation spectrum, showing intensity of radiation emitted by an activated substance as ordinate, and wavelength of the incident (activating) radiation as abscissa. As in absorption spectrophotometry, the important regions of the electromagnetic spectrum encompassed by the fluorescence of organic compounds are the UV, visible, and near-IR, i.e., the region from 250 to 800 nm. After a molecule has absorbed radiation, the energy can be lost as heat or released in the form of radiation of the same or longer wavelength as the absorbed radiation. Both absorption and emission of radiation are due to the transitions of electrons between different energy levels, or orbitals, of the molecule. There is a time delay between the absorption and emission of light; this interval, the duration of the excited state, has been measured to be about 10-9 second to 10-8 second for most organic fluorescent solutions. The short lifetime of fluorescence distinguishes this type of luminescence from phosphorescence, which is a long-lived afterglow having a lifetime of 10-3 second up to several minutes.

Turbidance [Symbol: S]—The light-scattering effect of suspended particles. The amount of suspended matter may be measured by observation of either the transmitted light (turbidimetry) or the scattered light (nephelometry).

Turbidity [Symbol: T]—In light-scattering measurements, the turbidity is the measure of the decrease in incident beam intensity per unit length of a given suspension.

Raman Scattering Activity—The molecular property (in units of  $\text{cm}^4 \text{ per g}$ ) governing the intensity of an observed Raman band for a randomly oriented specimen. The scattering activity is determined from the derivative of the molecular polarizability with respect to the molecular motion giving rise to the Raman shifted band. In general, the Raman band intensity is linearly proportional to the concentration of the analyte.

#### USE OF REFERENCE STANDARDS

With few exceptions, the Pharmacopeial spectrophotometric tests and assays call for comparison against a USP Reference Standard. This is to ensure measurement under conditions identical for the test specimen and the reference substance. These conditions include wavelength setting, slit-width adjustment, cell placement and correction, and transmittance levels. It should be noted that cells exhibiting identical transmittance at a given wavelength may differ considerably in transmittance at other wavelengths. Appropriate cell corrections should be established and used where required.

The expressions, "similar preparation" and "similar solution," as used in tests and assays involving spectrophotometry, indicate that the reference specimen, generally a USP Reference Standard, is to be prepared and observed in a manner identical for all practical purposes to that used for the test specimen. Usually in making up the solution of the



Specified Reference Standard, a solution of about (i.e., within 10%) the desired concentration is prepared and the absorptivity is calculated on the basis of the exact amount weighed out; if a previously dried specimen of the Reference Standard has not been used, the absorptivity is calculated on the anhydrous basis.

The expressions, "concomitantly determine" and "concomitantly measured," as used in tests and assays involving spectrophotometry, indicate that the absorbances of both the solution containing the test specimen and the solution containing the reference specimen, relative to the specified test blank, are to be measured in immediate succession.

#### APPARATUS

Many types of spectrophotometers are available. Fundamentally, most types, except those used for IR spectrophotometry, provide for passing essentially monochromatic radiant energy through a specimen in suitable form, and measuring the intensity of the fraction that is transmitted. Fourier transform IR spectrophotometers use an interferometric technique whereby polychromatic radiation passes through the analyte and onto a detector on an intensity and time basis. UV, visible, and dispersive IR spectrophotometers comprise an energy source, a dispersing device (e.g., a prism or grating), slits for selecting the wavelength band, a cell or holder for the test specimen, a detector of radiant energy, and associated amplifiers and measuring devices. In diode array spectrophotometers, the energy from the source is passed through the test specimen and then dispersed via a grating onto several hundred light-sensitive diodes, each of which in turn develops a signal proportional to the number of photons at its small wavelength interval; these signals then may be computed at rapid chosen intervals to represent a complete spectrum. Fourier transform IR systems utilize an interferometer instead of a dispersing device and a digital computer to process the spectral data. Some instruments are manually operated, whereas others are equipped for automatic and continuous recording. Instruments that are interfaced to a digital computer have the capabilities also of co-adding and storing spectra, performing spectral comparisons, and performing difference spectroscopy (accomplished with the use of a digital absorbance subtraction method).

Instruments are available for use in the visible; in the visible and UV; in the visible, UV, and near-IR; and in the IR regions of the spectrum. Choice of the type of spectrophotometric analysis and of the instrument to be used depends upon factors such as the composition and amount of available test specimen, the degree of accuracy, sensitivity, and selectivity desired, and the manner in which the specimen is handled.

The apparatus used in atomic absorption spectrophotometry has several unique features. For each element to be determined, a specific source that emits the spectral line to be absorbed should be selected. The source is usually a hollow-cathode lamp, the cathode of which is designed to emit the desired radiation when excited. Since the radiation to be absorbed by the test specimen element is usually of the same wavelength as that of its emission line, the element in the hollow-cathode lamp is the same as the element to be determined. The apparatus is equipped with an aspirator for introducing the test specimen into a flame, which is usually provided by air-acetylene, air-hydrogen, or, for refractory cases, nitrous oxide-acetylene. The flame, in effect, is a heated specimen chamber. A detector is used to read the signal from the chamber. Interfering radiation produced by the flame during combustion may be negated by the use of a chopped source lamp signal of a definite frequency. The detector should be tuned to this alternating current frequency so that the direct current signal arising from the flame is ignored. The detecting system, therefore, reads only the change in signal from the hollow-cathode source, which is directly proportional to the number of atoms to be determined in the test specimen. For Pharmacopeial purposes, apparatus that provides the readings directly in absorbance units is usually required.

However, instruments providing readings in percent transmission, percent absorption, or concentration may be used if the calculation formulas provided in the individual monographs are revised as necessary to yield the required quantitative results. Percent absorption or percent transmittance may be converted to absorbance, A, by the following two equations:

$$A = 2 - \log_{10} (100 - \% \text{ absorption})$$

or:

$$A = 2 - \log_{10} (\% \text{ transmittance})$$

Depending upon the type of apparatus used, the readout device may be a meter, digital counter, recorder, or printer. Both single-beam and double-beam instruments are commercially available, and either type is suitable.

Measurement of fluorescence intensity can be made with a simple filter fluorometer. Such an instrument consists of a radiation source, a primary filter, a specimen chamber, a secondary filter, and a fluorescence detection system. In most such fluorometers, the detector is placed on an axis at 90° from that of the exciting beam. This right-angle geometry permits the exciting radiation to pass through the test specimen and not contaminate the output signal received by the fluorescence detector. However, the detector unavoidably receives some of the exciting radiation as a result of the inherent scattering properties of the solutions themselves, or if dust or other solids are present. Filters are used to eliminate this residual scatter. The primary filter selects short-wavelength radiation capable of exciting the test specimen, while the secondary filter is normally a sharp cut-off filter that allows the longer-wavelength fluorescence to be transmitted but blocks the scattered excitation.

Most fluorometers use photomultiplier tubes as detectors, many types of which are available, each having special characteristics with respect to spectral region of maximum sensitivity, gain, and electrical noise. The photocurrent is amplified and read out on a meter or recorder.

A spectrofluorometer differs from a filter fluorometer in that filters are replaced by monochromators, of either the prism or the grating type. For analytical purposes, the spectrofluorometer is superior to the filter fluorometer in wavelength selectivity, flexibility, and convenience, in the same way in which a spectrophotometer is superior to a filter photometer.

Many radiation sources are available. Mercury lamps are relatively stable and emit energy mainly at discrete wavelengths. Tungsten lamps provide an energy continuum in the visible region. The high-pressure xenon arc lamp is often used in spectrofluorometers because it is a high-intensity source that emits an energy continuum extending from the UV into the IR.

In spectrofluorometers, the monochromators are equipped with slits. A narrow slit provides high resolution and spectral purity, while a large slit sacrifices these for high sensitivity. Choice of slit size is determined by the separation between exciting and emitting wavelengths as well as the degree of sensitivity needed.

Specimen cells used in fluorescence measurements may be round tubes or rectangular cells similar to those used in absorption spectrophotometry, except that they are polished on all four vertical sides. A convenient test specimen size is 2 to 3 mL, but some instruments can be fitted with small cells holding 100 to 300  $\mu$ L, or with a capillary holder requiring an even smaller amount of specimen.

Light-scattering instruments are available and consist in general of a mercury lamp, with filters for the strong green or blue lines, a shutter, a set of neutral filters with known transmittance, and a sensitive photomultiplier to be mounted on an arm that can be rotated around the solution cell and set at any angle from -135° to 0° to +135° by a dial outside the light-tight housing. Solution cells are of various shapes, such as square for measuring 90° scattering; semioctagonal for 45°, 90°, and 135° scattering; and cylindrical for scattering at all angles. Since the determination of molecular weight requires a precise measure of the difference in refractive index between the solution and solvent,  $[(n - n_0)/c]$ , a second instrument, a differential refractometer, is needed to measure this small difference.

Raman spectrometers include the following major components: a source of intense monochromatic radiation (invariably a laser); optics to collect the light scattered by the test specimen; a (double) monochromator to disperse the scattered light and reject the intense incident frequency; and a suitable light-detection and amplification system. Raman measurement is simple in that most specimens are examined directly in melting-point capillaries. Because the laser source can be focused sharply, only a few microliters of the specimen is required.

#### PROCEDURE

##### Absorption Spectrophotometry

Detailed instructions for operating spectrophotometers are supplied by the manufacturers. To achieve significant and valid results, the operator of a spectrophotometer should be aware of its limitations and of potential sources of error and variation. The instruction manual should be followed closely on such matters as care, cleaning, and calibration of the instrument, and techniques of handling absorption cells, as well as instructions for operation. The following points require special emphasis.

Check the instrument for accuracy of calibration. Where a continuous source of radiant energy is used, attention should be paid to both the wavelength and photometric scales; where a spectral line source is used, only the photometric scale need be checked. A number of sources of radiant energy have spectral lines of suitable intensity, adequately spaced throughout the spectral range selected. The best single source of UV and visible calibration spectra is the quartz-mercury arc, of which the lines at 253.7, 302.25, 313.16, 334.15,



48, 404.66, and 435.83 nm may be used. The glass-mercury arc is equally useful above 300 nm. The 486.13-nm and 656.28-nm lines of a hydrogen discharge lamp may be used also. The wavelength scale may be calibrated also by means of suitable glass filters, which have useful absorption bands through the visible and UV regions. Standard glasses containing didymium (a mixture of praseodymium and neodymium) have been used widely, although glasses containing holmium were found to be superior. Standard holmium oxide solution has superseded the use of holmium glass.<sup>1</sup> The wavelength scales of near-IR and IR spectrophotometers are readily checked by the use of absorption bands provided by polystyrene films, carbon dioxide, water vapor, or ammonia gas.

For checking the photometric scale, a number of standard inorganic glass filters as well as standard solutions of known transmittances such as potassium dichromate are available.<sup>2</sup>

Quantitative absorbance measurements usually are made on solutions of the substance in liquid-holding cells. Since both the solvent and the cell window absorb light, compensation must be made for their contribution to the measured absorbance. Matched cells are available commercially for UV and visible spectrophotometry for which no cell correction is necessary. In IR spectrophotometry, however, corrections for cell differences usually must be made. In such cases, pairs of cells are filled with the selected solvent and the difference in their absorbances at the chosen wavelength is determined. The cell exhibiting the greater absorbance is used for the solution of the test specimen and the measured absorbance is corrected by subtraction of the cell difference.

With the use of a computerized Fourier transform IR system, this correction need not be made, since the same cell can be used for both the solvent blank and the test solution. However, it must be ascertained that the transmission properties of the cell are constant.

Comparisons of a test specimen with a Reference Standard are best made at a peak of spectral absorption for the compound concerned. Assays prescribing spectrophotometry give the commonly accepted wavelength for peak spectral absorption of the substance in question. It is known that different spectrophotometers may show minor variation in the apparent wavelength of this peak. Good practice demands that comparisons be made at the wavelength at which peak absorption occurs. Should this differ by more than  $\pm 1$  nm from the wavelength specified in the individual monograph, recalibration of the instrument may be indicated.

#### test preparation

For determinations utilizing UV or visible spectrophotometry, the specimen generally is dissolved in a solvent. Unless otherwise directed in the monograph, determinations are made at room temperature using a path length of 1 cm. Many solvents are suitable for these ranges, including water, alcohols, chloroform, lower hydrocarbons, ethers, and dilute solutions of strong acids and alkalies. Precautions should be taken to utilize solvents free from contaminants absorbing in the spectral region being used. It is usually advisable to use water-free methanol or alcohol, or alcohol denatured by the addition of methanol but not containing benzene or other interfering impurities, as the solvent. Solvents of special spectrophotometric quality, guaranteed to be free from contaminants, are available commercially from several sources. Some other analytical reagent-grade organic solvents may contain traces of impurities that absorb strongly in the UV region. New lots of these solvents should be checked for their transparency, and care should be taken to use the same lot of solvent for preparation of the test solution and the standard solution and for the blank.

No solvent in appreciable thickness is completely transparent throughout the near-IR and IR spectrum. Carbon tetrachloride (up to 5 mm in thickness) is practically transparent to 6  $\mu$ m (1666  $\text{cm}^{-1}$ ). Carbon disulfide (1 mm in thickness) is suitable as a solvent to 40  $\mu$ m (250  $\text{cm}^{-1}$ ) with the exception of the 4.2- $\mu$ m to 5.0- $\mu$ m (2381- $\text{cm}^{-1}$  to 2000- $\text{cm}^{-1}$ ) and the 5.5- $\mu$ m to 7.5- $\mu$ m (1819- $\text{cm}^{-1}$  to 1333- $\text{cm}^{-1}$ ) regions, where it has strong absorption. Other solvents have relatively narrow regions of transparency. For IR spectrophotometry, an additional qualification for a suitable solvent is that it must not affect the material, usually sodium chloride, of which the cell is made. The test specimen may also be prepared by dispersing the finely ground solid specimen in mineral oil or by mixing it intimately with previously dried alkali halide salt (usually potassium bromide). Mixtures with alkali halide salts may be examined directly or as transparent disks or pellets obtained by pressing the mixture in a die. Typical drying conditions for potassium bromide are 105° in vacuum for 12 hours, although grades are commercially available that require no drying. Infrared microscopy or a mineral oil dispersion is preferable where disproportionation between the alkali halide and the test specimen is encountered. For suitable materials the test specimen may be prepared neat as a thin sample for IR microscopy or suspended neat as a thin film for mineral oil dispersion. For Raman spectrometry, most common solvents are suitable, and ordinary (nonfluorescing) glass specimen cells can be used. The IR region of the electromagnetic spectrum extends from 0.8 to 400  $\mu$ m. From 800 to 2500 nm (0.8 to 2.5  $\mu$ m) is generally considered to be the near-IR (NIR) region; from 2.5 to 25  $\mu$ m (4000 to 400  $\text{cm}^{-1}$ ) is generally considered to be the mid-range (mid-IR) region; and from 25 to 400  $\mu$ m is generally considered to be the far-IR (FIR) region. Unless otherwise specified in the individual monograph, the region from 3800 to 650  $\text{cm}^{-1}$  (2.6 to 15  $\mu$ m) should be used to ascertain compliance with monograph specifications for IR absorption.

Where values for IR line spectra are given in an individual monograph, the letters s, m, and w signify strong, medium, and weak absorption, respectively; sh signifies a shoulder, bd signifies a band, and v means very. The values may vary as much as 0.1  $\mu$ m or 10  $\text{cm}^{-1}$ , depending upon the particular instrument used. Polymorphism gives rise to variations in the IR spectra of many compounds in the solid state. Therefore, when conducting IR absorption tests, if a difference appears in the IR spectra of the analyte and the standard, dissolve equal portions of the test substance and the standard in equal volumes of a suitable solvent, evaporate the solutions to dryness in similar containers under identical conditions, and repeat the test on the residues.

In NIR spectroscopy much of the current interest centers around the ease of analysis. Samples can be analyzed in powder form or by means of reflectance techniques, with little or no preparation. Compliance with in-house specifications can be determined by computerized comparison of spectra with spectra previously obtained from reference materials. Many pharmaceutical materials exhibit low absorptivity in this spectral region, which allows incident near-IR radiation to penetrate samples more deeply than UV, visible, or IR radiation. NIR spectrophotometry may be used to observe matrix modifications and, with proper calibration, may be used in quantitative analysis.

In atomic absorption spectrophotometry, the nature of the solvent and the concentration of solids must be given special consideration. An ideal solvent is one that interferes to a minimal extent in the absorption or emission processes and one that produces neutral atoms in the flame. If there is a significant difference between the surface tension or viscosity of the test solution and standard solution, the solutions are aspirated or atomized at a different rate, causing significant differences in the signals generated. The acid concentration of the solutions also affects the absorption processes. Thus, the solvents used in preparing the test specimen and the standard should be the same or as much alike in these respects as possible, and should yield solutions that are easily aspirated via the specimen tube of the burner-aspirator. Since undissolved solids present in the solutions may give rise to matrix or bulk interferences, the total undissolved solids content in all solutions should be kept below 2% wherever possible.

#### calculations

The application of absorption spectrophotometry in an assay or a test generally requires the use of a Reference Standard. Where such a measurement is specified in an assay, a formula is provided in order to permit calculation of the desired result. A numerical constant is frequently included in the formula. The following derivation is provided to introduce a logical approach to the deduction of the constants appearing in formulas in the assays in many monographs.

The Beer's law relationship is valid for the solutions of both the Reference Standard (S) and the test specimen (U):

$$(1) AS = abCS$$

$$(2) AU = abCU$$

in which AS is the absorbance of the Standard solution of concentration CS; and AU is the absorbance of the test specimen solution of concentration CU. If CS and CU are expressed in the same units and the absorbances of both solutions are measured in matching cells having the same dimensions, the absorptivity, a, and the cell thickness, b, are the same; consequently, the two equations may be combined and rewritten to solve for CU:

$$(3) CU = CS (AU / AS)$$

Quantities of solid test specimens to be taken for analysis are generally specified in mg. Instructions for dilution are given in the assay and, since dilute solutions are used for absorbance measurements, concentrations are usually expressed for convenience in units of  $\mu$ g per mL. Taking a quantity, in mg, of a test specimen of a drug substance or solid dosage form for analysis, it therefore follows that a volume (VU), in L, of solution of concentration CU may be prepared from the amount of test specimen that contains a quantity WU, in mg, of the drug substance [note—CU is numerically the same whether expressed as  $\mu$ g per mL or mg per L], such that:

$$(4) WU = VUCU$$

The form in which the formula appears in the assay in a monograph for a solid article may be derived by substituting CU of equation (3) into equation (4). In summary, the use of equation (4), with due consideration for any unit conversions necessary to achieve equality in equation (5), permits the calculation of the constant factor (VU) occurring in the final formula:



## (5) WU = VUCS (AU / AS)

The same derivation is applicable to formulas that appear in monographs for liquid articles that are assayed by absorption spectrophotometry. For liquid dosage forms, results of calculations are generally expressed in terms of the quantity, in mg, of drug substance in each mL of the article. Thus it is necessary to include in the denominator an additional term, the volume (V), in mL, of the test preparation taken.

Assays in the visible region usually call for comparing concomitantly the absorbance produced by the Assay preparation with that produced by a Standard preparation containing approximately an equal quantity of a USP Reference Standard. In some situations, it is permissible to omit the use of a Reference Standard. This is true where spectrophotometric assays are made with routine frequency, and where a suitable standard curve is available, prepared with the respective USP Reference Standard, and where the substance assayed conforms to Beer's law within the range of about 75% to 125% of the final concentration used in the assay. Under these circumstances, the absorbance found in the assay may be interpolated on the standard curve, and the assay result calculated therefrom.

Such standard curves should be confirmed frequently, and always when a new spectrophotometer or new lots of reagents are put into use.

In spectrophotometric assays that direct the preparation and use of a standard curve, it is permissible and preferable, when the assay is employed infrequently, not to use the standard curve but to make the comparison directly against a quantity of the Reference Standard approximately equal to that taken of the specimen, and similarly treated.

## Fluorescence Spectrophotometry

The measurement of fluorescence is a useful analytical technique. Fluorescence is light emitted from a substance in an excited state that has been reached by the absorption of radiant energy. A substance is said to be fluorescent if it can be made to fluoresce. Many compounds can be assayed by procedures utilizing either their inherent fluorescence or the fluorescence of suitable derivatives.

Test specimens prepared for fluorescence spectrophotometry are usually one-tenth to one-hundredth as concentrated as those used in absorption spectrophotometry, for the following reason. In analytical applications, it is preferable that the fluorescence signal be linearly related to the concentration; but if a test specimen is too concentrated, a significant part of the incoming light is absorbed by the specimen near the cell surface, and the light reaching the center is reduced. That is, the specimen itself acts as an "inner filter." However, fluorescence spectrophotometry is inherently a highly sensitive technique, and concentrations of 10-5 M to 10-7 M frequently are used. It is necessary in any analytical procedure to make a working curve of fluorescence intensity versus concentration in order to establish a linear relationship. All readings should be corrected for a solvent blank.

Fluorescence measurements are sensitive to the presence of dust and other solid particles in the test specimen. Such impurities may reduce the intensity of the exciting beam or give misleading high readings because of multiple reflections in the specimen cell. It is, therefore, wise to eliminate solid particles by centrifugation; filtration also may be used, but some filter papers contain fluorescent impurities.

Temperature regulation is often important in fluorescence spectrophotometry. For some substances, fluorescence efficiency may be reduced by as much as 1% to 2% per degree of temperature rise. In such cases, if maximum precision is desired, temperature-controlled specimen cells are useful. For routine analysis, it may be sufficient to make measurements rapidly enough so that the specimen does not heat up appreciably from exposure to the intense light source. Many fluorescent compounds are light-sensitive. Exposed in a fluorometer, they may be photo-degraded into more or less fluorescent products. Such effects may be detected by observing the detector response in relationship to time, and may be reduced by attenuating the light source with filters or screens.

Change of solvent may markedly affect the intensity and spectral distribution of fluorescence. It is inadvisable, therefore, to alter the solvent specified in established methods without careful preliminary investigation. Many compounds are fluorescent in organic solvents but virtually nonfluorescent in water; thus, a number of solvents should be tried before it is decided whether or not a compound is fluorescent. In many organic solvents, the intensity of fluorescence is increased by elimination of dissolved oxygen, which has a strong quenching effect. Oxygen may be removed by bubbling an inert gas such as nitrogen or helium through the test specimen.

A semiquantitative measure of the strength of fluorescence is given by the ratio of the fluorescence intensity of a test specimen and that of a standard obtained with the same instrumental settings. Frequently, a solution of stated concentration of quinine in 0.1 N sulfuric acid or fluorescein in 0.1 N sodium hydroxide is used as a reference standard.

## Light-Scattering

Turbidity can be measured with a standard photoelectric filter photometer or spectrophotometer, preferably with illumination in the blue portion of the spectrum. Nephelometric measurements require an instrument with a photocell placed so as to receive scattered rather than transmitted light; this geometry applies also to fluorometers, so that, in general, fluorometers can be used as nephelometers, by proper selection of filters. A ratio turbidimeter combines the technology of 90° nephelometry and turbidimetry: it contains photocells that receive and measure scattered light at a 90° angle from the sample as well as receiving and measuring the forward scatter in front of the sample; it also measures light transmitted directly through the sample. Linearity is attained by calculating the ratio of the 90° angle scattered light measurement to the sum of the forward scattered light measurement and the transmitted light measurement. The benefit of using a ratio turbidimetry system is that the measurement of stray light becomes negligible.

In practice, it is advisable to ensure that settling of the particles being measured is negligible. This is usually accomplished by including a protective colloid in the liquid suspending medium. It is important that results be interpreted by comparison of readings with those representing known concentrations of suspended matter, produced under precisely the same conditions.

Turbidimetry or nephelometry may be useful for the measurement of precipitates formed by the interaction of highly dilute solutions of reagents, or other particulate matter, such as suspensions of bacterial cells. In order that consistent results may be achieved, all variables must be carefully controlled. Where such control is possible, extremely dilute suspensions may be measured.

The specimen solute is dissolved in the solvent at several different accurately known concentrations, the choice of concentrations being dependent on the molecular weight of the solute and ranging from 1% for  $M_w = 10,000$  to 0.01% for  $M_w = 1,000,000$ . Each solution must be very carefully cleaned before measurement by repeated filtration through fine filters. A dust particle in the solution vitiates the intensity of the scattered light measured. A criterion for a clear solution is that the dissymmetry, 45°/135° scattered intensity ratio, has attained a minimum.

The turbidity and refractive index of the solutions are measured. From the general 90° light-scattering equation, a plot of  $HC/\bar{M}$  versus C is made and extrapolated to infinite dilution, and the weight-average molecular weight, M, is calculated from the intercept, 1/M.

## Visual Comparison

Where a color or a turbidity comparison is directed, color-comparison tubes that are matched as closely as possible in internal diameter and in all other respects should be used. For color comparison, the tubes should be viewed downward, against a white background, with the aid of a light source directed from beneath the bottoms of the tubes, while for turbidity comparison the tubes should be viewed horizontally, against a dark background, with the aid of a light source directed from the sides of the tubes.

In conducting limit tests that involve a comparison of colors in two like containers (e.g., matched color-comparison tubes), a suitable instrument, rather than the unaided eye, may be used.

1 National Institute of Standards and Technology (NIST), Gaithersburg, MD 20899: "Spectral Transmittance Characteristics of Holmium Oxide in Perchloric Acid," J. Res. Natl. Bur. Stds. 90, No. 2, 115 (1985). The performance of an uncertified filter should be checked against a certified standard.

2 For further detail regarding checks on photometric scale of a spectrophotometer, reference may be made to the following NIST publications: J. Res. Nalt. Bur. Stds. 76A, 469 (1972) [re: SRM 931, "Liquid Absorbance Standards for Ultraviolet and Visible Spectrophotometry" as well as potassium chromate and potassium dichromate]; NIST Spec. Publ. 260-116 (1994) [re: SRM 930 and SRM 1930, "Glass Filters for Spectrophotometry"].

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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## 861 SUTURES—DIAMETER

The gauge for determining the diameter of sutures is of the dead-weight type, mechanical or electrical, and equipped with a direct-reading dial, a digital readout, or a printed readout. Use a gauge graduated to 0.002 mm or smaller. The anvil of the gauge is about 50 mm in diameter, and the presser foot is  $12.70 \pm 0.02$  mm in diameter. The presser foot and moving parts connected therewith are weighted so as to apply a total load of  $210 \pm 3$  g to the specimen. The presser foot and anvil surfaces are plane to within 0.005 mm and parallel to each other to within 0.005 mm. For measuring the diameter of sutures of metric size 0.4 and smaller, remove the additional weight from the presser foot so that the total load on the suture does not exceed 60 g.

**Collagen Absorbable Surgical Suture**— Determine the diameter immediately after removal from the immediate container and without stretching. Lay the strand across the center of the anvil and presser foot, and gently lower the foot until its entire weight rests upon the suture. Measure the diameter of each strand at three points corresponding roughly to one-fourth, one-half, and three-fourths of its length.

**Synthetic Absorbable Surgical Suture**— Proceed as directed for Nonabsorbable Surgical Suture.

**Nonabsorbable Surgical Suture**— Lay the strand across the center of the anvil and presser foot, and gently lower the foot until its entire weight rests upon the suture. Measure nonabsorbable sutures, whether packaged in dry form or in fluid, immediately after removal from the container, without prior drying or conditioning.

Measure the diameter of the suture at three points corresponding roughly to one-fourth, one-half, and three-fourths of its length. In the case of braided suture of sizes larger than 3-0 (metric size 2), make two measurements at each point at right angles to each other, and use the average as the observed diameter at that point.

In measuring multifilament sutures, attach a portion of the designated section of the strand in a fixed clamp in such a way that the strand lies across the center of the anvil. While holding the strand in the same plane as the surface of the anvil, place the strand under tension by suitable means, such as by passing the free end of the strand around a cylinder or a pulley and attaching to the free end a weight of about one-half of the knot-pull limit for the non-sterilized Class I suture of the size concerned, taking care not to permit the strand, if twisted, to untwist. Measure the diameter at the designated points on the strand, and calculate the average diameter likewise as directed.

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Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Radhakrishna S Tirumalai, Ph.D.</a> Senior Scientist 1-301-816-8339	(GTMDB05) General Toxicology and Medical Device Biocompatibility

## 871 SUTURES—NEEDLE ATTACHMENT

Absorbable (collagen) surgical sutures and nonabsorbable surgical sutures with Standard Needle Attachment are such that the needles are firmly attached and are not intended to be separated. Sutures supplied with eyeless needles attached fall into either the category of Standard Needle Attachment or the category of Removable Needle Attachment. Removable Needle Attachment of both absorbable and nonabsorbable surgical sutures is such that the needle may be deliberately separated from the suture by means of a quick tug. Both types

of attachments are tested on equipment as specified under [Tensile Strength](#) (881).

**Procedure**— Clamp each of 5 sutures in the tensiometer so that the needle is in the fixed clamp with all of the swaged portion exposed and in line with the direction of force applied to the suture by the moving clamp. Determine the force required to detach the suture from the needle. In the case of Standard Needle Attachment, the suture may break without needle detachment.

**Standard Needle Attachment**— The requirements are met if neither the average of the 5 values nor any individual value is less than the limit given for the designated size in [Table 1](#).

Table 1. Standard Needle Attachment for Absorbable and Nonabsorbable Sutures

Absorbable Collagen Suture	Nonabsorbable and Synthetic Absorbable Sutures	USP Size	Limits on Needle Attachment			
			Average (in kgf) (Min.)	Individual (in kgf) (Min.)	Average (in N) (Min.)	Individual (in N) (Min.)
0.4	0.1	11-0	0.007	0.005	0.069	0.049
	0.2	10-0	0.014	0.010	0.137	0.098
	0.3	9-0	0.021	0.015	0.206	0.147
	0.4	8-0	0.050	0.025	0.490	0.245
	0.5	7-0	0.080	0.040	0.784	0.392
	0.7	6-0	0.17	0.08	1.67	0.784
	1	5-0	0.23	0.11	2.25	1.08
	1.5	4-0	0.45	0.23	4.41	2.25
	2	3-0	0.68	0.34	6.67	3.33
	3	2-0	1.10	0.45	10.8	4.41
0.5	3.5	0	1.50	0.45	14.7	4.41
	4	3.5	1.80	0.60	17.6	5.88
	5	4	1.80	0.70	17.6	6.86
	6 and larger	5 and larger	2 and larger			

**Removable Needle Attachment**— The requirements are met if the individual values of the 5 sutures are within the limits shown in [Table 2](#). [note—For either type of attachment, if not more than 1 of the individual values falls outside the prescribed limits, repeat the test on an additional 10 sutures: the requirements of the test are met if none of the 10 additional values falls outside the individual limit requirements.]

Table 2. Removable Needle Attachment for Absorbable and Nonabsorbable Sutures

Absorbable Collagen Suture	Nonabsorbable and Synthetic Absorbable Sutures	USP Size	Limits on Needle Attachment			
			Minimum (in kgf)	Maximum (in kgf)	Minimum (in N)	Maximum (in N)
1.5	1	5-0	0.028	1.59	0.274	15.6

2	1.5	4-0	0.028	1.59	0.274	15.6
3	2	3-0	0.028	1.59	0.274	15.6
3.5	3	2-0	0.028	1.59	0.274	15.6
4	3.5	0	0.028	1.59	0.274	15.6
5	4	1	0.028	1.59	0.274	15.6
6	5	2	0.028	1.59	0.274	15.6

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General Chapter	<a href="#">Radhakrishna S Tirumalai, Ph.D.</a> Senior Scientist 1-301-816-8339	(GTMDB05) General Toxicology and Medical Device Biocompatibility

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### 881 TENSILE STRENGTH

Devices for measurement of tensile strength used in the United States may be calibrated in the English units of measure. The following directions are given in metric units with the understanding that the corresponding English equivalents may be used.

#### Surgical Suture

Determine the tensile strength of surgical suture on a motor-driven tensile strength testing machine having suitable clamps for holding the specimen firmly and using either the principle of constant rate of load on specimen or the principle of constant rate of elongation of specimen, as described below. The apparatus has two clamps for holding the strand. One of these clamps is mobile. The clamps are designed so that the strand being tested can be attached without any possibility of slipping. Gauge length is defined as the interior distance between the two clamps. For gauge lengths of 125 to 200 mm, the mobile clamp is driven at a constant rate of elongation of  $30 \pm 5$  cm per minute. For gauge lengths of less than 125 mm, the rate of elongation per minute is adjusted to equal 2 times the gauge length per minute. For example, a 5-cm gauge length has a rate of elongation of 10 cm per minute.

Determine the tensile strength of the suture, whether packaged in dry form or in fluid, promptly after removal from the container, without prior drying or conditioning. Attach one end of the suture to the clamp at the load end of the machine, pass the other end through the opposite clamp, applying sufficient tension so that the specimen is taut between the clamps, and engage the second clamp. Perform as many breaks as are specified in the individual monograph. If the break occurs at the clamp, discard the reading on the specimen.

Procedure for a machine operating on the principle of constant rate of load on specimen— This description applies to the machine known as the Incline Plane Tester.

The carriage used in any test is of a weight such that when the break occurs, the position of the recording pen on the chart is between 20% and 80% of the capacity that may be recorded on the chart. The friction in the carriage is low enough to permit the recording pen to depart from the zero line of the chart at a point not exceeding 2.5% of the capacity of the chart when no specimen is held in the clamps.

For surgical sutures of intermediate and larger sizes, the clamp for holding the specimen is of the roll type, with a flat gripping surface. The roll has a diameter of 19 mm and the flat gripping surface is not less than 25 mm in length. The length of the specimen, when inserted in the clamps, is at least 127 mm from nip to nip. The speed of inclination of the plane of the tester is such that it reaches its full inclination of  $30^\circ$  from the horizontal in  $20 \pm 1$  seconds from the start of the test.

For surgical sutures of small sizes, the suitable clamp has a flat gripping surface of not less than 13 mm in length. The speed of inclination of the plane is such that it reaches its full inclination of  $30^\circ$  from the horizontal in  $60 \pm 5$  seconds from the start of the test.

Except where straight pull (no knot required) is indicated in the suture monograph, tie the test suture into a surgeon's knot with one turn of suture around flexible rubber tubing of 6.5-mm inside diameter and 1.6-mm wall thickness. The surgeon's knot is a square knot in which the free end is first passed twice, instead of once, through the loop, and pulled taut, then passed once through a second loop, and the ends are drawn taut so that a single throw is superimposed upon a double throw. Start the first knot with the left end over the right end, exerting sufficient tension to tie the knot securely. Where the test specimen includes a knot, place the specimen in the testing device with the knot approximately midway between the clamps. Leave the flexible rubber tubing in place for the duration of the test.

Procedure for a machine operating on the principle of constant rate of elongation of specimen— This description applies to any suitable tensile testing machine that operates on the principle of constant rate of elongation of specimen.

Except where straight pull (no knot required) is indicated in the suture monographs, tie the test suture into a simple knot formed by placing one end of a strand held in the right hand over the other end held in the left hand, passing one end over the strand and through the loop so formed, and pulling the knot tight. The specimen is placed in the testing device with the knot approximately midway between the clamps.

#### Textile Fabrics and Films

Determine the tensile strength of textile fabrics, including adhesive tape, on a constant-speed or pendulum type of testing machine of the following general description.

The clamps for holding the specimen are smooth, flat, parallel jaws that are not less than 25 mm in length in the dimension parallel to the direction of application of the load. When the width of the strip being tested does not exceed 19 mm, the jaws of the clamp should be at least 25 mm wide. If the width of the strip is greater than 19 mm and not greater than 44 mm, the width of the jaws of the clamp should be at least 50 mm. If the width of the specimen is greater than 44 mm, cut a 25-mm strip, and use a clamp with jaws not less than 50 mm wide. Round all edges that might have a cutting action on the specimen to a radius of 0.4 mm. The jaws are 76.2 mm apart at the beginning of the test, and they separate at the rate of  $30.5 \text{ cm} \pm 13 \text{ mm}$  per minute. The machine is of such capacity that when the break occurs, the deviation of the pendulum from the vertical is between  $9^\circ$  and  $45^\circ$ .

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General Chapter	<a href="#">Radhakrishna S Tirumalai, Ph.D.</a> Senior Scientist 1-301-816-8339	(GTMDB05) General Toxicology and Medical Device Biocompatibility

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### 891 THERMAL ANALYSIS

Precisely determined thermodynamic events, such as a change of state, can indicate the identity and purity of drugs. Compendial standards have long been established for the melting or boiling temperatures of substances. These transitions occur at characteristic temperatures, and the compendial standards therefore contribute to the identification of the substances.

Because impurities affect these changes in predictable ways, the same compendial standards contribute to the control of the purity of the substances.

Thermal analysis in the broadest sense is the measurement of physical-chemical properties of materials as a function of temperature. Instrumental methods have largely supplanted older methods dependent on visual inspection and on measurements under fixed or arbitrary conditions, because they are objective, they provide more information, they afford permanent records, and they are generally more sensitive, more precise, and more accurate. Furthermore, they may provide information on crystal perfection, polymorphism, melting

temperature, sublimation, glass transitions, dehydration, evaporation, pyrolysis, solid-solid interactions, and purity. Such data are useful in the characterization of substances with respect to compatibility, stability, packaging, and quality control. The measurements used most often in thermal analysis, i.e., transition temperature, thermogravimetry, and impurity analysis, are described here.

**Transition Temperature**— As a specimen is heated, its uptake (or evolution) of heat can be measured [differential scanning calorimetry (DSC)] or the resulting difference in temperature from that of an inert reference heated identically [differential thermal analysis (DTA)] can be measured. Either technique provides a record of the temperature at which phase changes, glass transitions, or chemical reactions occur. In the case of melting, both an “onset” and a “peak” temperature can be determined objectively and reproducibly, often to within a few tenths of a degree. While these temperatures are useful for characterizing substances, and the difference between the two temperatures is indicative of purity, the values cannot be correlated with subjective, visual “melting-range” values or with constants such as the triple point of the pure material.

A complete description of the conditions employed should accompany each thermogram, including make and model of instrument; record of last calibration; specimen size and identification (including previous thermal history); container; identity, flow rate, and pressure of gaseous atmosphere; direction and rate of temperature change; and instrument and recorder sensitivity.

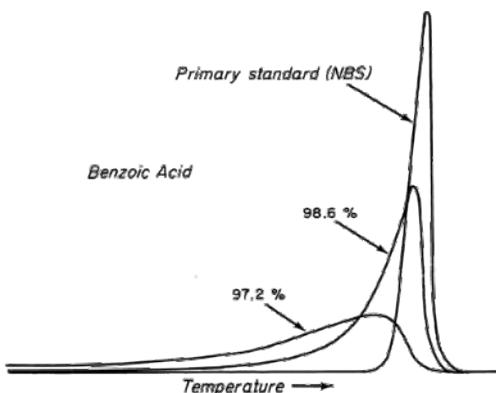
It is appropriate to make a preliminary examination over a wide range of temperature (typically room temperature to decomposition temperature or about 10° to 20° above the melting point) and over a wide range of heating rates (2° to 20° per minute), which may reveal unexpected effects; then a single examination or replicate examinations over a narrow range, bracketing the transition of interest at one or more lower heating rates, can be made. In examining pure crystalline materials, rates as low as 1° per minute may be appropriate, whereas rates of up to 10° per minute are more appropriate for polymeric and other semi-crystalline materials. As the reliability of the measurements varies from one substance to another, statements of the number of significant figures to be used in the reporting of intralaboratory repeatability and of interlaboratory reproducibility cannot be given here, but should be included in the individual monograph.

**Thermogravimetric Analysis**— Thermogravimetric analysis involves the determination of the mass of a specimen as a function of temperature, or time of heating, or both, and when properly applied, provides more useful information than does loss on drying at fixed temperature, often for a fixed time and in what is usually an ill-defined atmosphere. Usually, loss of surface-absorbed solvent can be distinguished from solvent in the crystal lattice and from degradation losses. The measurements can be carried out in atmospheres having controlled humidity and oxygen concentration to reveal interactions with the drug substance, between drug substances, and between active substances and excipients or packaging materials.

While the details depend on the manufacturer, the essential features of the equipment are a recording balance and a programmable heat source. Equipment differs in the ability to handle specimens of various sizes, the means of sensing specimen temperature, and the range of atmosphere control. Calibration is required with all systems, i.e., the mass scale is calibrated by the use of standard weights; calibration of the temperature scale, which is more difficult, involving either variations in positioning of thermocouples and their calibration; or in other systems, calibration involves the use of standard materials because it is assumed that the specimen temperature is the furnace temperature.

Procedural details are specified in order to provide for valid interlaboratory comparison of results. The specimen weight, source, and thermal history are noted. The equipment description covers dimensions and geometry, the materials of the test specimen holder, and the location of the temperature transducer. Alternatively, the make and model number of commercial equipment are specified. In all cases, the calibration record is specified. Data on the temperature environment include the initial and final temperatures and the rate of change or other details if nonlinear. The test atmosphere is critical; the volume, pressure, composition, whether static or dynamic, and if the latter, the flow rate and temperature are specified.

**Eutectic Impurity Analysis**— The basis of any calorimetric purity method is the relationship between the melting and freezing point depression, and the level of impurity. The melting of a compound is characterized by the absorption of latent heat of fusion,  $\Delta H_f$ , at a specific temperature,  $T_0$ . In theory, a melting transition for an absolutely pure crystalline compound should occur within an infinitely narrow range. A broadening of the melting range, due to impurities, provides a sensitive criterion of purity. The effect is apparently visually by examination of thermograms of specimens differing by a few tenths percent in impurity content. A material that is 99% pure is about 20% molten at 3° below the melting point of the pure material (see [accompanying figure](#)).



Superimposed Thermograms Illustrating the Effect of Impurities on DSC Melting Peak Shape

The parameters of melting (melting range,  $\Delta H_f$ , and calculated eutectic purity) are readily obtained from the thermogram of a single melting event using a small test specimen, and the method does not require multiple, precise actual temperature measurements. Thermogram units are directly convertible to heat transfer, millicalories per second.

The lowering of the freezing point in dilute solutions by molecules of nearly equal size is expressed by a modified van't Hoff equation:

$$\frac{dT}{dX_2} = \frac{RT^2}{\Delta H_f} \cdot (K - 1) \quad (1)$$

in which  $T$  = absolute temperature in degrees Kelvin (°K),  $X_2$  = mole fraction of minor component (solute; impurity),  $\Delta H_f$  = molar heat of fusion of the major component,  $R$  = gas constant, and  $K$  = distribution ratio of solute between the solid and liquid phases.

Assuming that the temperature range is small and that no solid solutions are formed ( $K = 0$ ), integration of the van't Hoff equation yields the following relationship between mole fraction of impurity and the melting-point depression:

$$X_2 = \frac{(T_0 - T_m)\Delta H_f}{RT_0^2} \quad (2)$$

in which  $T_o$  = melting point of the pure compound, in  $^{\circ}\text{K}$ , and  $T_m$  = melting point of the test specimen, in  $^{\circ}\text{K}$ .

With no solid solution formation, the concentration of impurity in the liquid phase at any temperature during the melting is inversely proportional to the fraction melted at that temperature, and the melting-point depression is directly proportional to the mole fraction of impurity. A plot of the observed test specimen temperature,  $T_s$ , versus the reciprocal of the fraction melted,  $1/F$ , at temperature  $T_s$ , should yield a straight line with the slope equal to the melting-point depression ( $T_o - T_m$ ). The theoretical melting point of the pure compound is obtained by extrapolation to  $1/F = 0$ :

$$T_s = T_o - \frac{RT_o^2 X_2 (1/F)}{\Delta H_f} \quad (3)$$

Substituting the experimentally obtained values for  $T_o - T_m$ ,  $\Delta H_f$ , and  $T_o$  in Equation 2 yields the mole fraction of the total eutectic impurity, which, when multiplied by 100, gives the mole percentage of total eutectic impurities.

Deviations from the theoretical linear plot also may be due to solid solution formation ( $K \neq 0$ ), so that care must be taken in interpreting the data.

To observe the linear effect of the impurity concentration on the melting-point depression, the impurity must be soluble in the liquid phase or melt of the compound, but insoluble in the solid phase, i.e., no solid solutions are formed. Some chemical similarities are necessary for solubility in the melt. For example, the presence of ionic compounds in neutral organic compounds and the occurrence of thermal decomposition may not be reflected in purity estimates. The extent of these theoretical limitations has been only partially explored.

Impurities present from the synthetic route often are similar to the end product, hence there usually is no problem of solubility in the melt. Impurities consisting of molecules of the same shape, size, and character as those of the major component can fit into the matrix of the major component without disruption of the lattice, forming solid solutions or inclusions; such impurities are not detectable by DSC. Purity estimates are too high in such cases. This is more common with less-ordered crystals as indicated by low heats of fusion.

Impurity levels calculated from thermograms are reproducible and probably reliable within 0.1% for ideal compounds. Melting-point determinations by scanning calorimetry have a reproducibility with a standard deviation of about  $0.2^{\circ}\text{C}$ . Calibration against standards may allow about  $1^{\circ}\text{C}$  accuracy for the melting point, so that this technique is comparable to other procedures.

Compounds that exist in polymorphic form cannot be used in purity determination unless the compound is completely converted to one form. On the other hand, DSC and DTA are inherently useful for detecting, and therefore monitoring, polymorphism.

**Procedure**— The actual procedure and the calculations to be employed are dependent on the particular instrument used. Consult the manufacturer's literature and/or the thermal analysis literature for the most appropriate technique for a given instrument. In any event, it is imperative to keep in mind the limitations of solid solution formation, insolubility in the melt, polymorphism, and decomposition during the analysis.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Antonio Hernandez-Cardoso, B.S.</a> Scientist, Latin American Specialist 1-301-816-8308	(GC05) General Chapters 05

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#### 905 UNIFORMITY OF DOSAGE UNITS

[note—In this chapter, unit and dosage unit are synonymous.]

To ensure the consistency of dosage units, each unit in a batch should have a drug substance content within a narrow range around the label claim. Dosage units are defined as dosage forms containing a single dose or a part of a dose of drug substance in each unit. The uniformity of dosage units specification is not intended to apply to suspensions, emulsions, or gels in unit-dose containers intended for topical administration.

The term "uniformity of dosage unit" is defined as the degree of uniformity in the amount of the drug substance among dosage units. Therefore, the requirements of this chapter apply to each drug substance being comprised in dosage units containing one or more drug substances, unless otherwise specified in the individual monograph.

The uniformity of dosage units can be demonstrated by either of two methods, Content Uniformity or Weight Variation (see [Table 1](#)). The test for Content Uniformity is based on the assay of the individual content of drug substance(s) in a number of individual dosage units to determine whether the individual content is within the limits set. The Content Uniformity method may be applied in all cases. The test for Content Uniformity is required for those dosage forms described in (C1)–(C6) below:

- (C1) coated tablets, other than film-coated tablets containing 25 mg or more of a drug substance that comprises 25% or more (by weight) of one tablet;
- (C2) transdermal systems;
- (C3) suspensions or emulsions or gels in single-unit containers or in soft capsules that are intended for systemic administration only (not for those drug products that are intended for topical administration);
- (C4) inhalations (other than solutions for inhalation packaged in glass or plastic ampuls and intended for use in nebulizers) packaged in premetered dosage units. For inhalers and premetered dosage units labeled for use with a named inhalation device, also see [Aerosols, Nasal Sprays, Metered-Dose Inhalers, and Dry Powder Inhalers](#) (601);
- (C5) solids (including sterile solids) that are packaged in single-unit containers and that contain active or inactive added substances, except that the test for Weight Variation may be applied in the special cases stated in (W3) below; and
- (C6) suppositories.

The test for Weight Variation is applicable for the following dosage forms:

- (W1) solutions for inhalation that are packaged in glass or plastic ampuls and intended for use in nebulizers, and oral solutions packaged in unit-dose containers and into soft capsules;
- (W2) solids (including sterile solids) that are packaged in single-unit containers and contain no added substances, whether active or inactive;
- (W3) solids (including sterile solids) that are packaged in single-unit containers, with or without added substances, whether active or inactive, that have been prepared from true solutions and freeze-dried in the final containers and are labeled to indicate this method of preparation; and
- (W4) hard capsules, uncoated tablets, or film-coated tablets, containing 25 mg or more of a drug substance comprising 25% or more, by weight, of the dosage unit or, in the case of hard capsules, the capsule contents, except that uniformity of other drug substances present in lesser proportions is demonstrated by meeting Content Uniformity requirements.

The test for Content Uniformity is required for all dosage forms not meeting the above conditions for the Weight Variation test. Where compliance with the Content Uniformity test is required, then, by application of the provision for use of alternative methods provided in the General Notices section of this Pharmacopeia, it is possible for manufacturers to ensure this compliance by application of the Weight Variation test where the concentration relative standard deviation (RSD) of the drug substance in the final dosage units is not more than 2%. This RSD determination may be based on the manufacturer's process validation and product development data. The concentration RSD is the RSD of the concentration per dosage unit (w/w or w/v), where concentration per dosage unit equals the assay result per dosage unit divided by the individual dosage unit weight. See the RSD formula in [Table 2](#).

Where the Weight Variation test is used in this way, the product must, if tested, nevertheless comply with the official compendial test for Content Uniformity.



Table 1. Application of Content Uniformity (CU) and Weight Variation (WV) Tests for Dosage Forms

Dosage Form	Type	Subtype	Dose & Ratio of Drug Substance	
			$\geq 25$ mg & $\geq 25\%$	<25 mg or <25%
Tablets	Uncoated		WV	CU
	Coated	Film	WV	CU
		Others	CU	CU
Capsules	Hard		WV	CU
	Soft	Suspension, emulsion, or gel	CU	CU
		Solutions	WV	WV
Solids in single-unit containers	Single component		WV	WV
	Multiple components	Solution freeze-dried in final container	WV	WV
		Others	CU	CU
Suspension, emulsion, or gel for systemic use only, packaged in single-unit containers			CU	CU
Solutions for inhalation packaged in glass or plastic ampuls and intended for use in nebulizers, and oral solutions packaged in unit-dose containers and into soft capsules			WV	WV
Inhalations (other than solutions for inhalation packaged in glass or plastic ampuls and intended for use in nebulizers) packaged in premetered dosage units			CU	CU
Transdermal systems			CU	CU
Suppositories			CU	CU
Others			CU	CU

## CONTENT UNIFORMITY

Select not fewer than 30 units, and proceed as follows for the dosage form designated. Where the amount of drug substance in a single dosage unit differs from that required in the Assay, adjust the degree of dilution of the solutions and/or the volume of aliquots so that the concentration of the drug substances in the final solution is of the same order as that obtained in the Assay procedure; or, in the case of a titrimetric assay, use a titrant of a different concentration, if necessary, so that an adequate volume of titrant is required (see [Titrimetry](#) (541)); see also Procedures under Tests and Assays in the General Notices and Requirements. If any such modifications are made in the Assay procedure set forth in the individual monograph, make the appropriate corresponding changes in the calculation formula and titration factor.

Where a special Procedure for content uniformity is specified in the test for Uniformity of dosage units in the individual monograph, make any necessary correction of the results obtained as follows.

1. Prepare a composite specimen of a sufficient number of dosage units to provide the amount of specimen called for in the Assay in the individual monograph plus the amount required for the special Procedure for content uniformity in the monograph by finely powdering tablets or mixing the contents of capsules or oral solutions, suspensions, emulsions, gels, or solids in single-unit containers to obtain a homogeneous mixture. If a homogeneous mixture cannot be obtained in this manner, use suitable solvents or other procedures to prepare a solution containing all of the drug substance, and use appropriate aliquot portions of this solution for the specified procedures.
2. Assay separate, accurately measured portions of the composite specimen of capsules or tablets or suspensions or inhalations or solids in single-unit containers, both (a) as directed in the Assay, and (b) using the special Procedure for content uniformity in the monograph.
3. Calculate the weight of drug substance equivalent to 1 average dosage unit, by (a) using the results obtained by the Assay procedure, and by (b) using the results obtained by the special procedure.
4. Calculate the correction factor, F, by the formula:

$$F = W/P$$

in which W is the weight of drug substance equivalent to 1 average dosage unit obtained by the Assay procedure, and P is the weight of drug substance equivalent to 1 average dosage unit obtained by the special procedure. If

$$\frac{100|W - P|}{W}$$

is greater than 10, the use of a correction factor is not valid.

5. The correction factor is to be applied only if F is not less than 1.030 nor greater than 1.100, or not less than 0.900 nor greater than 0.970. If F is between 0.970 and 1.030, no correction is required.
6. If F lies between 1.030 and 1.100, or between 0.900 and 0.970, calculate the weight of drug substance in each dosage unit by multiplying each of the weights found using the special procedure by F.

Uncoated, Coated, or Molded Tablets, Capsules, Oral Solutions in Unit-Dose Containers, Suspensions or Emulsions or Gels in Single-Unit Containers (that are intended for systemic administration only), and Solids (including Sterile Solids) in Single-Unit Containers— Assay 10 units individually as directed in the Assay in the individual monograph, unless otherwise specified in the Procedure for content uniformity in the individual monograph. Calculate the acceptance value as directed below.

For oral solutions in unit-dose containers, and for suspensions, emulsions, or gels in single-unit containers that are intended for systemic administration only, conduct the Assay on the amount of well-mixed material that drains from an individual container in not more than 5 seconds, or for highly viscous products, conduct the Assay on the amount of well-mixed material that is obtained by quantitatively removing the contents from an individual container, and express the results as the delivered dose.

Calculation of Acceptance Value— Calculate the acceptance value by the formula:



$$|M - \bar{X}| + ks$$

in which the terms are as defined in [Table 2](#).

Table 2

Variable	Definition	Conditions	Value
X	Mean of individual contents ( $\chi_1, \chi_2, \dots, \chi_n$ ), expressed as a percentage of the label claim		
$\chi_1, \chi_2, \dots, \chi_n$	Individual contents of the units tested, expressed as a percentage of the label claim		
n	Sample size (number of units in a sample)		
k	Acceptability constant	If $n = 10$ , then $k =$ If $n = 30$ , then $k =$	2.4 2.0
s	Sample standard deviation		$\left[ \frac{\sum_{i=1}^n (\chi_i - \bar{X})^2}{n-1} \right]^{\frac{1}{2}}$
RSD	Relative standard deviation (the sample standard deviation expressed as a percentage of the mean)		$\frac{100s}{\bar{X}}$
M (case 1) to be applied when $T \leq 101.5$	Reference value	If $98.5 \leq X \leq 101.5$ , then	$M = X$ ( $AV = ks$ )
		If $X < 98.5$ , then	$M = 98.5\%$ ( $AV = 98.5 - X + ks$ )
		If $X > 101.5$ , then	$M = 101.5\%$ ( $AV = X - 101.5 + ks$ )
M (case 2) to be applied when $T > 101.5$	Reference value	If $98.5 \leq X \leq T$ , then	$M = X$ ( $AV = ks$ )
		If $X < 98.5$ , then	$M = 98.5\%$ ( $AV = 98.5 - X + ks$ )
		If $X > T$ , then	$M = T\%$ ( $AV = X - T + ks$ )
Acceptance value (AV)			general formula: $ M - \bar{X}  + ks$ (Calculations are specified above for the different cases.)
L1	Maximum allowed acceptance value		$L1 = 15.0$ unless otherwise specified in the individual monograph
L2	Maximum allowed range for deviation of each dosage unit tested from the calculated value of M	On the low side, no dosage unit result can be less than $[1 - (0.01)(L2)]M$ , while on the high side no dosage unit result can be greater than $[1 + (0.01)(L2)]M$ . (This is based on an L2 value of 25.0.)	$L2 = 25.0$ unless otherwise specified in the individual monograph
T	Target content per dosage unit at the time of manufacture, expressed as a percentage of the label claim. For purposes of this Pharmacopeia, unless otherwise specified in the individual monograph, T is the average of the limits specified in the potency definition in the individual monograph.		

Suppositories, Transdermal Systems, and Inhalations Packaged in Premetered Dosage Units— [note—Acceptance value calculations are not required for these dosage forms.] Assay 10 units individually as directed in the Assay in the individual monograph, unless otherwise specified in the Procedure for content uniformity.



## WEIGHT VARIATION

Select not fewer than 30 dosage units, and proceed as follows for the dosage form designated. The result of the Assay, obtained as directed in the individual monograph, is designated as result A, expressed as % of label claim (see Calculation of Acceptance Value). Assume that the concentration (weight of drug substance per weight of dosage unit) is uniform. [note—Specimens other than these test units may be drawn from the same batch for assay determinations.]

Uncoated or Film-Coated Tablets— Accurately weigh 10 tablets individually. Calculate the drug substance content, expressed as % of label claim, of each tablet from the weight of the individual tablet and the result of the Assay. Calculate the acceptance value.

Hard Capsules— Accurately weigh 10 capsules individually, taking care to preserve the identity of each capsule. Remove the contents of each capsule by a suitable means. Accurately weigh the emptied shells individually, and calculate for each capsule the net weight of its contents by subtracting the weight of the shell from the respective gross weight. Calculate the drug substance content, expressed as % of label claim, of each capsule from the net weight of the individual capsule content and the result of the Assay. Calculate the acceptance value.

Soft Capsules— Accurately weigh 10 intact capsules individually to obtain their gross weights, taking care to preserve the identity of each capsule. Then cut open the capsules by means of a suitable clean, dry cutting instrument such as scissors or a sharp open blade, and remove the contents by washing with a suitable solvent. Allow the occluded solvent to evaporate from the shells at room temperature over a period of about 30 minutes, taking precautions to avoid uptake or loss of moisture. Weigh the individual shells, and calculate the net contents. Calculate the drug substance content, expressed as % of label claim, in each capsule from the net weight of product removed from the individual capsules and the result of the Assay. Calculate the acceptance value.

Solids (Including Sterile Solids) in Single-Unit Containers— Proceed as directed for Hard Capsules, treating each unit as described therein. Calculate the acceptance value.

Oral Solutions Packaged in Unit-Dose Containers— Accurately weigh the amount of liquid that drains in not more than 5 seconds from each of 10 individual containers. If necessary, compute the equivalent volume after determining the density. Calculate the drug substance content, expressed as % of label claim, in the liquid drained from each unit from the net weight of the individual container content and the result of the Assay. Calculate the acceptance value.

Calculation of Acceptance Value— Calculate the acceptance value as shown in Content Uniformity, except that the individual contents of the units are replaced with the individual estimated contents defined below.

$\bar{x}_1, \bar{x}_2, \dots, \bar{x}_n$  = individual estimated contents of the units tested, where  $\bar{x}_i = w_i \times A / W$ ,  
 $w_1, w_2, \dots, w_n$  = individual weights of the units tested, for weight variation,  
 $A$  = content of drug substance (% of label claim) determined as described in the Assay, and  
 $W$  = mean of individual weights ( $w_1, w_2, \dots, w_n$ ) of the units used in the Assay.

Solutions for Inhalation Packaged in Glass or Plastic Ampuls and Intended for Use in Nebulizers— [note—Acceptance value calculations are not required for these dosage forms.] Accurately weigh 10 containers individually, taking care to preserve the identity of each container. Remove the contents of each container by a suitable means. Accurately weigh the emptied containers individually, and calculate for each container the net weight of its contents by subtracting the weight of the container from the respective gross weight. From the results of the Assay, obtained as directed in the individual monograph, calculate the drug substance content, expressed as % of label claim, in each of the containers.

## CRITERIA

Apply the following criteria, unless otherwise specified in the individual monograph.

Uncoated, Coated, or Molded Tablets, Capsules, Oral Solutions in Unit-Dose Containers, Suspensions or Emulsions or Gels in Single-Unit Containers (that are intended for systemic administration only), and Solids (including Sterile Solids) in Single-Unit Containers— The requirements for dosage uniformity are met if the acceptance value of the first 10 dosage units is less than or equal to L1%. If the acceptance value is greater than L1%, test the next 20 units, and calculate the acceptance value. The requirements are met if the final acceptance value of the 30 dosage units is less than or equal to L1%, and no individual content of any dosage unit is less than  $[1 - (0.01)(L2)] M$  nor more than  $[1 + (0.01)(L2)] M$  as specified in the Calculation of Acceptance Value under Content Uniformity or under Weight Variation. Unless otherwise specified in the individual monograph, L1 is 15.0 and L2 is 25.0.

## Suppositories—

Limit A (if the average of the limits specified in the potency definition in the individual monograph is 100.0 percent or less)—Unless otherwise specified in the individual monograph, the requirements for dosage uniformity are met if the amount of the drug substance in each of the 10 dosage units as determined from the Content Uniformity method lies within the range of 85.0% to 115.0% of the label claim, and the RSD is less than or equal to 6.0%.

If 1 unit is outside the range of 85.0% to 115.0% of label claim, and no unit is outside the range of 75.0% to 125.0% of label claim, or if the RSD is greater than 6.0%, or if both conditions prevail, test 20 additional units. The requirements are met if not more than 1 unit of the 30 is outside the range of 85.0% to 115.0% of label claim, and no unit is outside the range of 75.0% to 125.0% of label claim and the RSD of the 30 dosage units does not exceed 7.8%.

Limit B (if the average of the limits specified in the potency definition in the individual monograph is greater than 100.0 percent)—

1. If the average value of the dosage units tested is 100.0 percent or less, the requirements are as in Limit A.
2. If the average value of the dosage units tested is greater than or equal to the average of the limits specified in the potency definition in the individual monograph, the requirements are as specified under Limit A, except that the words "label claim" are replaced by the words "label claim multiplied by the average of the limits specified in the potency definition in the monograph divided by 100".
3. If the average value of the dosage units tested is between 100 percent and the average of the limits specified in the potency definition in the individual monograph, the requirements are as specified under Limit A, except that the words "label claim" are replaced by the words "label claim multiplied by the average value of the dosage units tested (expressed as a percent of label claim) divided by 100".

## Transdermal Systems and Inhalations Packaged in Premetered Dosage Units—

Limit A (if the average of the limits specified in the potency definition in the individual monograph is 100.0 percent or less)—Unless otherwise specified in the individual monograph, the requirements for dosage uniformity are met if the amount of the drug substance in not fewer than 9 of the 10 dosage units as determined from the Content Uniformity method (or, in the case of solutions for inhalation packaged in glass or plastic ampuls and intended for use in nebulizers, from either the Content Uniformity or the Weight Variation method) lies within the range of 85.0% to 115.0% of label claim, and no unit is outside the range of 75.0% to 125.0% of label claim, and the RSD of the 10 dosage units is less than or equal to 6.0%.

If 2 or 3 dosage units are outside the range of 85.0% to 115.0% of label claim, but not outside the range of 75.0% to 125.0% of label claim, or if the RSD is greater than 6.0% or if both conditions prevail, test 20 additional units. The requirements are met if not more than 3 units of the 30 are outside the range of 85.0% to 115.0% of label claim and no unit is outside the range of 75.0% to 125.0% of label claim, and the RSD of the 30 dosage units does not exceed 7.8%.

Limit B (if the average of the limits specified in the potency definition in the individual monograph is greater than 100.0 percent)—

1. If the average value of the dosage units tested is 100.0 percent or less, the requirements are as in Limit A.
2. If the average value of the dosage units tested is greater than or equal to the average of the limits specified in the potency definition in the individual monograph, the requirements are as specified under Limit A, except that the words "label claim" are replaced by the words "label claim multiplied by the average of the limits specified in the potency definition in the monograph divided by 100".
3. If the average value of the dosage units tested is between 100 percent and the average of the limits specified in the potency definition in the individual monograph, the requirements are as specified under Limit A, except that the words "label claim" are replaced by the words "label claim multiplied by the average value of the dosage units tested (expressed as a percent of label claim) divided by 100".

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.



Topic/Question	Contact	Expert Committee
General Chapter	William E. Brown Senior Scientist 1-301-816-8380	(PDF05) Pharmaceutical Dosage Forms 05

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## 911 VISCOSITY

Viscosity is a property of liquids that is closely related to the resistance to flow. It is defined in terms of the force required to move one plane surface continuously past another under specified steady-state conditions when the space between is filled by the liquid in question. It is defined as the shear stress divided by the rate of shear strain. The basic unit is the poise; however, viscosities commonly encountered represent fractions of the poise, so that the centipoise (1 poise = 100 centipoises) proves to be the more convenient unit. The specifying of temperature is important because viscosity changes with temperature; in general, viscosity decreases as temperature is raised. While on the absolute scale viscosity is measured in poises or centipoises, for convenience the kinematic scale, in which the units are stokes and centistokes (1 stoke = 100 centistokes) commonly is used. To obtain the kinematic viscosity from the absolute viscosity, the latter is divided by the density of the liquid at the same temperature, i.e., kinematic viscosity = (absolute viscosity)/(density). The sizes of the units are such that viscosities in the ordinary ranges are conveniently expressed in centistokes. The approximate viscosity in centistokes at room temperature of ether is 0.2; of water, 1; of kerosene, 2.5; of mineral oil, 20 to 70; and of honey, 10,000.

Absolute viscosity can be measured directly if accurate dimensions of the measuring instruments are known, but it is more common practice to calibrate the instrument with a liquid of known viscosity and to determine the viscosity of the unknown fluid by comparison with that of the known.

Many substances, such as the gums employed in pharmacy, have variable viscosity, and most of them are less resistant to flow at higher flow rates. In such cases, a given set of conditions is selected for measurement, and the measurement obtained is considered to be an apparent viscosity. Since a change in the conditions of measurement would yield a different value for the apparent viscosity of such substances, the instrument dimensions and conditions for measurement must be closely adhered to by the operator.

**Measurement of Viscosity**— The usual method for measurement of viscosity involves the determination of the time required for a given volume of liquid to flow through a capillary. Many capillary-tube viscosimeters have been devised, but Ostwald and Ubbelohde viscosimeters are among the most frequently used. Several types are described, with directions for their use, by the American Society for Testing and Materials (ASTM, D-445). The viscosity of oils is expressed on arbitrary scales that vary from one country to another, there being several corresponding instruments. The most widely used are the Redwood No. I and No. II, the Engler, the Saybolt Universal, and the Saybolt Furol. Each of these instruments uses arbitrary units that bear the name of the instrument. Standard temperatures are adopted as a matter of convenience with these instruments. For the Saybolt instruments, measurements usually are made at 100°F and 210°F; Redwood instruments may be used at several temperatures up to 250°F; and values obtained on the Engler instrument usually are reported at 20°C and 50°C. A particularly convenient and rapid type of instrument is a rotational viscosimeter, which utilizes a bob or spindle immersed in the test specimen and measures the resistance to movement of the rotating part. Different spindles are available for given viscosity ranges, and several rotational speeds generally are available. Other rotational instruments may have a stationary bob and a rotating cup. The Brookfield, Rotovisco, and Stormer viscosimeters are examples of rotating-bob instruments, and the MacMichael is an example of the rotating-cup instrument. Numerous other rotational instruments of advanced design with special devices for reading or recording, and with wide ranges of rotational speed, have been devised.

Where only a particular type of instrument is suitable, the individual monograph so indicates.

For measurement of viscosity or apparent viscosity, the temperature of the substance being measured must be accurately controlled, since small temperature changes may lead to marked changes in viscosity. For usual pharmaceutical purposes, the temperature should be held to within  $\pm 0.1^\circ$ .

**Procedure for Cellulose Derivatives**— Measurement of the viscosity of solutions of the high-viscosity types of methylcellulose is a special case, since they are too viscous for the commonly available viscosimeters. The Ubbelohde viscosimeter may be adapted (cf. ASTM, D-1347) to the measurement of the ranges of viscosity encountered in methylcellulose solutions.

**Calibration of Capillary-Type Viscosimeters**— Determine the viscosimeter constant,  $k$ , for each viscosimeter by the use of an oil of known viscosity.\*

**Ostwald-Type Viscosimeter**— Fill the tube with the exact amount of oil (adjusted to  $20.0 \pm 0.1^\circ$ ) as specified by the manufacturer. Adjust the meniscus of the column of liquid in the capillary tube to the level of the top graduation line with the aid of either pressure or suction. Open both the filling and capillary tubes in order to permit the liquid to flow into the reservoir against atmospheric pressure. [note—Failure to open either of these tubes will yield false values.] Record the time, in seconds, for liquid to flow from the upper mark to the lower mark in the capillary tube.

**Ubbelohde-Type Viscosimeter**— Place a quantity of the oil (adjusted to  $20.0 \pm 0.1^\circ$ ) in the filling tube, and transfer to the capillary tube by gentle suction, taking care to prevent bubble formation in the liquid by keeping the air vent tube closed. Adjust the meniscus of the column of liquid in the capillary tube to the level of the top graduation line. Open both the vent and capillary tubes in order to permit the liquid to flow into the reservoir against atmospheric pressure. [note—Failure to open the vent tube before releasing the capillary tube will yield false values.] Record the time, in seconds, for the liquid to flow from the upper mark to the lower mark in the capillary tube.

## Calculations—

Calculate the viscosimeter constant,  $k$ , from the equation:

$$k = v / d t$$

in which  $v$  is the known viscosity of the liquid in centipoises,  $d$  is the specific gravity of the liquid tested at  $20^\circ/20^\circ$ , and  $t$  is the time in seconds for the liquid to pass from the upper mark to the lower mark.

If a viscosimeter is repaired, it must be recalibrated, since even minor repairs frequently cause significant changes in the value of its constant,  $k$ .

\* Oils of known viscosities may be obtained from the Cannon Instrument Co., Box 16, State College, PA 16801. For methylcellulose, choose an oil the viscosity of which is as close as possible to that of the type of methylcellulose to be determined.

**Auxiliary Information**— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	Hong Wang, Ph.D. Scientist 1-301-816-8351	(EGC05) Excipient General Chapters

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## 921 WATER DETERMINATION

Many Pharmacopeial articles either are hydrates or contain water in adsorbed form. As a result, the determination of the water content is important in demonstrating compliance with



Pharmacopeial standards. Generally one of the methods given below is called for in the individual monograph, depending upon the nature of the article. In rare cases, a choice is allowed between two methods. When the article contains water of hydration, the Method I (Titrimetric), the Method II (Azeotropic), or the Method III (Gravimetric) is employed, as directed in the individual monograph, and the requirement is given under the heading Water.

The heading Loss on drying (see [Loss on Drying](#) 731) is used in those cases where the loss sustained on heating may be not entirely water.

#### METHOD I (TITRIMETRIC)

Determine the water by Method Ia, unless otherwise specified in the individual monograph.

##### Method Ia (Direct Titration)

**Principle**— The titrimetric determination of water is based upon the quantitative reaction of water with an anhydrous solution of sulfur dioxide and iodine in the presence of a buffer that reacts with hydrogen ions.

In the original titrimetric solution, known as Karl Fischer Reagent, the sulfur dioxide and iodine are dissolved in pyridine and methanol. The test specimen may be titrated with the Reagent directly, or the analysis may be carried out by a residual titration procedure. The stoichiometry of the reaction is not exact, and the reproducibility of a determination depends upon such factors as the relative concentrations of the Reagent ingredients, the nature of the inert solvent used to dissolve the test specimen, and the technique used in the particular determination. Therefore, an empirically standardized technique is used in order to achieve the desired accuracy. Precision in the method is governed largely by the extent to which atmospheric moisture is excluded from the system. The titration of water is usually carried out with the use of anhydrous methanol as the solvent for the test specimen; however, other suitable solvents may be used for special or unusual test specimens.

**Apparatus**— Any apparatus may be used that provides for adequate exclusion of atmospheric moisture and determination of the endpoint. In the case of a colorless solution that is titrated directly, the endpoint may be observed visually as a change in color from canary yellow to amber. The reverse is observed in the case of a test specimen that is titrated residually. More commonly, however, the endpoint is determined electrometrically with an apparatus employing a simple electrical circuit that serves to impress about 200 mV of applied potential between a pair of platinum electrodes immersed in the solution to be titrated. At the endpoint of the titration a slight excess of the reagent increases the flow of current to between 50 and 150 microamperes for 30 seconds to 30 minutes, depending upon the solution being titrated. The time is shortest for substances that dissolve in the reagent. With some automatic titrators, the abrupt change in current or potential at the endpoint serves to close a solenoid-operated valve that controls the buret delivering the titrant. Commercially available apparatus generally comprises a closed system consisting of one or two automatic burets and a tightly covered titration vessel fitted with the necessary electrodes and a magnetic stirrer. The air in the system is kept dry with a suitable desiccant, and the titration vessel may be purged by means of a stream of dry nitrogen or current of dry air.

**Reagent**— Prepare the Karl Fischer Reagent as follows. Add 125 g of iodine to a solution containing 670 mL of methanol and 170 mL of pyridine, and cool. Place 100 mL of pyridine in a 250-mL graduated cylinder, and, keeping the pyridine cold in an ice bath, pass in dry sulfur dioxide until the volume reaches 200 mL. Slowly add this solution, with shaking, to the cooled iodine mixture. Shake to dissolve the iodine, transfer the solution to the apparatus, and allow the solution to stand overnight before standardizing. One mL of this solution when freshly prepared is equivalent to approximately 5 mg of water, but it deteriorates gradually; therefore, standardize it within 1 hour before use, or daily if in continuous use. Protect from light while in use. Store any bulk stock of the reagent in a suitably sealed, glass-stoppered container, fully protected from light, and under refrigeration.

A commercially available, stabilized solution of Karl Fischer type reagent may be used. Commercially available reagents containing solvents or bases other than pyridine or alcohols other than methanol may be used also. These may be single solutions or reagents formed in situ by combining the components of the reagents present in two discrete solutions. The diluted Reagent called for in some monographs should be diluted as directed by the manufacturer. Either methanol or other suitable solvent, such as ethylene glycol monomethyl ether, may be used as the diluent.

**Test Preparation**— Unless otherwise specified in the individual monograph, use an accurately weighed or measured amount of the specimen under test estimated to contain 2 to 250 mg of water. The amount of water depends on the water equivalency factor of the Reagent and on the method of endpoint determination. In most cases, the minimum amount of specimen, in mg, can be estimated using the formula:

$$FCV / KF$$

in which F is the water equivalency factor of the Reagent, in mg per mL; C is the used volume, in percent, of the capacity of the buret; V is the buret volume, in mL; and KF is the limit or reasonable expected water content in the sample, in percent. C is between 30% and 100% for manual titration, and between 10% and 100% for the instrumental method endpoint determination.

Where the specimen under test is an aerosol with propellant, store it in a freezer for not less than 2 hours, open the container, and test 10.0 mL of the well-mixed specimen. In titrating the specimen, determine the endpoint at a temperature of 10° or higher.

Where the specimen under test is capsules, use a portion of the mixed contents of not fewer than 4 capsules.

Where the specimen under test is tablets, use powder from not fewer than 4 tablets ground to a fine powder in an atmosphere of temperature and relative humidity known not to influence the results.

Where the monograph specifies that the specimen under test is hygroscopic, use a dry syringe to inject an appropriate volume of methanol, or other suitable solvent, accurately measured, into a tared container, and shake to dissolve the specimen. Using the same syringe, remove the solution from the container and transfer it to a titration vessel prepared as directed for Procedure. Repeat the procedure with a second portion of methanol, or other suitable solvent, accurately measured, add this washing to the titration vessel, and immediately titrate. Determine the water content, in mg, of a portion of solvent of the same total volume as that used to dissolve the specimen and to wash the container and syringe, as directed for Standardization of Water Solution for Residual Titrations, and subtract this value from the water content, in mg, obtained in the titration of the specimen under test. Dry the container and its closure at 100° for 3 hours, allow to cool in a desiccator, and weigh. Determine the weight of specimen tested from the difference in weight from the initial weight of the container.

**Standardization of the Reagent**— Place enough methanol or other suitable solvent in the titration vessel to cover the electrodes, and add sufficient Reagent to give the characteristic endpoint color, or 100 ± 50 microamperes of direct current at about 200 mV of applied potential.

For determination of trace amounts of water (less than 1%), it is preferable to use Reagent with a water equivalency factor of not more than 2.0. Sodium tartrate may be used as a convenient water reference substance. Quickly add 75 to 125 mg of sodium tartrate ( $C_4H_4Na_2O_6 \cdot 2H_2O$ ), accurately weighed by difference, and titrate to the endpoint. The water equivalence factor F, in mg of water per mL of reagent, is given by the formula:

$$2(18.02/230.08)(W/V)$$

in which 18.02 and 230.08 are the molecular weights of water and sodium tartrate dihydrate, respectively; W is the weight, in mg, of sodium tartrate dihydrate; and V is the volume, in mL, of the Reagent consumed in the second titration.

For the precise determination of significant amounts of water (1% or more), use Purified Water as the reference substance. Quickly add between 25 and 250 mg of water, accurately weighed by difference, from a weighing pipet or from a precalibrated syringe or micropipet, the amount taken being governed by the reagent strength and the buret size, as referred to under [Volumetric Apparatus](#) 31. Titrate to the endpoint. Calculate the water equivalence factor, F, in mg of water per mL of reagent, by the formula:

$$W/V$$

in which W is the weight, in mg, of the water; and V is the volume, in mL, of the reagent required.

**Procedure**— Unless otherwise specified, transfer 35 to 40 mL of methanol or other suitable solvent to the titration vessel, and titrate with the Reagent to the electrometric or visual endpoint to consume any moisture that may be present. (Disregard the volume consumed, since it does not enter into the calculations.) Quickly add the Test Preparation, mix, and again titrate with the Reagent to the electrometric or visual endpoint. Calculate the water content of the specimen, in mg, taken by the formula:

$$SF$$



in which S is the volume, in mL, of the Reagent consumed in the second titration; and F is the water equivalence factor of the Reagent.

#### Method Ib (Residual Titration)

**Principle**— See the information given in the section Principle under Method Ia. In the residual titration, excess Reagent is added to the test specimen, sufficient time is allowed for the reaction to reach completion, and the unconsumed Reagent is titrated with a standard solution of water in a solvent such as methanol. The residual titration procedure is applicable generally and avoids the difficulties that may be encountered in the direct titration of substances from which the bound water is released slowly.

**Apparatus, Reagent, and Test Preparation**— Use Method Ia.

**Standardization of Water Solution for Residual Titration**— Prepare a Water Solution by diluting 2 mL of water with methanol or other suitable solvent to 1000 mL. Standardize this solution by titrating 25.0 mL with the Reagent, previously standardized as directed under Standardization of the Reagent. Calculate the water content, in mg per mL, of the Water Solution taken by the formula:

$$VF/25$$

in which V' is the volume of the Reagent consumed, and F is the water equivalence factor of the Reagent. Determine the water content of the Water Solution weekly, and standardize the Reagent against it periodically as needed.

**Procedure**— Where the individual monograph specifies that the water content is to be determined by Method Ib, transfer 35 to 40 mL of methanol or other suitable solvent to the titration vessel, and titrate with the Reagent to the electrometric or visual endpoint. Quickly add the Test Preparation, mix, and add an accurately measured excess of the Reagent. Allow sufficient time for the reaction to reach completion, and titrate the unconsumed Reagent with standardized Water Solution to the electrometric or visual endpoint. Calculate the water content of the specimen, in mg, taken by the formula:

$$F(X' - XR)$$

in which F is the water equivalence factor of the Reagent; X' is the volume, in mL, of the Reagent added after introduction of the specimen; X is the volume, in mL, of standardized Water Solution required to neutralize the unconsumed Reagent; and R is the ratio, V/25 (mL Reagent/mL Water Solution), determined from the Standardization of Water Solution for Residual Titration.

#### Method Ic (Coulometric Titration)

**Principle**— The Karl Fischer reaction is used in the coulometric determination of water. Iodine, however, is not added in the form of a volumetric solution but is produced in an iodide-containing solution by anodic oxidation. The reaction cell usually consists of a large anode compartment and a small cathode compartment that are separated by a diaphragm. Other suitable types of reaction cells (e.g., without diaphragms) may also be used. Each compartment has a platinum electrode that conducts current through the cell. Iodine, which is produced at the anode electrode, immediately reacts with water present in the compartment. When all the water has been consumed, an excess of iodine occurs, which usually is detected electrometrically, thus indicating the endpoint. Moisture is eliminated from the system by pre-electrolysis. Changing the Karl Fischer solution after each determination is not necessary since individual determinations can be carried out in succession in the same reagent solution. A requirement for this method is that each component of the test specimen is compatible with the other components, and no side reactions take place. Samples are usually transferred into the vessel as solutions by means of injection through a septum. Gases can be introduced into the cell by means of a suitable gas inlet tube. Precision in the method is predominantly governed by the extent to which atmospheric moisture is excluded from the system; thus, the introduction of solids into the cell is not recommended, unless elaborate precautions are taken, such as working in a glove-box in an atmosphere of dry inert gas. Control of the system may be monitored by measuring the amount of baseline drift. This method is particularly suited to chemically inert substances like hydrocarbons, alcohols, and ethers. In comparison with the volumetric Karl Fischer titration, coulometry is a micro-method.

**Apparatus**— Any commercially available apparatus consisting of an absolutely tight system fitted with the necessary electrodes and a magnetic stirrer is appropriate. The instrument's microprocessor controls the analytical procedure and displays the results. Calibration of the instrument is not necessary, as the current consumed can be measured absolutely.

**Reagent**— See Reagent under Method Ia.

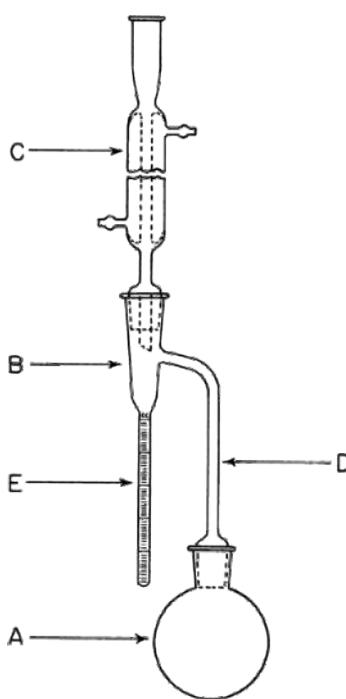
**Test Preparation**— Where the specimen is a soluble solid, dissolve an appropriate quantity, accurately weighed, in anhydrous methanol or other suitable solvents. Liquids may be used as such or as accurately prepared solutions in appropriate anhydrous solvents.

Where the specimen is an insoluble solid, the water may be extracted using a suitable anhydrous solvent from which an appropriate quantity, accurately weighed, may be injected into the anolyte solution. Alternatively an evaporation technique may be used in which water is released and evaporated by heating the specimen in a tube in a stream of dry inert gas, this gas being then passed into the cell.

**Procedure**— Using a dry syringe, quickly inject the Test Preparation, accurately measured and estimated to contain 0.5 to 5 mg of water, or as recommended by the instrument manufacturer into the anolyte, mix, and perform the coulometric titration to the electrometric endpoint. Read the water content of the Test Preparation directly from the instrument's display, and calculate the percentage that is present in the substance. Perform a blank determination, and make any necessary corrections.

#### METHOD II (AZEOTROPIC—TOLUENE DISTILLATION)

**Apparatus**— Use a 500-mL glass flask A connected by means of a trap B to a reflux condenser C by ground glass joints (see [Figure](#)).



Toluene Moisture Apparatus

The critical dimensions of the parts of the apparatus are as follows. The connecting tube D is 9 to 11 mm in internal diameter. The trap is 235 to 240 mm in length. The condenser, if of the straight-tube type, is approximately 400 mm in length and not less than 8 mm in bore diameter. The receiving tube E has a 5-mL capacity, and its cylindrical portion, 146 to 156 mm in length, is graduated in 0.1-mL subdivisions, so that the error of reading is not greater than 0.05 mL for any indicated volume. The source of heat is preferably an electric heater with rheostat control or an oil bath. The upper portion of the flask and the connecting tube may be insulated.

Clean the receiving tube and the condenser with chromic acid cleansing mixture, thoroughly rinse with water, and dry in an oven. Prepare the toluene to be used by first shaking with a small quantity of water, separating the excess water, and distilling the toluene.

Procedure— Place in the dry flask a quantity of the substance, weighed accurately to the nearest centigram, which is expected to yield 2 to 4 mL of water. If the substance is of a pasty character, weigh it in a boat of metal foil of a size that will just pass through the neck of the flask. If the substance is likely to cause bumping, add enough dry, washed sand to cover the bottom of the flask, or a number of capillary melting-point tubes, about 100 mm in length, sealed at the upper end. Place about 200 mL of toluene in the flask, connect the apparatus, and fill the receiving tube E with toluene poured through the top of the condenser. Heat the flask gently for 15 minutes and, when the toluene begins to boil, distill at the rate of about 2 drops per second until most of the water has passed over, then increase the rate of distillation to about 4 drops per second. When the water has apparently all distilled over, rinse the inside of the condenser tube with toluene while brushing down the tube with a tube brush attached to a copper wire and saturated with toluene. Continue the distillation for 5 minutes,

then remove the heat, and allow the receiving tube to cool to room temperature. If any droplets of water adhere to the walls of the receiving tube, scrub them down with a brush consisting of a rubber band wrapped around a copper wire and wetted with toluene. When the water and toluene have separated completely, read the volume of water, and calculate the percentage that was present in the substance.

#### METHOD III (GRAVIMETRIC)

Procedure for Chemicals— Proceed as directed in the individual monograph preparing the chemical as directed under [Loss on Drying \(731\)](#).

Procedure for Biologics— Proceed as directed in the individual monograph.

Procedure for Articles of Botanical Origin— Place about 10 g of the drug, prepared as directed (see Methods of Analysis under [Articles of Botanical Origin \(561\)](#)) and accurately weighed, in a tared evaporating dish. Dry at 105° for 5 hours, and weigh. Continue the drying and weighing at 1-hour intervals until the difference between two successive weighings corresponds to not more than 0.25%.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Gary E. Ritchie, M.Sc.</a> Scientific Fellow 1-301-816-8353	(GC05) General Chapters 05

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#### 941 X-RAY DIFFRACTION

Every crystal form of a compound produces its own characteristic X-ray diffraction pattern. These diffraction patterns can be derived either from a single crystal or from a powdered specimen (containing numerous crystals) of the material. The spacings between and the relative intensities of the diffracted maxima can be used for qualitative and quantitative analysis of crystalline materials. Powder diffraction techniques are most commonly employed for routine identification and the determination of relative purity of crystalline materials. Small amounts of impurity, however, are not normally detectable by the X-ray diffraction method, and for quantitative measurements it is necessary to prepare the sample carefully to avoid preferred orientation effects.

The powder methods provide an advantage over other means of analysis in that they are usually nondestructive in nature (specimen preparation is usually limited to grinding to ensure a randomly oriented sample, and deleterious effects of X-rays on solid pharmaceutical compounds are not commonly encountered). The principal use of single-crystal diffraction data is for the determination of molecular weights and analysis of crystal structures at the atomic level. However, diffraction established for a single crystal can be used to support a specific powder pattern as being truly representative of a single phase.

Solids— A solid substance can be classified as being crystalline, noncrystalline, or a mixture of the two forms. In crystalline materials, the molecular or atomic species are ordered in a

...ree-dimensional array, called a lattice, within the solid particles. This ordering of molecular components is lacking in noncrystalline material. Noncrystalline solids sometimes are referred to as glasses or amorphous solids when repetitive order is nonexistent in all three dimensions. It is also possible for order to exist in only one or two dimensions, resulting in mesomorphic phases (liquid crystals). Although crystalline materials are usually considered to have well-defined visible external morphologies (their habits), this is not a necessity for X-ray diffraction analysis.

The relatively random arrangement of molecules in noncrystalline substances makes them poor coherent scatterers of X-rays, resulting in broad, diffuse maxima in diffraction patterns. Their X-ray patterns are quite distinguishable from crystalline specimens, which give sharply defined diffraction patterns.

Many compounds are capable of crystallizing in more than one type of crystal lattice. At any particular temperature and pressure, only one crystalline form (polymorph) is thermodynamically stable. Since the rate of phase transformation of a metastable polymorph to the stable one can be quite slow, it is not uncommon to find several polymorphs of crystalline pharmaceutical compounds existing under normal handling conditions.

In addition to exhibiting polymorphism, many compounds form crystalline solvates in which the solvent molecule is an integral part of the crystal structure. Just as every polymorph has its own characteristic X-ray patterns, so does every solvate. Sometimes the differences in the diffraction patterns of different polymorphs are relatively minor, and must be very carefully evaluated before a definitive conclusion is reached. In some instances, these polymorphs and/or solvates show varying dissolution rates. Therefore, on the time scale of pharmaceutical bioavailability, different total amounts of drug are dissolved, resulting in potential bioinequivalence of the several forms of the drug.

**Fundamental Principles**— A collimated beam of monochromatic X-rays is diffracted in various directions when it impinges upon a rotating crystal or randomly oriented powdered crystal. The crystal acts as a three-dimensional diffraction grating to this radiation. This phenomenon is described by Braggs law, which states that diffraction (constructive interference) can occur only when waves that are scattered from different regions of the crystal, in a specific direction, travel distances differing by integral numbers (n) of the wavelength ( $\lambda$ ). Under such circumstances, the waves are in phase. This condition is described by the Braggs equation:

$$\frac{n\lambda}{2 \sin \theta} = d_{hkl}$$

in which  $d_{hkl}$  denotes the interplanar spacings and  $\theta$  is the angle of diffraction.

A family of planes in space can be indexed by three whole numbers, usually referred to as Miller indices. These indices are the reciprocals, reduced to smallest integers, of the intercepts that a plane makes along the axes corresponding to three nonparallel edges of the unit cell (basic crystallographic unit). The unit cell dimensions are given by the lengths of the spacings along the three axes, a, b, c, and the angles between them,  $\alpha$ ,  $\beta$ , and  $\gamma$ . The interplanar spacing for a specific set of parallel planes  $hkl$  is denoted by  $d_{hkl}$ . Each such family of planes may show higher orders of diffraction where the d values for the related families of planes  $nh$ ,  $nk$ ,  $nl$  are diminished by the factor 1/n (n being an integer: 2, 3, 4, etc.).

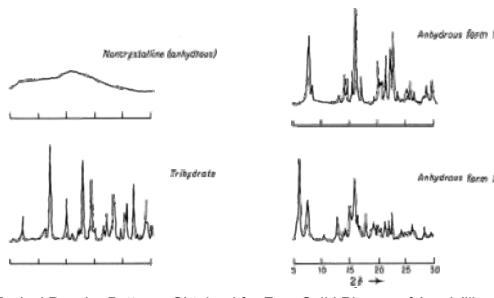
Every set of planes throughout a crystal has a corresponding Braggs diffraction angle associated with it (for a specific  $\lambda$ ).

The amplitude of a diffracted X-ray beam from any set of planes is dependent upon the following atomic properties of the crystal: (1) position of each atom in the unit cell; (2) the respective atomic scattering factors; and (3) the individual thermal motions. Other factors that directly influence the intensities of the diffracted beam are: (1) the intensity and wavelength of the incident radiation; (2) the volume of crystalline specimen; (3) the absorption of the X-radiation by the specimen; and (4) the experimental arrangement utilized to record the intensity data. Thus, the experimental conditions are especially important for measurement of diffraction intensities.

Only a limited number of Braggs planes are in a position to diffract when monochromatized X-rays pass through a single crystal. Techniques of recording the intensities of all of the possible diffracting  $hkl$  planes involve motion of the single crystal and the recording media. Recording of these data is accomplished by photographic techniques (film) or with radiation detectors.

A beam passing through a very large number of small, randomly oriented crystals produces continuous cones of diffracted rays from each set of lattice planes. Each cone corresponds to the diffraction from various planes having a similar interplanar spacing. The intensities of these Braggs reflections are recorded by either film or radiation detectors. The Braggs angle can be measured easily from a film, but the advent of radiation detectors has made possible the construction of diffractometers that read this angle directly. The intensities and d spacings are more conveniently determined with powder diffractometers employing radiation detectors than by film methods. Microphotometers are frequently used for precise intensity measurements of films.

An example of the type of powder patterns obtained for four different solid phases of ampicillin are shown in the accompanying figure.



Typical Powder Patterns Obtained for Four Solid Phases of Ampicillin

These diffraction patterns were derived from a powder diffractometer equipped with a Geiger-Müller detector; nickel-filtered Cu K $\alpha$  radiation was used.

**Radiation**— The principal radiation sources utilized for X-ray diffraction are vacuum tubes utilizing copper, molybdenum, iron, and chromium as anodes; copper X-rays are employed most commonly for organic substances. For each of these radiations there is an element that will filter off the K $\beta$  radiation and permit the K $\alpha$  radiation to pass (nickel is used, in the case of copper radiation). In this manner the radiation is practically monochromatized. The choice of radiation to be used depends upon the absorption characteristics of the material and possible fluorescence by atoms present in the specimen.

**Caution**—Care must be taken in the use of such radiation. Those not familiar with the use of X-ray equipment should seek expert advice. Improper use can result in harmful effects to the operator.

**Test Preparation**— In an attempt to improve randomness in the orientation of crystallites (and, for film techniques, to avoid a grainy pattern), the specimen may be ground in a mortar to a fine powder. Grinding pressure has been known to induce phase transformations; therefore, it is advisable to check the diffraction pattern of the unground sample.

In general, the shapes of many crystalline particles tend to give a specimen that exhibits some degree of preferred orientation in the specimen holder. This is especially evident for needle-like or plate-like crystals where size reduction yields finer needles or platelets. Preferred orientation in the specimen influences the relative intensities of various reflections.

Several specialized handling techniques may be employed to minimize preferred orientation, but further reduction of particle size is often the best approach.



here very accurate measurement of the Braggs angles is necessary, a small amount of an internal standard can be mixed into the specimen. This enables the film or recorder tracing to be calibrated. If comparisons to literature values (including compendial limits) of  $d$  are being made, calibrate the diffractometer. NIST standards are available covering to a  $d$ -value of 0.998 nm. Tetradecanol<sup>1</sup> may be used ( $d$  is 3.963 nm) for larger spacing.

The absorption of the radiation by any specimen is determined by the number and kinds of atoms through which the X-ray beam passes. An organic matrix usually absorbs less of the diffracted radiation than does an inorganic matrix. Therefore, it is important in quantitative studies that standard curves relating amount of material to the intensity of certain  $d$  spacings for that substance be determined in a matrix similar to that in which the substance will be analyzed.

In quantitative analyses of materials, a known amount of standard usually is added to a weighed amount of specimen to be analyzed. This enables the amount of the substance to be determined relative to the amount of standard added. The standard used should have approximately the same density as the specimen and similar absorption characteristics. More important, its diffraction pattern should not overlap to any extent with that of the material to be analyzed. Under these conditions a linear relationship between line intensity and concentration exists. In favorable cases, amounts of crystalline materials as small as 10% may be determined in solid matrices.

Identification of crystalline materials can be accomplished by comparison of X-ray powder diffraction patterns obtained for known<sup>2</sup> materials with those of the unknown. The intensity ratio (ratio of the peak intensity of a particular  $d$  spacing to the intensity of the strongest maxima in the diffraction pattern) and the  $d$  spacing are used in the comparison. If a reference material (e.g., USP Reference Standard) is available, it is preferable to generate a primary reference pattern on the same equipment used for running the unknown sample, and under

the same conditions. For most organic crystals, it is appropriate to record the diffraction pattern to include values for  $2\theta$  that range from as near zero degrees as possible to 40 degrees. Agreement between sample and reference should be within the calibrated precision of the diffractometer for diffraction angle ( $2\theta$ ) values should typically be reproducible to  $\pm 0.10$  degrees), while relative intensities between sample and reference may vary considerably. For other types of samples (e.g., inorganic salts), it may be necessary to extend the  $2\theta$  region scanned to well beyond 40 degrees. It is generally sufficient to scan past the ten strongest reflections identified in the Powder Diffraction File.<sup>2</sup>

1 Brindley, GW and Brown, G, eds., *Crystal Structures of Clay Minerals and Their X-ray Identification*, Mineralogical Society Monograph No. 5, London, 1980, pp. 318 ff.

2 The International Centre for Diffraction Data, Newtown Square Corporate Campus, 12 Campus Boulevard, Newtown Square, PA 19073, maintains a file on more than 60,000 crystalline materials, both organic and inorganic, suitable for such comparisons.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Gary E. Ritchie, M.Sc.</a> Scientific Fellow 1-301-816-8353	(GC05) General Chapters 05

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General Information

The chapters in this section are information, and aside from excerpts given herein from Federal Acts and regulations that may be applicable, they contain no standards, tests, assays, nor other mandatory specifications, with respect to any Pharmacopeial articles. The excerpts from pertinent Federal Acts and regulations included in this section are placed here inasmuch as they are not of Pharmacopeial authorship. Revisions of the federal requirements that affect these excerpts will be included in USP Supplements as promptly as practical. The official requirements for Pharmacopeial articles are set forth in the General Notices, the individual monographs, and the General Tests and Assays chapters of this Pharmacopeia.

## 1005 ACOUSTIC EMISSION

### INTRODUCTION

Ultrasound techniques can be categorized into two distinct types: acoustic emission (passive mode) and ultrasound spectroscopy (active mode). Both of these techniques have many applications.

The technique of acoustic emission is based on the detection and analysis of sound produced by a process or system. This is essentially equivalent to listening to the process or system, although these sounds are often well above the frequencies that can be detected by the human ear. Generally, frequencies up to about 15 kHz are audible.

In the case of ultrasound spectroscopy, the instrument is designed to generate ultrasound waves across a defined frequency range. These waves travel through the sample and are measured using a receiver. An analogy can be drawn with UV-visible or IR spectroscopy in that the detected ultrasound spectrum reflects changes in velocity or sound attenuation due to the interaction with a sample across a range of frequencies. However, as the scope of this chapter is limited to acoustic emission, ultrasound spectroscopy will not be discussed further.

Acoustic emission is well-known in the study of fracture mechanics and therefore is used extensively by material scientists. It is also widely used as a nondestructive testing technique and is applied routinely for the inspection of aircraft wings, pressure vessels, load-bearing structures, and components. Acoustic emission is also used in the engineering industry for the monitoring of machine tool wear.

In terms of pharmaceutical applications, the dependence of the acoustic emission measurement on physical properties such as particle size, mechanical strength, and cohesivity of solid materials allows the technique to be used for the control and endpoint detection of processes such as high shear granulation, fluid bed drying, milling, and micronization.

### General Principles

Acoustic emissions can propagate by a number of modes. In solids, compressional and shear or transverse modes are important. Compressional modes have the highest velocity and thus reach the acoustic detector (or acoustic emission transducer) first. However, in most process applications of acoustic emission, there are many sources—each producing short bursts of energy—and, consequently, the different modes cannot easily be resolved. The detected signal, for example on the wall of a vessel, is a complex mixture of many overlapping waveforms resulting from many sources and many propagation modes.

At interfaces, depending on the relative acoustic impedance of the two materials, much of the energy is reflected back towards the source. In a fluidized bed, for example, acoustic emissions will only be detected from particles directly impacting the walls of the bed close to the transducer.

A convenient method of studying acoustic emission from processes is to use the “average signal level”. A root mean square-to-direct current (RMS-to-DC) converter may be used to convert the amplitude-modulated (AM) carrier into a more slowly varying DC signal. This is referred to as the average signal level (ASL). The ASL can then be digitally sampled (typically at a sampling frequency of about 50 Hz) and stored electronically for further signal processing.

The simplest way of studying the acoustic data is to examine changes in the ASL. However, other information can be derived from examining the power spectrum of the ASL. The power spectrum is calculated by taking the complex square of the amplitude spectrum and can be obtained by performing a Fast Fourier Transform (FFT) on the digitized raw data record. Power spectra may be averaged to produce a reliable estimate of power spectral density or to give a “fingerprint” of a particular process regime. Interpretation of the power spectrum is complicated by the fact that the acoustic signal originating in the system is distorted by several factors including transmission, reflection, and signal transfer characteristics.

The shape of the power spectrum of the ASL record is a function of the process dynamics. Periodic processes (e.g., mechanical stirring or periodic bubbling of a fluidized bed) show high power at certain discrete frequencies. Random processes show either flicker type properties, where power is inversely proportional to frequency, or white noise type properties in which power is independent of frequency. The amplitude of the power spectrum is also affected by the energy of the acoustic emissions produced by the process. For example, if hard material is being processed, the acoustic emission produced by particle impact will be greater than that produced by soft material.

## INSTRUMENTATION

Generally, piezoelectric sensors are used to detect and quantify the acoustic signals produced by a process. Piezoelectric transducers are constructed from piezoelectric crystalline solids connected to transducer control circuitry by electrical leads. When configured as a detector, an acoustic wave that impinges on the piezoelectric element is transformed into an electrical signal in the transducer control circuitry. When configured as an acoustic generator, an electrical signal applied to the piezoelectric element by the control circuitry creates an acoustic wave that can propagate into the medium to which the transducer is attached. Typically, this means that acoustic emission detectors can also be operated as acoustic wave generators and this feature is used to ensure good sensor performance as described later (see Qualification and Verification of Acoustic Emission Instruments).

In general acoustic emission applications, sensors with different resonance frequencies are often used (e.g., 70 and 190 kHz, although higher frequencies may be more appropriate at smaller scales of operation), incorporating various band-passes. As sound (ultrasound) of the appropriate frequency range reaches these sensors, an electrical signal is generated, the amplitude of which is directly proportional to the energy (amplitude) of the incident sound waves.

These signals are processed through the following:

1. a pre-amplifier (which incorporates signal filtering),
2. an RMS-to-DC converter,
3. a variable gain amplifier, and
4. a PC-based data acquisition board.

The controlling software is also incorporated into the PC.

Acoustic emission equipment generally allows several sensors to be used simultaneously by incorporating multiple electronic channels into a single instrument.

### Signal Processing

The signal from a resonant transducer resembles an AM radio signal. At the resonance frequency of the transducer, the signal consists of a carrier wave that is modulated in amplitude by the process. An RMS-to-DC converter is used to demodulate the signal. The output of this device is the modulation signal or envelope.

The envelope is digitally resampled at a frequency appropriate for the process. For example, 50 Hz is a typical digital sampling rate for a fluid bed drier or high shear granulator.

## FACTORS AFFECTING MEASUREMENT

The following factors can affect the acoustic data obtained and should be considered when installing an acoustic emission system.

1. Failure or Physical Damage—As with any other type of sensor, acoustic emission sensors can fail with time or as a result of physical damage. It is important to check the sensor function as part of routine maintenance of the instrument. If multiple sensors are installed on the same vessel, an active signal can be generated from one sensor and this can be used to check the detection on another sensor. This exercise would ensure that the sensors are detecting the acoustic signals generated by the process. A statistically valid “minimum acceptable acoustic signal” for the sensor(s) should also be determined and monitored at the start, middle, and end of a process to ensure the performance of the sensor(s) during a process run. This may be established from the routine maintenance signal experiments or on the basis of historical data for the sensors.
2. Issues of Sensor Interfacing—Sensors are typically installed on the outer wall of the process vessel. Several types of adhesives (temporary or permanent) can be used to attach the sensor to the vessel wall. Through repeated cleaning and vessel movement, it is possible for the bonding between the sensor and vessel to be compromised. Checking the integrity of the installation should be part of routine maintenance. Similar to item 1 above, an active signal can be used to ensure proper bonding between sensor and vessel and helps to confirm the matching of acoustic impedance.
3. Influence of Mechanical Noise—The use of high frequencies significantly reduces the contribution of mechanical noise to the acoustic signal detected, especially at smaller scales of operation, although it does not eliminate it completely. Testing the effect of various motor settings, for example, can determine if the acoustic signal detected is a function of mechanical noise. If the effect is significant, using higher frequencies may be necessary. Awareness of the contribution of the mechanical noise, no matter how small, is important to consider as the motors age or are replaced.
4. Influence of Vessel Wall Characteristics—Because the sensors are often placed on the outer vessel wall, wall thickness can affect the quality of the signal detected. If the vessel is jacketed, the amplitude of the acoustic signal may be reduced. Adding more sensors on the vessel can improve signal quality. Alternatively, an increase in signal may be obtained by positioning sensor(s) at a location where contact exists between the inner and outer walls, essentially providing a waveguide between the sensor and sound source. Waveguides may also be incorporated into the design of manufacturing equipment to enable utilization of acoustic emission monitoring. Appropriate validation is required to ensure that this does not adversely affect the performance of equipment.
5. Effect of Material Properties—During operation, the acoustic signal collected is a summation of various events occurring within the process. For example, the acoustic signal generated as particles hit the wall in a granulator is a function of the material properties of the granules (i.e., density, size, porosity). Therefore, significant changes to any of these parameters can affect the acoustic signal and the quality of the ensuing prediction.
6. Influence of Process-Related Factors—Similar to item 5 above, the process-related properties (i.e., force of impact, frequency of impact, amount of material) can also affect the acoustic signal and the quality of the ensuing prediction.
7. Impact of Environmental Conditions—Finally, the influence of environmental factors (i.e., temperature, humidity) must also be considered.

The acoustic emission data collected is vessel/equipment specific. It is not advisable to apply a model generated on one piece of equipment to another because the acoustic information can differ as a result of the issues discussed in items 3, 4, and 5 above.

### Qualification and Verification of Acoustic Emission Instruments

A system suitability approach should be taken around instrument performance, establishing optimum measurement configuration, then comparing the instrument performance to the values obtained during routine use to those obtained during installation qualification (IQ).

This approach effectively answers the issues related to sampling because, unlike other on-line analytical systems, the transducers can be optimally positioned and attached to receive the maximum signal without vessel modification.

Sample rates need to comply with the Nyquist sampling theorem, which states that a signal must be sampled at a rate that is twice the highest frequency component in the signal. A low-pass filter should be used to remove the frequency components greater than half the sampling frequency (Nyquist frequency). Failure to comply with this criterion will result in aliasing.

Owing to the nature of the piezoelectric transducers and because resonance frequencies are natural properties of the crystals, it is not necessary to test the variation (reproducibility) or drift in the frequency domain. If other types of transducers are used, this may be necessary. Any gross change in the frequency domain will be recorded as a drop in the power intensity at the resonance frequency, and therefore is covered by the power intensity tests.

The two main areas for instrument performance verification are power intensity and timings. Any change in the signal intensity will affect the raw signal and the ASL and, therefore, will also affect the power spectrum. Changes in power intensity can occur as a result of changes in the process (e.g., variation in hardness or moisture in the particles impacting the vessel wall) or changes in the acoustic conduit from the process to transducer.

Reproducibility of the acoustic conduit should be tested using a second transducer to input a pulse or “ping” at the resonance frequency of the receiving sensor. This reproducibility value represents the noise of the signal and can be used in calculations of limit of detection (LOD) and limit of quantitation (LOQ), where LOD is defined as three times the noise of the signal and LOQ is ten times the noise of the signal. The noise on the background signal level (in acoustic emission this background signal is mainly due to amplifier noise) should be calculated from twenty sequential ASL values acquired at the sampling frequency used for normal operation. This test should be repeated in reverse in order to establish that



statistically similar intensity values can be obtained on both channels.

Short term reproducibility allows the calculation of noise. However, it does not give a measure of integrity of acoustic conduit over time or, more specifically, of changes caused by the process (e.g., variations in adhesive properties with process changes such as heating/cooling). The noise test should be repeated while executing the normal processing parameters (using an empty vessel) and the drift in the ASL should be calculated. Care should be taken to make sure that signal drift (due to normal variation in processing parameters) does not impact chemometric models used for endpoint determination. For trend plots, it should be shown that drift is not statistically significant; otherwise, drift correction will need to be applied. Values for noise, drift, and absolute ASL should be recorded and logged, and the tests re-executed if changes are made to the processing equipment or to the acoustic emission system. If no changes are made, then the tests should be re-executed every month. In this way the quality of the acoustic conduit can be shown to be intact and any changes to the signal intensity isolated and attributed to the process itself.

During routine use, it is recommended that the noise test be executed (as above) before each process run, and that power intensity and noise be calculated. These values should be logged and compared to those generated both during previous use and during installation. Impact of the deviation from previous values will be a function of the prediction model and should be addressed by method validation.

The noise data (from above) can also be used to calculate the time of flight of the pulse. If the pulse activation and signal reception are synchronized, the time taken for the pulse to transmit across the vessel can be measured. This is a good indication of the measurement electronics as well as the overall condition of the acoustic conduit. However, this test should be regarded as a measure of the "system" condition and needs to be executed only if changes have been made to the process equipment or the acoustic emission system, or every 6 months. Correlation of the measured timings with the historical ones should be statistically valid. If not, it is an indicator that the acoustic emission system may need requalification by the instrument manufacturer or supplier, or that there are changes in the acoustic conduit.

All of these tests require the use of an acoustic pulse generated electrically. Failure in any of the above tests could be attributed to the signal generation itself. It is recommended that the electrical pulse generation system be requalified and certified against National Institute of Standards and Technology (NIST) traceable standards every 12 months.

#### DATA ANALYSIS

Acoustic emission from granulators and fluid bed driers is known as continuous acoustic emission. Continuous acoustic emission is aphasic (i.e., there are no starts or stops to the signal). This means that it is unnecessary to use signal processing techniques that preserve phase. Power spectral analysis is a useful technique in processing acoustic emission signals. The information in the power spectra, unlike the raw acoustic emission signals, is coherent in the short term, allowing signal averaging to be performed. This provides a better estimate of power spectral density than that provided by a single power spectrum.

To detect endpoints in batch processes (e.g., granulation or drying endpoint), a qualitative multivariate model is appropriate (e.g., PCA or SIMCA). The following sequence of operations is performed:

1. Training/Calibration—Acoustic emission spectra that are representative of the endpoint condition are obtained.
2. Modeling—A multivariate model describing the distribution of acoustic emission signals at the endpoint condition is created.
3. Prediction—Acoustic emission spectra are compared against the model. The fit to the model (usually expressed in terms of a number of standard deviations) is monitored. As the system approaches the endpoint, the fit improves and completion of the process is established once the model fits predefined criteria. The prediction model is generated from acoustic emission spectra obtained from the process operating under normal conditions. Upsets (e.g., unwanted agglomeration in coaters) are detected by observing statistically valid deviations from the model.

Adaptive modeling has also been proposed for upset detection. This involves generating multivariate models continuously as the acoustic emission signals are acquired. Unusual deviation of the acoustic emission signal indicates the occurrence of a process upset. The advantage of adaptive modeling is that it is not necessary to perform a separate calibration step.

#### GLOSSARY

**Acoustic Emission Transducer**—A solid state device usually incorporating a piezoelectric element to convert the acoustic emission wave to an electrical signal.

**Acoustic Impedance**—Acoustic impedance ( $Z$ ) is defined as  $Z = \rho v$  (where  $\rho$  is density and  $v$  is the sound velocity). It is an important quantity and gives the proportion of sound energy transmitted from one medium to another and the amount of energy reflected at the interface.

**Adaptive Modeling**—A method that predicts the state of a process without the use of a previously generated model (i.e., there is no prior training or calibration step).

**Aliasing**—Spurious low frequency components, appearing in the signal, that are really frequencies above the Nyquist frequency.

**Amplitude**—The magnitude or strength of a varying waveform.

**Average Signal Level (ASL)**—A measure of the average power in an acoustic emission signal.

**Band-Pass**—The range of frequencies within which a component operates.

**Compressional Mode**—A longitudinal mode of acoustic transmission encountered in solids, liquids, and gases.

**Continuous Acoustic Emission**—Acoustic emission signals that cannot be separated in time and are typical of pharmaceutical processes such as granulation and fluid bed drying.

**Flicker Type Properties**—A type of signal associated with many natural processes. The characteristics of flicker noise are that the power of the noise is directly proportional to the signal and has approximately a  $1/f$  ( $f$  = frequency) spectral density distribution.

**Gain**—The amplification factor for a component usually expressed in terms of decibels (dB).

**Gain in dB** =  $20 \log_{10} (\text{Voltageout} / \text{Voltagein})$ .

**Nyquist Frequency**—The Nyquist frequency is defined as half the digital sampling rate and is the highest frequency that can be reproduced faithfully.

**Piezoelectric**—A material which generates an electric field when compressed. Piezoelectric materials are used in the construction of acoustic emission sensors. A common material is PZT (lead zirconium titanate).

**Power Spectrum**—A power spectrum of a signal is a representation of the signal power as a function of frequency. A power spectrum is calculated from the time domain signal by means of the Fast Fourier Transform (FFT) algorithm. It is useful to study acoustic emission signals in the frequency or spectral domain, as the spectrum is often characteristic of the mechanism. Improvements in signal-to-noise ratio can be obtained by averaging a number of power spectra, as they are coherent.

**Power Spectral Density**—The measure of acoustic emission power in each resolution element of the power spectrum.

**Resonance Frequency**—The frequency at which an acoustic emission sensor is most sensitive. Resonant acoustic emission sensors have a clearly defined resonance frequency, but are usually sensitive to other frequencies.

**RMS-to-DC Converter**—An electronic device that converts an alternating signal to a voltage level proportional to the average power in the signal.

**Shear Mode**—A transverse mode of acoustic transmission, encountered only in solids.

**Signal Filtering**—Filtering a signal means attenuating frequencies outside a prescribed range. In acoustic emission work, band-pass filtering is used to improve the signal-to-noise ratio by attenuating noise outside the bandwidth of the sensor. Low-pass filtering is used to remove frequencies higher than the Nyquist frequency in order to prevent aliasing.

**Transverse Mode**—A mode of wave propagation where the displacement of the material is perpendicular to the direction of propagation. These modes are only encountered in solid

materials.

White Noise—The characteristic of white noise is a power spectrum of uniform spectral density and is associated with purely random processes.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Gary E. Ritchie, M.Sc.</a> Scientific Fellow 1-301-816-8353	(GC05) General Chapters 05

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## 1010 ANALYTICAL DATA—INTERPRETATION AND TREATMENT

### INTRODUCTION

This chapter provides information regarding acceptable practices for the analysis and consistent interpretation of data obtained from chemical and other analyses. Basic statistical approaches for evaluating data are described, and the treatment of outliers and comparison of analytical methods are discussed in some detail.

NOTE—It should not be inferred that the analysis tools mentioned in this chapter form an exhaustive list. Other, equally valid, statistical methods may be used at the discretion of the manufacturer and other users of this chapter.

Assurance of the quality of pharmaceuticals is accomplished by combining a number of practices, including robust formulation design, validation, testing of starting materials, in-process testing, and final-product testing. Each of these practices is dependent on reliable test methods. In the development process, test procedures are developed and validated to ensure that the manufactured products are thoroughly characterized. Final-product testing provides further assurance that the products are consistently safe, efficacious, and in compliance with their specifications.

Measurements are inherently variable. The variability of biological tests has long been recognized by the USP. For example, the need to consider this variability when analyzing biological test data is addressed under [Design and Analysis of Biological Assays](#) 111. The chemical analysis measurements commonly used to analyze pharmaceuticals are also inherently variable, although less so than those of the biological tests. However, in many instances the acceptance criteria are proportionally tighter, and thus, this smaller allowable variability has to be considered when analyzing data generated using analytical procedures. If the variability of a measurement is not characterized and stated along with the result of the measurement, then the data can only be interpreted in the most limited sense. For example, stating that the difference between the averages from two laboratories when testing a common set of samples is 10% has limited interpretation, in terms of how important such a difference is, without knowledge of the intralaboratory variability.

This chapter provides direction for scientifically acceptable treatment and interpretation of data. Statistical tools that may be helpful in the interpretation of analytical data are described.

Many descriptive statistics, such as the mean and standard deviation, are in common use. Other statistical tools, such as outlier tests, can be performed using several different, scientifically valid approaches, and examples of these tools and their applications are also included. The framework within which the results from a compendial test are interpreted is clearly outlined in Test Results, Statistics, and Standards under General Notices and Requirements. Selected references that might be helpful in obtaining additional information on the statistical tools discussed in this chapter are listed in Appendix F at the end of the chapter. USP does not endorse these citations, and they do not represent an exhaustive list. Further information about many of the methods cited in this chapter may also be found in most statistical textbooks.

### PREREQUISITE LABORATORY PRACTICES AND PRINCIPLES

The sound application of statistical principles to laboratory data requires the assumption that such data have been collected in a traceable (i.e., documented) and unbiased manner. To ensure this, the following practices are beneficial.

#### Sound Record Keeping

Laboratory records are maintained with sufficient detail, so that other equally qualified analysts can reconstruct the experimental conditions and review the results obtained. When collecting data, the data should generally be obtained with more decimal places than the specification requires and rounded only after final calculations are completed as per the General Notices and Requirements.

#### Sampling Considerations

Effective sampling is an important step in the assessment of a quality attribute of a population. The purpose of sampling is to provide representative data (the sample) for estimating the properties of the population. How to attain such a sample depends entirely on the question that is to be answered by the sample data. In general, use of a random process is considered the most appropriate way of selecting a sample. Indeed, a random and independent sample is necessary to ensure that the resulting data produce valid estimates of the properties of the population. Generating a nonrandom or "convenience" sample risks the possibility that the estimates will be biased. The most straightforward type of random sampling is called simple random sampling, a process in which every unit of the population has an equal chance of appearing in the sample. However, sometimes this method of selecting a random sample is not optimal because it cannot guarantee equal representation among factors (i.e., time, location, machine) that may influence the critical properties of the population. For example, if it requires 12 hours to manufacture all of the units in a lot and it is vital that the sample be representative of the entire production process, then taking a simple random sample after the production has been completed may not be appropriate because there can be no guarantee that such a sample will contain a similar number of units made from every time period within the 12-hour process. Instead, it is better to take a systematic random sample whereby a unit is randomly selected from the production process at systematically selected times or locations (e.g., sampling every 30 minutes from the units produced at that time) to ensure that units taken throughout the entire manufacturing process are included in the sample. Another type of random sampling procedure is needed if, for example, a product is filled into vials using four different filling machines. In this case it would be important to capture a random sample of vials from each of the filling machines. A stratified random sample, which randomly samples an equal number of vials from each of the four filling machines, would satisfy this requirement. Regardless of the reason for taking a sample (e.g., batch-release testing), a sampling plan should be established to provide details on how the sample is to be obtained to ensure that the sample is representative of the entirety of the population and that the resulting data have the required sensitivity. The optimal sampling strategy will depend on knowledge of the manufacturing and analytical measurement processes. Once the sampling scheme has been defined, it is likely that the sampling will include some element of random selection. Finally, there must be sufficient sample collected for the original analysis, subsequent verification analyses, and other analyses.

Tests discussed in the remainder of this chapter assume that simple random sampling has been performed.

#### Use of Reference Standards

Where the use of the USP Reference Standard is specified, the USP Reference Standard, or a secondary standard traceable to the USP Reference Standard, is used. Because the assignment of a value to a standard is one of the most important factors that influences the accuracy of an analysis, it is critical that this be done correctly.

#### System Performance Verification

Verifying an acceptable level of performance for an analytical system in routine or continuous use can be a valuable practice. This may be accomplished by analyzing a control sample at appropriate intervals, or using other means, such as, variation among the standards, background signal-to-noise ratios, etc. Attention to the measured parameter, such as charting the results obtained by analysis of a control sample, can signal a change in performance that requires adjustment of the analytical system.

#### Method Validation

All methods are appropriately validated as specified under [Validation of Compendial Procedures](#) 1225. Methods published in the USP-NF have been validated and meet the Current Good Manufacturing Practices regulatory requirement for validation as established in the Code of Federal Regulations. A validated method may be used to test a new

...ulation (such as a new product, dosage form, or process intermediate) only after confirming that the new formulation does not interfere with the accuracy, linearity, or precision of the method. It may not be assumed that a validated method could correctly measure the active ingredient in a formulation that is different from that used in establishing the original validity of the method.

#### MEASUREMENT PRINCIPLES AND VARIATION

All measurements are, at best, estimates of the actual ("true" or "accepted") value for they contain random variability (also referred to as random error) and may also contain systematic variation (bias). Thus, the measured value differs from the actual value because of variability inherent in the measurement. If an array of measurements consists of individual results that are representative of the whole, statistical methods can be used to estimate informative properties of the entirety, and statistical tests are available to investigate whether it is likely that these properties comply with given requirements. The resulting statistical analyses should address the variability associated with the measurement process as well as that of the entity being measured. Statistical measures used to assess the direction and magnitude of these errors include the mean, standard deviation, and expressions derived therefrom, such as the coefficient of variation (CV, also called the relative standard deviation, RSD). The estimated variability can be used to calculate confidence intervals for the mean, or measures of variability, and tolerance intervals capturing a specified proportion of the individual measurements.

The use of statistical measures must be tempered with good judgment, especially with regard to representative sampling. Most of the statistical measures and tests cited in this chapter rely on the assumptions that the distribution of the entire population is represented by a normal distribution and that the analyzed sample is a representative subset of this population. The normal (or Gaussian) distribution is bell-shaped and symmetric about its center and has certain characteristics that are required for these tests to be valid. If the assumption of a normal distribution for the population is not warranted, then normality can often be achieved (at least approximately) through an appropriate transformation of the measurement values. For example, there exist variables that have distributions with longer right tails than left. Such distributions can often be made approximately normal through a log transformation. An alternative approach would be to use "distribution-free" or "nonparametric" statistical procedures that do not require that the shape of the population be that of a normal distribution. When the objective is to construct a confidence interval for the mean or for the difference between two means, for example, then the normality assumption is not as important because of the central limit theorem. However, one must verify normality of data to construct valid confidence intervals for standard deviations and ratios of standard deviations, perform some outlier tests, and construct valid statistical tolerance limits. In the latter case, normality is a critical assumption. Simple graphical methods, such as dot plots, histograms, and normal probability plots, are useful aids for investigating this assumption.

A single analytical measurement may be useful in quality assessment if the sample is from a whole that has been prepared using a well-validated, documented process and if the analytical errors are well known. The obtained analytical result may be qualified by including an estimate of the associated errors. There may be instances when one might consider the use of averaging because the variability associated with an average value is always reduced as compared to the variability in the individual measurements. The choice of whether to use individual measurements or averages will depend upon the use of the measure and its variability. For example, when multiple measurements are obtained on the same sample aliquot, such as from multiple injections of the sample in an HPLC method, it is generally advisable to average the resulting data for the reason discussed above.

Variability is associated with the dispersion of observations around the center of a distribution. The most commonly used statistic to measure the center is the sample mean ( $\bar{x}$ ):

$$\bar{x} = \frac{\sum_{i=1}^n x_i}{n} = \frac{x_1 + x_2 + \dots + x_n}{n}$$

Method variability can be estimated in various ways. The most common and useful assessment of a method's variability is the determination of the standard deviation based on repeated independent<sup>4</sup> measurements of a sample. The sample standard deviation,  $s$ , is calculated by the formula:

$$s = \sqrt{\sum_{i=1}^n (x_i - \bar{x})^2 / (n-1)}$$

in which  $x_i$  is the individual measurement in a set of  $n$  measurements; and  $\bar{x}$  is the mean of all the measurements. The relative standard deviation (RSD) is then calculated as:

$$RSD = \frac{s}{x} \cdot 100\%$$

and expressed as a percentage. If the data requires log transformation to achieve normality (e.g., for biological assays), then alternative methods are available.<sup>2</sup>

A control sample is defined as a homogeneous and stable sample that is tested at specific intervals sufficient to monitor the performance of the method for which it was established. Test data from a control sample can be used to monitor the method variability or be used as part of system suitability requirements.<sup>3</sup> The control sample should be essentially the same as the test sample and should be treated similarly whenever possible. A control chart can be constructed and used to monitor the method performance on a continuing basis as shown under Appendix A.

A precision study should be conducted to provide a better estimate of method variability. The precision study may be designed to determine intermediate precision (which includes the components of both "between run" and "within-run" variability) and repeatability ("within-run" variability). The intermediate precision studies should allow for changes in the experimental conditions that might be expected, such as different analysts, different preparations of reagents, different days, and different instruments. To perform a precision study, the test is repeated several times. Each run must be completely independent of the others to provide accurate estimates of the various components of variability. In addition, within each run, replicates are made in order to estimate repeatability. See an example of a precision study under Appendix B.

A confidence interval for the mean may be considered in the interpretation of data. Such intervals are calculated from several data points using the sample mean ( $\bar{x}$ ) and sample standard deviation ( $s$ ) according to the formula:

$$\left( \bar{x} - t_{\alpha/2, n-1} \frac{s}{\sqrt{n}}, \bar{x} + t_{\alpha/2, n-1} \frac{s}{\sqrt{n}} \right)$$

in which  $t_{\alpha/2, n-1}$  is a statistical number dependent upon the sample size ( $n$ ), the number of degrees of freedom ( $n - 1$ ), and the desired confidence level ( $1 - \alpha$ ). Its values are obtained from published tables of the Student t-distribution. The confidence interval provides an estimate of the range within which the "true" population mean ( $\mu$ ) falls, and it also evaluates the reliability of the sample mean as an estimate of the true mean. If the same experimental set-up were to be replicated over and over and a 95% (for example) confidence



interval for the true mean is calculated each time, then 95% of such intervals would be expected to contain the true mean,  $\mu$ . One cannot say with certainty whether or not the confidence interval derived from a specific set of data actually collected contains  $\mu$ . However, assuming the data represent mutually independent measurements randomly generated from a normally distributed population the procedure used to construct the confidence interval guarantees that 95% of such confidence intervals contain  $\mu$ . Note that it is important to define the population appropriately so that all relevant sources of variation are captured.

## OUTLYING RESULTS

Occasionally, observed analytical results are very different from those expected. Aberrant, anomalous, contaminated, discordant, spurious, suspicious or wild observations; and flyers, rogues, and mavericks are properly called outlying results. Like all laboratory results, these outliers must be documented, interpreted, and managed. Such results may be accurate measurements of the entity being measured, but are very different from what is expected. Alternatively, due to an error in the analytical system, the results may not be typical, even though the entity being measured is typical. When an outlying result is obtained, systematic laboratory and process investigations of the result are conducted to determine if an assignable cause for the result can be established. Factors to be considered when investigating an outlying result include—but are not limited to—human error, instrumentation error, calculation error, and product or component deficiency. If an assignable cause that is not related to a product or component deficiency can be identified, then retesting may be performed on the same sample, if possible, or on a new sample. The precision and accuracy of the method, the Reference Standard, process trends, and the specification limits should all be examined. Data may be invalidated, based on this documented investigation, and eliminated from subsequent calculations.

If no documentable, assignable cause for the outlying laboratory result is found, the result may be tested, as part of the overall investigation, to determine whether it is an outlier. However, careful consideration is warranted when using these tests. Two types of errors may occur with outlier tests: (a) labeling observations as outliers when they really are not; and (b) failing to identify outliers when they truly exist. Any judgment about the acceptability of data in which outliers are observed requires careful interpretation.

"Outlier labeling" is informal recognition of suspicious laboratory values that should be further investigated with more formal methods. The selection of the correct outlier identification technique often depends on the initial recognition of the number and location of the values. Outlier labeling is most often done visually with graphical techniques. "Outlier identification" is the use of statistical significance tests to confirm that the values are inconsistent with the known or assumed statistical model.

When used appropriately, outlier tests are valuable tools for pharmaceutical laboratories. Several tests exist for detecting outliers. Examples illustrating three of these procedures, the Extreme Studentized Deviate (ESD) Test, Dixon's Test, and Hampel's Rule, are presented in [Appendix C](#).

Choosing the appropriate outlier test will depend on the sample size and distributional assumptions. Many of these tests (e.g., the ESD Test) require the assumption that the data generated by the laboratory on the test results can be thought of as a random sample from a population that is normally distributed, possibly after transformation. If a transformation is made to the data, the outlier test is applied to the transformed data. Common transformations include taking the logarithm or square root of the data. Other approaches to handling single and multiple outliers are available and can also be used. These include tests that use robust measures of central tendency and spread, such as the median and median absolute deviation and exploratory data analysis (EDA) methods. "Outlier accommodation" is the use of robust techniques, such as tests based on the order or rank of each data value in the data set instead of the actual data value, to produce results that are not adversely influenced by the presence of outliers. The use of such methods reduces the risks associated with both types of error in the identification of outliers.

"Outlier rejection" is the actual removal of the identified outlier from the data set. However, an outlier test cannot be the sole means for removing an outlying result from the laboratory data. An outlier test may be useful as part of the evaluation of the significance of that result, along with other data. Outlier tests have no applicability in cases where the variability in the product is what is being assessed, such as content uniformity, dissolution, or release-rate determination. In these applications, a value determined to be an outlier may in fact be an accurate result of a nonuniform product. All data, especially outliers, should be kept for future review. Unusual data, when seen in the context of other historical data, are often not unusual after all but reflect the influences of additional sources of variation.

In summary, the rejection or retention of an apparent outlier can be a serious source of bias. The nature of the testing as well as scientific understanding of the manufacturing process and analytical method have to be considered to determine the source of the apparent outlier. An outlier test can never take the place of a thorough laboratory investigation. Rather, it is performed only when the investigation is inconclusive and no deviations in the manufacture or testing of the product were noted. Even if such statistical tests indicate that one or more values are outliers, they should still be retained in the record. Including or excluding outliers in calculations to assess conformance to acceptance criteria should be based on scientific judgment and the internal policies of the manufacturer. It is often useful to perform the calculations with and without the outliers to evaluate their impact.

Outliers that are attributed to measurement process mistakes should be reported (i.e., footnoted), but not included in further statistical calculations. When assessing conformance to a particular acceptance criterion, it is important to define whether the reportable result (the result that is compared to the limits) is an average value, an individual measurement, or something else. If, for example, the acceptance criterion was derived for an average, then it would not be statistically appropriate to require individual measurements to also satisfy the criterion because the variability associated with the average of a series of measurements is smaller than that of any individual measurement.

## COMPARISON OF ANALYTICAL METHODS

It is often necessary to compare two methods to determine if their average results or their variabilities differ by an amount that is deemed important. The goal of a method comparison experiment is to generate adequate data to evaluate the equivalency of the two methods over a range of concentrations. Some of the considerations to be made when performing such comparisons are discussed in this section.

### Precision

Precision is the degree of agreement among individual test results when the analytical method is applied repeatedly to a homogeneous sample. For an alternative method to be considered to have "comparable" precision to that of a current method, its precision (see Analytical Performance Characteristics under [Validation of Compendial Procedures \(1225\)](#)) must not be worse than that of the current method by an amount deemed important. A decrease in precision (or increase in variability) can lead to an increase in the number of results expected to fail required specifications. On the other hand, an alternative method providing improved precision is acceptable.

One way of comparing the precision of two methods is by estimating the variance for each method (the sample variance,  $s^2$ , is the square of the sample standard deviation) and calculating a one-sided upper confidence interval for the ratio of (true) variances, where the ratio is defined as the variance of the alternative method to that of the current method. An example, with this assumption, is outlined under [Appendix D](#). The one-sided upper confidence limit should be compared to an upper limit deemed acceptable, a priori, by the analytical laboratory. If the one-sided upper confidence limit is less than this upper acceptable limit, then the precision of the alternative method is considered acceptable in the sense that the use of the alternative method will not lead to an important loss in precision. Note that if the one-sided upper confidence limit is less than one, then the alternative method has been shown to have improved precision relative to the current method.

The confidence interval method just described is preferred to applying the two-sample F-test to test the statistical significance of the ratio of variances. To perform the two-sample F-test, the calculated ratio of sample variances would be compared to a critical value based on tabulated values of the F distribution for the desired level of confidence and the number of degrees of freedom for each variance. Tables providing F-values are available in most standard statistical textbooks. If the calculated ratio exceeds this critical value, a statistically significant difference in precision is said to exist between the two methods. However, if the calculated ratio is less than the critical value, this does not prove that the methods have the same or equivalent level of precision; but rather that there was not enough evidence to prove that a statistically significant difference did, in fact, exist.

### Accuracy

Comparison of the accuracy (see Analytical Performance Characteristics under [Validation of Compendial Procedures \(1225\)](#)) of methods provides information useful in determining if the new method is equivalent, on the average, to the current method. A simple method for making this comparison is by calculating a confidence interval for the difference in true means, where the difference is estimated by the sample mean of the alternative method minus that of the current method.

The confidence interval should be compared to a lower and upper range deemed acceptable, a priori, by the laboratory. If the confidence interval falls entirely within this acceptable range, then the two methods can be considered equivalent, in the sense that the average difference between them is not of practical concern. The lower and upper limits of the confidence interval only show how large the true difference between the two methods may be, not whether this difference is considered tolerable. Such an assessment can only be



made within the appropriate scientific context.

The confidence interval method just described is preferred to the practice of applying a t-test to test the statistical significance of the difference in averages. One way to perform the t-test is to calculate the confidence interval and to examine whether or not it contains the value zero. The two methods have a statistically significant difference in averages if the confidence interval excludes zero. A statistically significant difference may not be large enough to have practical importance to the laboratory because it may have arisen as a result of highly precise data or a larger sample size. On the other hand, it is possible that no statistically significant difference is found, which happens when the confidence interval includes zero, and yet an important practical difference cannot be ruled out. This might occur, for example, if the data are highly variable or the sample size is too small. Thus, while the outcome of the t-test indicates whether or not a statistically significant difference has been observed, it is not informative with regard to the presence or absence of a difference of practical importance.

#### Determination of Sample Size

Sample size determination is based on the comparison of the accuracy and precision of the two methods<sup>4</sup> and is similar to that for testing hypotheses about average differences in the former case and variance ratios in the latter case, but the meaning of some of the input is different. The first component to be specified is  $\delta$ , the largest acceptable difference between the two methods that, if achieved, still leads to the conclusion of equivalence. That is, if the two methods differ by no more than  $\delta$ , they are considered acceptably similar. The comparison can be two-sided as just expressed, considering a difference of  $\delta$  in either direction, as would be used when comparing means. Alternatively, it can be one-sided as in the case of comparing variances where a decrease in variability is acceptable and equivalency is concluded if the ratio of the variances (new/current, as a proportion) is not more than 1.0 +  $\delta$ . A researcher will need to state  $\delta$  based on knowledge of the current method and/or its use, or it may be calculated. One consideration, when there are specifications to satisfy, is that the new method should not differ by so much from the current method as to risk generating out-of-specification results. One then chooses  $\delta$  to have a low likelihood of this happening by, for example, comparing the distribution of data for the current method to the specification limits. This could be done graphically or by using a tolerance interval, an example of which is given in [Appendix E](#). In general, the choice for  $\delta$  must depend on the scientific requirements of the laboratory.

The next two components relate to the probability of error. The data could lead to a conclusion of similarity when the methods are unacceptably different (as defined by  $\delta$ ). This is called a false positive or Type I error. The error could also be in the other direction; that is, the methods could be similar, but the data do not permit that conclusion. This is a false negative or Type II error. With statistical methods, it is not possible to completely eliminate the possibility of either error. However, by choosing the sample size appropriately, the probability of each of these errors can be made acceptably small. The acceptable maximum probability of a Type I error is commonly denoted as  $\alpha$  and is commonly taken as 5%, but may be chosen differently. The desired maximum probability of a Type II error is commonly denoted by  $\beta$ . Often,  $\beta$  is specified indirectly by choosing a desired level of  $1 - \beta$ , which is called the "power" of the test. In the context of equivalency testing, power is the probability of correctly concluding that two methods are equivalent. Power is commonly taken to be 80% or 90% (corresponding to a  $\beta$  of 20% or 10%), though other values may be chosen. The protocol for the experiment should specify  $\delta$ ,  $\alpha$ , and power. The sample size will depend on all of these components. An example is given in [Appendix E](#). Although [Appendix E](#) determines only a single value, it is often useful to determine a table of sample sizes corresponding to different choices of  $\delta$ ,  $\alpha$ , and power. Such a table often allows for a more informed choice of sample size to better balance the competing priorities of resources and risks (false negative and false positive conclusions).

#### APPENDIX A: CONTROL CHARTS

[Figure 1](#) illustrates a control chart for individual values. There are several different methods for calculating the upper control limit (UCL) and lower control limit (LCL). One method involves the moving range, which is defined as the absolute difference between two consecutive measurements ( $|x_i - x_{i-1}|$ ). These moving ranges are averaged ( MR ) and used in the following formulae:

$$UCL = \bar{x} + 3 \frac{\overline{MR}}{d_2}$$

$$LCL = \bar{x} - 3 \frac{\overline{MR}}{d_2}$$

where  $x$  is the sample mean, and  $d_2$  is a constant commonly used for this type of chart and is based on the number of observations associated with the moving range calculation.

Where  $n = 2$  (two consecutive measurements), as here,  $d_2 = 1.128$ . For the example in [Figure 1](#), the MR was 1.7:

$$UCL = 102.0 + 3 \frac{1.7}{1.128} = 106.5$$

$$LCL = 102.0 - 3 \frac{1.7}{1.128} = 97.5$$

Other methods exist that are better able to detect small shifts in the process mean, such as the cumulative sum (also known as "CUSUM") and exponentially weighted moving average ("EWMA").

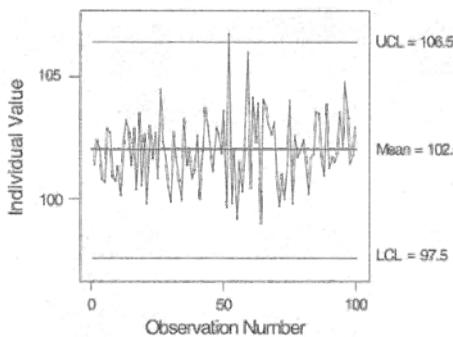


Fig. 1. Individual X or individual measurements control chart for control samples. In this particular example, the mean for all the samples (x) is 102.0, the UCL is 106.5, and the LCL is 97.5.

#### APPENDIX B: PRECISION STUDY

[Table 1](#) displays data collected from a precision study. This study consisted of five independent runs and, within each run, results from three replicates were collected.

Performing an analysis of variance (ANOVA) on the data in [Table 1](#) leads to the ANOVA table ([Table 1A](#)). Because there were an equal number of replicates per run in the precision study, values for VarianceRun and VarianceRep can be derived from the ANOVA table in a straightforward manner. The equations below calculate the variability associated with both the runs and the replicates where the MSwithin represents the “error” or “within-run” mean square, and MSbetween represents the “between-run” mean square.

$$\text{VarianceRep} = \text{MSwithin} = 0.102$$

$$\text{Variance}_{\text{Run}} = \frac{\text{MS}_{\text{between}} - \text{MS}_{\text{within}}}{\# \text{of runs per run}} = \frac{3.550 - 0.102}{3} = 1.149$$

Estimates can still be obtained with unequal replication, but the formulas are more complex. Studying the relative magnitude of the two variance components is important when designing and interpreting a precision study. For example, for these data the between-run component of variability is much larger than the within-run component. This suggests that performing additional runs would be more beneficial to reducing variability than performing more replication per run (see [Table 2](#)).

[Table 2](#) shows the computed variance and RSD of the mean (i.e., of the reportable value) for different combinations of number of runs and number of replicates per run using the following formulas:

*Variance of the mean =*

$$\frac{\text{Variance}_{\text{Run}}}{(\# \text{of runs})} + \frac{\text{Variance}_{\text{Rep}}}{(\# \text{of runs})(\# \text{of reps per run})}$$

*Standard deviation of the mean =  $\sqrt{\text{Variance of the mean}}$*

$$\text{RSD} = \frac{\text{Standard deviation of the mean}}{\text{Average of all results}} \times 100\%$$

For example, the Variance of the mean, Standard deviation of the mean, and RSD of a test involving two runs and three replicates per each run are 0.592, 0.769, and 0.76% respectively, as shown below.

$$\text{Variance of the mean} = \frac{1.149}{2} + \frac{0.102}{(2 \cdot 3)} = 0.592$$

$$\text{Standard deviation of the mean} = \sqrt{0.592} = 0.769$$

$$\text{RSD} = \frac{0.769}{100.96} \times 100\% = 0.76\%$$

Where 100.96 is the mean for all the data points in [Table 1](#). As illustrated in [Table 2](#), increasing the number of runs from one to two provides a more dramatic reduction in the variability of the reportable value than does increasing the number of replicates per run.

No distributional assumptions were made on the data in [Table 1](#) as the purpose of this Appendix is to illustrate the calculations involved in a precision study.

#### APPENDIX C: EXAMPLES OF OUTLIER TESTS FOR ANALYTICAL DATA

Given the following set of 10 measurements: 100.0, 100.1, 100.3, 100.0, 99.7, 99.9, 100.2, 99.5, 100.0, and 95.7 (mean = 99.5, standard deviation = 1.369) are there any outliers?

Generalized Extreme Studentized Deviate (ESD) Test

This is a modified version of the ESD Test that allows for testing up to a previously specified number, r, of outliers from a normally distributed population. Let r equal 2, and n equal 10.

Stage 1 (n = 10)—Normalize each result by subtracting the mean from each value and dividing this difference by the standard deviation (see [Table 3](#)).<sup>5</sup>

Take the absolute value of these results, select the maximum value ( $|R_1| = 2.805$ ), and compare it to a previously specified tabled critical value  $\lambda_1$  (2.290) based on the selected significance level (for example, 5%). The maximum value is larger than the tabled value and is identified as being inconsistent with the remaining data. Sources for  $\lambda$ -values are included in many statistical textbooks. Caution should be exercised when using any statistical table to ensure that the correct notations (i.e., level of acceptable error) are used when extracting table values.

Stage 2 (n = 9)—Remove the observation corresponding to the maximum absolute normalized result from the original data set, so that n is now 9. Again, find the mean and standard



uation ([Table 3](#), right two columns), normalize each value, and take the absolute value of these results. Find the maximum of the absolute values of the 9 normalized results ( $|R_1| = 1.905$ ), and compare it to  $\lambda_2$  (2.215). The maximum value is not larger than the tabled value.

Conclusion— The result from the first stage, 95.7, is declared to be an outlier, but the result from the second stage, 99.5, is not an outlier.

#### Dixon-Type Tests

Similar to the ESD test, the two smallest values will be tested as outliers; again assuming the data come from a single normal population.

Stage 1 ( $n = 10$ )— The results are ordered on the basis of their magnitude (i.e.,  $X_n$  is the largest observation,  $X_{n-1}$  is the second largest, etc., and  $X_1$  is the smallest observation).

Dixon's Test has different ratios based on the sample size (in this example, with  $n = 10$ ), to declare  $X_1$  an outlier, the following ratio,  $r_{11}$ , is calculated by the formula:

$$r_{11} = \frac{X_2 - X_1}{X_{n-1} - X_1}$$

A different ratio would be employed if the largest data point was tested as an outlier. The  $r_{11}$  result is compared to an  $r_{11}, 0.05$  value in a table of critical values. If  $r_{11}$  is greater than  $r_{11}, 0.05$ , then it is declared an outlier. For the above set of data,  $r_{11} = (99.5 - 95.7)/(100.2 - 95.7) = 0.84$ . This ratio is greater than  $r_{11}, 0.05$ , which is 0.534 at the 5% significance level for a two-sided Dixon's Test. Sources for  $r_{11}, 0.05$  values are included in many statistical textbooks.

Stage 2— Remove the smallest observation from the original data set, so that  $n$  is now 9. The same  $r_{11}$  equation is used, but a new critical  $r_{11}, 0.05$  value for  $n = 9$  is needed ( $r_{11}, 0.05 = 0.570$ ). Now  $r_{11} = (99.7 - 99.5)/(100.2 - 99.5) = 0.29$ , which is less than  $r_{11}, 0.05$  and not significant at the 5% level.

Conclusion— Therefore, 95.7 is declared to be an outlier. This stepwise procedure is not an exact procedure for testing for the second outlier as the result of the second test is conditional upon the first. Because the sample size is also reduced in the second stage, the end result is a procedure that usually lacks the sensitivity of the exact procedures that Dixon provides for testing for two outliers simultaneously; however, these procedures are beyond the scope of this Appendix.

#### Hampel's Rule

Step 1— The first step in applying Hampel's Rule is to normalize the data. However, instead of subtracting the mean from each data point and dividing the difference by the standard deviation, the median is subtracted from each data value and the resulting differences are divided by MAD (see below). The calculation of MAD is done in three stages. First, the median is subtracted from each data point. Next, the absolute values of the differences are obtained. These are called the absolute deviations. Finally, the median of the absolute deviations is calculated and multiplied by the constant 1.483 to obtain MAD<sup>6</sup>.

Step 2— The second step is to take the absolute value of the normalized data. Any such result that is greater than 3.5 is declared to be an outlier. [Table 4](#) summarizes the calculations.

The value of 95.7 is again identified as an outlier. This value can then be removed from the data set and Hampel's Rule re-applied to the remaining data. The resulting table is displayed as [Table 5](#). Similar to the previous examples, 99.5 is not considered an outlier.

#### APPENDIX D: COMPARISON OF METHODS—PRECISION

The following example illustrates the calculation of a 90% confidence interval for the ratio of (true) variances for the purpose of comparing the precision of two methods. It is assumed that the underlying distribution of the sample measurements are well-characterized by normal distributions. For this example, assume the laboratory will accept the alternative method if its precision (as measured by the variance) is no more than four-fold greater than that of the current method.

To determine the appropriate sample size for precision, one possible method involves a trial and error approach using the following formula:

$$\text{Power} = \Pr\left[F > \frac{1}{4} F_{\alpha, n-1, n-1}\right]$$

where  $n$  is the smallest sample size required to give the desired power, which is the likelihood of correctly claiming the alternative method has acceptable precision when in fact the two methods have equal precision;  $\alpha$  is the risk of wrongly claiming the alternative method has acceptable precision; and the 4 is the allowed upper limit for an increase in variance.  $F$ -values are found in commonly available tables of critical values of the F-distribution.  $F_{\alpha, n-1, n-1}$  is the upper  $\alpha$  percentile of an F-distribution with  $n - 1$  numerator and  $n - 1$  denominator degrees of freedom; that is, the value exceeded with probability  $\alpha$ . Suppose initially the laboratory guessed a sample size of 11 per method was necessary (10 numerator and denominator degrees of freedom); the power calculation would be as follows:<sup>7</sup>

$$\begin{aligned} \Pr\left[F > \frac{1}{4} F_{\alpha, n-1, n-1}\right] &= \Pr\left[F > \frac{1}{4} F_{.05, 10, 10}\right] = \\ \Pr\left[F > \frac{2.978}{4}\right] &= 0.6311 \end{aligned}$$

In this case the power was only 63%; that is, even if the two methods had exactly equal variances, with only 11 samples per method, there is only a 63% chance that the experiment will lead to data that permit a conclusion of no more than a four-fold increase in variance. Most commonly, sample size is chosen to have at least 80% power, with choices of 90% power or higher also used. To determine the appropriate sample size, various numbers can be tested until a probability is found that exceeds the acceptable limit (e.g., power > 0.90).

For example, the power determination for sample sizes of 12–20 are displayed in [Table 6](#). In this case, the initial guess at a sample size of 11 was not adequate for comparing precision, but 15 samples per method would provide a large enough sample size if 80% power were desired, or 20 per method for 90% power.

Typically the sample size for precision comparisons will be larger than for accuracy comparisons. If the sample size for precision is so large as to be impractical for the laboratory to conduct the study, there are some options. The first is to reconsider the choice of an allowable increase in variance. For larger allowable increases in variance, the required sample size for a fixed power will be smaller. Another alternative is to plan an interim analysis at a smaller sample size, with the possibility of proceeding to a larger sample size if needed. In this case, it is strongly advisable to seek professional help from a statistician.

Now, suppose the laboratory opts for 90% power and obtains the results presented in [Table 7](#) based on the data generated from 20 independent runs per method.

Ratio = Alternative Method Variance/Current Method Variance = 45.0/25.0 = 1.8

Lower Limit of Confidence Interval = Ratio/F.05 = 1.8/2.168 = 0.83

Upper Limit of Confidence Interval = Ratio/F.95 = 1.8/0.461 = 3.90



For this application, a 90% (two-sided) confidence interval is used when a 5% one-sided test is sought. The test is one-sided, because only an increase in standard deviation of the alternative method is of concern. Some care must be exercised in using two-sided intervals in this way, as they must have the property of equal tails—most common intervals have this property. Because the one-side upper confidence limit, 3.90, is less than the allowed limit, 4.0, the study has demonstrated that the alternative method has acceptable precision. If the same results had been obtained from a study with a sample size of 15—as if 80% power had been chosen—the laboratory would not be able to conclude that the alternative method had acceptable precision (upper confidence limit of 4.47).

#### APPENDIX E: COMPARISON OF METHODS—DETERMINING THE LARGEST ACCEPTABLE DIFFERENCE, $\delta$ , BETWEEN TWO METHODS

This Appendix describes one approach to determining the difference,  $\delta$ , between two methods (alternative-current), a difference that, if achieved, still leads to the conclusion of equivalence between the two methods. Without any other prior information to guide the laboratory in the choice of  $\delta$ , it is a reasonable way to proceed. Sample size calculations under various scenarios are discussed in this Appendix.

##### Tolerance Interval Determination

Suppose the process mean and the standard deviation are both unknown, but a sample of size 50 produced a mean and standard deviation of 99.5 and 2.0, respectively. These values were calculated using the last 50 results generated by this specific method for a particular (control) sample. Given this information, the tolerance limits can be calculated by the following formula:

$$x \pm Ks$$

in which  $x$  is the mean;  $s$  is the standard deviation; and  $K$  is based on the level of confidence, the proportion of results to be captured in the interval, and the sample size,  $n$ . Tables providing  $K$  values are available. In this example, the value of  $K$  required to enclose 95% of the population with 95% confidence for 50 samples is 2.382<sup>8</sup>. The tolerance limits are calculated as follows:

$$99.5 \pm 2.382 \times 2.0;$$

hence, the tolerance interval is (94.7, 104.3).

##### Comparison of the Tolerance Limits to the Specification Limits

Assume the specification interval for this method is (90.0, 110.0) and the process mean and standard deviation have not changed since this interval was established. The following quantities can be defined: the lower specification limit (LSL) is 90.0, the upper specification limit (USL) is 110.0, the lower tolerance limit (LTL) is 94.7, and the upper tolerance limit (UTL) is 104.3. Calculate the acceptable difference, ( $\delta$ ), in the following manner:

$$A = LTL - LSL \text{ for } LTL \geq LSL$$

$$(A = 94.7 - 90.0 = 4.7);$$

$$B = USL - UTL \text{ for } USL \geq UTL$$

$$(B = 110.0 - 104.3 = 5.7); \text{ and}$$

$$\delta = \text{minimum } (A, B) = 4.7.$$



Fig. 2. A graph of the quantities calculated above.

With this choice of  $\delta$ , and assuming the two methods have comparable precision, the confidence interval for the difference in means between the two methods (alternative-current) should fall within  $-4.7$  and  $+4.7$  to claim that no important difference exists between the two methods.

Quality control analytical laboratories sometimes deal with 99% tolerance limits, in which cases the interval will widen. Using the previous example, the value of  $K$  required to enclose 99% of the population with 99% confidence for 50 samples is 3.390. The tolerance limits are calculated as follows:

$$99.5 \pm 3.390 \times 2.0;$$

the resultant wider tolerance interval is (92.7, 106.3). Similarly, the new LTL of 92.7 and UTL of 106.3 would produce a smaller  $\delta$ :

$$A = LTL - LSL \text{ for } LTL \geq LSL$$

$$(A = 92.7 - 90.0 = 2.7);$$

$$B = USL - UTL \text{ for } USL \geq UTL$$

$$(B = 110.0 - 106.3 = 3.7); \text{ and}$$

$$\delta = \text{minimum } (A, B) = 2.7.$$

Choosing a larger  $\delta$  leads to a smaller  $n$  but at the cost of increasing the risk of reaching the wrong conclusion.

##### Sample Size

Formulas are available that can be used for a specified  $\delta$ , under the assumption that the population variances are known and equal, to calculate the number of samples required to be tested per method,  $n$ . The level of confidence and power must also be specified. [note—Power refers to the probability of correctly concluding that two identical methods are equivalent.] For example, if  $\delta = 4.7$ , and the two population variances are assumed to equal 4.0, then, for a 5% level test<sup>9</sup> and 80% power (with associated z-values of 1.645 and 1.282, respectively), the sample size is approximated by the following formula:

$$n \geq \frac{2\sigma^2}{\delta^2} (z_{\alpha} + z_{\beta/2})^2$$

$$n \geq \frac{2(4)}{(4.7)^2} (1.645 + 1.282)^2 = 3.10$$



Thus, assuming each method has a population variance,  $\sigma^2$ , of 4.0, the number of samples,  $n$ , required to conclude with 80% probability that the two methods are equivalent (90% confidence interval for the difference in the true means falls between -4.7 and +4.7) when in fact they are identical (the true mean difference is zero) is 4. Because the normal distribution was used in the above formula, 4 is actually a lower bound on the needed sample size. If feasible, one might want to use a larger sample size. Values for  $z$  for common confidence levels are presented in [Table 8](#). The formula above makes three assumptions: 1) the variance used in the sample size calculation is based on a sufficiently large amount of prior data to be treated as known; 2) the prior known variance will be used in the analysis of the new experiment, or the sample size for the new experiment is sufficiently large so that the normal distribution is a good approximation to the t distribution; and 3) the laboratory is confident that there is no actual difference in the means, the most optimistic case. It is not common for all three of these assumptions to hold. The formula above should be treated most often as an initial approximation. Deviations from the three assumptions will lead to a larger required sample size. In general, we recommend seeking assistance from someone familiar with the necessary methods.

When a log transformation is required to achieve normality, the sample size formula needs to be slightly adjusted as shown below. Instead of formulating the problem in terms of the population variance and the largest acceptable difference,  $\delta$ , between the two methods, it now is formulated in terms of the population RSD and the largest acceptable proportional difference between the two methods.

$$n \geq \frac{2\sigma_L^2}{\delta_L^2} \left( z_\alpha + z_{\beta/2} \right)^2$$

where

$$\sigma_L^2 = \log((RSD)^2 + 1)$$

$$\delta_L^2 = (\log(\rho + 1))^2$$

and  $\rho$  represents the largest acceptable proportional difference between the two methods ((alternative-current)/current) and the population RSDs are assumed known and equal.

#### APPENDIX F: ADDITIONAL SOURCES OF INFORMATION

There may be a variety of statistical tests that can be used to evaluate any given set of data. This chapter presents several tests for interpreting and managing analytical data, but many other similar tests could also be employed. The chapter simply illustrates the analysis of data using statistically acceptable methods. As mentioned in the Introduction, specific tests are presented for illustrative purposes, and USP does not endorse any of these tests as the sole approach for handling analytical data. Additional information and alternative tests can be found in the references listed below or in many statistical textbooks.

##### Control Charts:

1. Manual on Presentation of Data and Control Chart Analysis, 6th ed., American Society for Testing and Materials (ASTM), Philadelphia, 1996.
2. Grant, E.L., Leavenworth, R.S., Statistical Quality Control, 7th ed., McGraw-Hill, New York, 1996.
3. Montgomery, D.C., Introduction to Statistical Quality Control, 3rd ed., John Wiley and Sons, New York, 1997.
4. Ott, E., Schilling, E., Neubauer, D., Process Quality Control: Troubleshooting and Interpretation of Data, 3rd ed., McGraw-Hill, New York, 2000.

##### Detectable Differences and Sample Size Determination:

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TABLES

Table 1. Data from a Precision Study

Replicate Number	Run Number				
	1	2	3	4	5
1	100.70	99.46	99.96	101.80	101.91
2	101.05	99.37	100.17	102.16	102.00
3	101.15	99.59	101.01	102.44	101.67
Mean	100.97	99.47	100.38	102.13	101.86
Standard Deviation	0.236	0.111	0.556	0.321	0.171
RSD <sup>1</sup>	0.234%	0.111%	0.554%	0.314%	0.167%

1 RSD (relative standard deviation) =  $100\% \times (\text{standard deviation}/\text{mean})$

Table 1A. Analysis Variance Table for Data Presented in Table 1

Source of Variation	Degrees of Freedom (df)	Sum of Squares (SS)	Mean Squares <sup>1</sup> (MS)	F = MSB / MSW
Between Runs	4	14.200	3.550	34.80
Within Runs	10	1.018	0.102	
Total	14	15.217		

1 The Mean Squares Between (MSB) =  $SS_{\text{Between}}/df_{\text{Between}}$  and the Mean Squares Within (MSW) =  $SS_{\text{Within}}/df_{\text{Within}}$

Table 2. Computed Variance and RSD of the Mean

No. of Runs	No. of Replicates	Precision of the Mean Corresponding to Various Test Plans (# of Runs, # of Reps per Run)			
		Variance of the Mean	SD of the Mean	Mean <sup>1</sup>	RSD (%)
1	1	1.251	1.118	100.96	1.11
1	2	1.200	1.095	100.96	1.09
1	3	1.183	1.088	100.96	1.08
2	1	0.625	0.791	100.96	0.78
2	2	0.600	0.775	100.96	0.77
2	3	0.592	0.769	100.96	0.76

1 Sample mean is based on the 15 data points presented in Table 1.

Table 3. Generalized ESD Test Results

	n = 10		n = 9	
	Data	Normalized	Data	Normalized
	100.3	+0.555	100.3	+1.361



100.2	+0.482	100.2	+0.953
100.1	+0.409	100.1	+0.544
100.0	+0.336	100.0	+0.136
100.0	+0.336	100.0	+0.136
100.0	+0.336	100.0	+0.136
99.9	+0.263	99.9	-0.272
99.7	+0.117	99.7	-1.089
99.5	-0.029	99.5	-1.905
95.7	-2.805		
Mean =	99.54	99.95	
SD =	1.369	0.245	

Table 4. Test Results Using Hampel's Rule

n = 10

	Data	Deviations from the Median	Absolute Deviations	Absolute Normalized
	100.3	0.3	0.3	1.35
	100.2	0.2	0.2	0.90
	100.1	0.1	0.1	0.45
	100	0	0	0
	100	0	0	0
	100	0	0	0
	99.9	-0.1	0.1	0.45
	99.7	-0.3	0.3	1.35
	99.5	-0.5	0.5	2.25
	95.7	-4.3	4.3	19.33
Median =	100		0.15	
MAD =			0.22	

Table 5. Test Results of Re-Applied Hampel's Rule

n = 9

	Data	Deviations from the Median	Absolute Deviations	Absolute Normalized
	100.3	0.3	0.3	2.02
	100.2	0.2	0.2	1.35
	100.1	0.1	0.1	0.67
	100	0	0	0
	100	0	0	0
	100	0	0	0
	99.9	-0.1	0.1	0.67
	99.7	-0.3	0.3	2.02
	99.5	-0.5	0.5	3.37
Median =	100		0.1	
MAD =			0.14	

Table 6. Power Determinations for Various Sample Sizes (Specific to the Example in Appendix D)

Sample Size	Pr[F > 1/4 F0.05, n-1, n-1]
12	0.7145
13	0.7495
14	0.7807
15	0.8083
16	0.8327
17	0.8543
18	0.8732
19	0.8899
20	0.9044

Table 7. Example of Measures of Variance for Independent Runs (Specific to the Example in Appendix D)

Method	Variance (standard deviation)	Sample Size	Degrees of Freedom
Alternative	45.0 (6.71)	20	19
Current	25.0 (5.00)	20	19

Table 8. Common Values for a Standard Normal Distribution

	z-values	
Confidence level	One-sided ( $\alpha$ )	Two-sided ( $\alpha/2$ )



99%	2.326	2.576
95%	1.645	1.960
90%	1.282	1.645
80%	0.842	1.282

1 Multiple measurements (or, equivalently, the experimental errors associated with the multiple measurements) are independent from one another when they can be assumed to represent a random sample from the population. In such a sample, the magnitude of one measurement is not influenced by, nor does it influence the magnitude of, any other measurement. Lack of independence implies the measurements are correlated over time or space. Consider the example of a 96-well microtiter plate. Suppose that whenever the unknown causes that produce experimental error lead to a low result (negative error) when a sample is placed in the first column and these same causes would also lead to a low result for a sample placed in the second column, then the two resulting measurements would not be statistically independent. One way to avoid such possibilities would be to randomize the placement of the samples on the plate.

2 When data have been log (base e) transformed to achieve normality, the RSD is:

$$RSD = 100\% \cdot \sqrt{e^{s^2} - 1}$$

This can be reasonably approximated by:

$$RSD = 100\% \cdot (e^s - 1)$$

where  $s$  is the standard deviation of the log (base e) transformed data.

3 See System Suitability under [Chromatography](#) (621).

4 In general, the sample size required to compare the precision of two methods will be greater than that required to compare the accuracy of the methods.

5 The difference between each value and the mean is termed the residual. Other Studentized residual outlier tests exist where the residual, instead of being divided by the standard deviation, can be divided by the standard deviation times the square root of  $n - 1$  divided by  $n$ .

6 Assuming an underlying normal distribution, 1.483 is a constant used so that the resulting MAD is a consistent estimator of the population standard deviation. This means that as the sample size gets larger,  $1.483 \times \text{MAD}$  gets closer to the population standard deviation.

7 This could be calculated using a computer spreadsheet. For example, in Microsoft® Excel the formula would be:  $\text{FDIST}((R/A) * \text{FINV}(\text{alpha}, n - 1, n - 1), n - 1, n - 1)$ , where  $R$  is the ratio of variances at which to determine power (e.g.,  $R = 1$ , which was the value chosen in the power calculations provided in the above table) and  $A$  is the maximum ratio for acceptance (e.g.,  $A = 4$ ).  $\text{alpha}$  is the significance level, typically 0.05.

8 There are existing tables of tolerance factors that give approximate values and thus differ slightly from the values reported here.

9 When testing equivalence, a 5% level test corresponds to a 90% confidence interval.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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#### 1015 AUTOMATED RADIOCHEMICAL SYNTHESIS APPARATUS

The preparation and quality control of diagnostic radiopharmaceuticals labeled with the very short-lived positron-emitting nuclides (e.g.,  $^{15}\text{O}$ ,  $^{13}\text{N}$ ,  $^{11}\text{C}$  and  $^{18}\text{F}$  having half-lives of 2, 10, 20, and 110 minutes, respectively), are subject to constraints different from those applicable to therapeutic drugs: (1) Synthesis must be rapid, yet must be arranged to protect the chemist or pharmacist from excessive radiation exposure. (2) Except to a limited extent for  $^{18}\text{F}$ , synthesis must occur at the time of use. (3) With the exception of  $^{18}\text{F}$ , each batch of radiopharmaceutical generally leads to only a single administration.

These factors raise the importance of quality control of the final drug product relative to validation of the synthesis process. Since with few exceptions every dose is individually manufactured, ideally every dose should be subjected to quality control tests for radiochemical purity and other key aspects of quality before administration. Because quality testing of every batch is not possible, batches are selected at regular intervals for examination to establish and completely characterize their radiopharmaceutical purity. This routine and thorough quality testing of selected batches forms the basis of process validation, which is absolutely essential for prospective assessment of batch quality and purity when dealing with such extremely short-lived radiopharmaceuticals. Since radiopharmaceuticals used in positron emission tomography (PET) are administered intravenously or (for radioactive gases) by inhalation, batch-to-batch variability in bioavailability is not an issue. Furthermore, the very small scale of radiopharmaceutical syntheses (almost always less than 1 milligram and often in the microgram range) and the fact that patients generally receive only a single dose of radioactive drug minimize the likelihood of administering harmful amounts of chemical impurities. These statements are not intended to contest the need for quality control in the operation of automated synthesis equipment, but to place the manufacture of positron-emitting radiopharmaceuticals in an appropriate perspective and to reemphasize the overwhelming importance of prospective process validation and finished product quality control.

The routine synthesis of radiopharmaceuticals can result in unnecessarily high radiation doses to personnel. Automated radiochemical synthesis devices have been developed, partly to comply with the concept of reducing personnel radiation exposures to "as low as reasonably achievable" (ALARA). These automated synthesis devices can be more efficient and precise than existing manual methods. Such automated methods are especially useful where a radiochemical synthesis requires repetitive, uniform manipulations on a daily basis.

The products from these automated radiosynthesis devices must meet the same quality assurance criteria as the products obtained by conventional manual syntheses. In the case of positron-emitting radiopharmaceuticals, these criteria will include many of the same determinations used for conventional nuclear medicine radiopharmaceuticals, for example, tests for sterility and bacterial endotoxins. Many of the same limitations apply. Typical analytical procedures such as spectroscopy are not generally applicable because the small amount of product is below the minimum detection level of the method. In all cases, the applicable Pharmacopeial method is the conclusive arbiter (see Procedures under Tests and Assays in the General Notices).

Preparation of Fludeoxyglucose F 18 Injection and other positron-emitting radiopharmaceuticals can be adapted readily to automated synthesis. In general, the equipment required for the manual methods is simpler and less expensive than that used in automated methods but is more labor-intensive. Of special concern are the methods involved in validating the correct performance of an automated apparatus. For a manual procedure, human intervention and correction by inspection can nullify many procedural errors. In an automated system, effective feedback also can begin during the synthesis. For example, radiation detectors can monitor activity at various stages of radiosynthesis. Failure to obtain the appropriate activity could activate an alarm system that would lead to human intervention.

Radiochemicals versus Radiopharmaceuticals— It is appropriate to draw a distinction between a radiochemical and a corresponding radiopharmaceutical. In research PET centers, automated equipment is used to prepare labeled compounds for animal experiments. These radiochemicals are not regarded as radiopharmaceuticals if (1) they are not prepared according to a validated process that provides a high degree of assurance that the preparation meets all established requirements for quality and purity; and (2) have not been certified by qualified personnel (licensed pharmacists and approved physicians) in accordance with published Pharmacopeial methods for individual radiopharmaceuticals.



Automated Equipment— The considerations in this chapter apply to synthesis conducted by general purpose robots and by special purpose apparatus. Both are automated devices used in the synthesis of radiochemicals. The exact method of synthesis device control is variable. Both hard-wired and software-controlled synthesis devices fall under the general designation, and there is a spectrum ranging from traditional manual equipment through semi-automated devices to completely automatic devices.

Common Elements of Automated Synthesis Equipment— To manipulate a chemical apparatus to effect the synthesis of a radiochemical, control of parameters such as time, temperature, pressure, volume, and sequencing are needed. These parameters can be monitored and constrained to fall within certain bounds.

Equipment Quality Assurance— The goal of quality assurance is to help ensure that the subsequent radiopharmaceutical meets Pharmacopeial standards. Although the medical device good manufacturing practice regulations (21 CFR 820) are not applicable, they may be helpful in developing a quality assurance program. As a practical matter this involves documented measurement and control of all relevant physical parameters controlled by the synthesis apparatus.

Routine Quality Control Testing— Routine quality control testing of automated equipment implies periodic testing of all parameters initially certified during the quality assurance qualification. Depending on the criticality and the stability of the parameter setting, testing may be as often as daily. This process performance assessment must be augmented by regular end product testing. For example, variations in the temperature of an oil bath may be acceptable if the radiochemical (end product) can be shown to meet all relevant testing criteria.

Reagent Audit Trail— Materials and reagents used for the synthesis of radiopharmaceuticals should conform to established quality control specifications and tests. Procedures for the testing, storage, and use of these materials should be established. In this context, a reagent is defined as any chemical used in the procedure leading to the final radiochemical product, whereas materials are defined as ancillary supplies (tubing, glassware, vials, etc.). For example, in some processes compressed nitrogen is used to move liquid reagents. In this case, both the nitrogen and the tubing should meet established specifications.

Documentation of Apparatus Parameters— Key synthesis variables should be identified, monitored, and documented. These characteristics include meaningful physical, chemical, electrical, and performance attributes. A method for specifying, testing, and documenting computer software and hardware is especially important for microprocessor- and computer-controlled devices. This program should include periodic generalized testing of the computer hardware. In addition, the software program code should be periodically examined to determine that it has not been modified and that it continues to result in the final product's meeting all specifications. In-process feedback is one means of confirming that the synthesis is under control. Changes to the software code should involve a formal authorization procedure, and changes should be documented.

Each type of radiochemical synthesis device requires a set of specific procedures for testing and monitoring the reliability and reproducibility of the various subsystems that make up the total synthesis system.

It is essential that calibration of each of the components be confirmed according to an established maintenance timetable and that measurements or monitoring be made under actual synthesis conditions.

Delivery times, reagent volumes, temperatures, gas pressures, and rates of flow need to be measured and shown to be stable and reproducible within established limits. Delivery of the reagents and solvents needs to be calibrated periodically. Other components to be routinely calibrated include the radiation detection system and process monitoring sensors and system.

For illustration, elements of system validation of several representative components of an automatic synthetic device are as follows:

Reaction vessels may be cleaned and inspected by an established documented method. The vessels themselves may be numbered and their performance tracked.

Heating and cooling systems (such as oil baths) may be monitored by thermometers or thermocouples. The temperatures may be recorded in a batch sheet, or they may be automatically printed out as part of a computerized log. Maintenance involves periodic calibration.

Gases and gas delivery system performance may be tracked by pressure gauges and flowmeters. Gas purity may be established via supplier certificates of analysis or may be verified by independent testing. Maintenance of gauges and flowmeters involves periodic calibration with standards.

Position-dependent motor performance may be verified by limit switches. Maintenance could involve actual measurement of distance traversed and elapsed time.

Solenoid valves may be checked electrically, by flow and pressure tests.

Heater output is evidenced by proper thermocouple readings. Additional tests could involve resistance measurements.

Reagents may be accepted on the basis of suppliers' certificates of analysis. Alternatively, the chemical could be tested in-house or sent to an independent testing laboratory. Periodic retesting may be necessary depending on stability.

Computer programs may be tested by documenting elapsed time of synthesis, with printouts verifying that all appropriate manipulations occurred, including printing of relevant parameters such as times, temperature, pressures, and activities.

Patterns of activity distribution such as absolute amount of product, percentage yield, and individual impurity activity levels afford the experienced user an opportunity to discern systems failures.

Changes in the Synthesis Method— Some changes in the synthesis apparatus can be considered to be trivial. This category would often include changes not affecting any of the monitored parameters. However, it is important that care be taken to ensure that seemingly innocuous changes do not have an unexpected impact. For example, changes in a comment line of a computer program may result in inadvertently changing or deleting a vital instruction. Any changes in monitored parameters have the potential for changing the process output. If the resultant radiochemical does not meet specifications or if the subsequent radiopharmaceutical does not meet Pharmacopeial criteria, the process change is unacceptable; the fault must be corrected and the process revalidated.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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#### 1031 THE BIOCOMPATIBILITY OF MATERIALS USED IN DRUG CONTAINERS, MEDICAL DEVICES, AND IMPLANTS

This chapter provides guidance on the identification and performance of procedures for evaluating the biocompatibility of drug containers, elastomeric closures, medical devices, and implants. Biocompatibility refers to the tendency of these products to remain biologically inert throughout the duration of their contact with the body. The biocompatibility testing procedures referenced in this chapter are designed to detect the nonspecific, biologically reactive, physical or chemical characteristics of medical products or the materials used in their construction. In combination with chemical assays, these biological procedures can be used to detect and identify the inherent or acquired toxicity of medical products prior to or during their manufacturing and processing.

Preclinical testing procedures to evaluate the safety of the elastomers, plastics, or other polymers used in the construction of medical products are referenced or described in the following general chapters: [Injections](#) 1, [Biological Reactivity Tests, In Vitro](#) 87, [Biological Reactivity Tests, In Vivo](#) 88, [Transfusion and Infusion Assemblies and Similar Medical Devices](#) 161, [Elastomeric Closures for Injections](#) 381, and [Containers—Plastics](#) 661. Specific in vitro and in vivo testing procedures to evaluate the biocompatibility



of medical products in patients are described under [Biological Reactivity Tests, In Vitro](#) 87 and under [Biological Reactivity Tests, In Vivo](#) 88.

The procedures used to evaluate the biocompatibility of a medical product or its construction materials have been categorized as a panel of biological effects (toxicity procedures): cytotoxicity, sensitization, irritation or intracutaneous reactivity, acute systemic toxicity, subchronic toxicity (subacute toxicity), genotoxicity, implantation, hemocompatibility, chronic toxicity (extending beyond 10% of the life span of the test animal or beyond 90 days), carcinogenicity, reproductive or developmental toxicity, and biodegradation.<sup>1</sup> The USP general chapters referring to the toxicity procedures for these categories are indicated in [Table 1](#). In addition, pyrogenicity, an area of special toxicity, is evaluated under [Pyrogen Test](#) 151 and under [Bacterial Endotoxins Test](#) 85. There are currently no general chapters that detail sensitization, subchronic toxicity, genotoxicity, chronic toxicity, carcinogenicity, hemotoxicity, reproductive toxicity, or biodegradation testing requirements.

Table 1. Toxicity Procedures in the USP General Chapters

Biological Effect	USP General Chapter
Cytotoxicity	<a href="#">Biological Reactivity Tests, In Vitro</a> 87*
Sensitization	[to come]
Irritation or intracutaneous reactivity	<a href="#">Biological Reactivity Tests, In Vivo</a> 88‡
Systemic toxicity (acute toxicity)	<a href="#">Biological Reactivity Tests, In Vivo</a> 88
Subchronic toxicity (subacute toxicity)	[to come]
Genotoxicity	[to come]
Implantation	<a href="#">Biological Reactivity Tests, In Vivo</a> 88
Hemocompatibility	Under development in the USP monograph Sterile Single-Use Plastic Large-Volume Containers for Human Blood and Blood Components
Chronic toxicity	[to come]
Carcinogenicity	[to come]
Reproductive or developmental toxicity	[to come]
Biodegradation	[to come]

\* Additional general chapters referring to this biological effect include [Transfusion and Infusion Assemblies and Similar Medical Devices](#) 161, [Elastomeric Closures for Injections](#) 381, and [Containers—Plastics](#) 661.

† Additional general chapters referring to this biological effect include [Injections](#) 1, [Transfusion and Infusion Assemblies and Similar Medical Devices](#) 161, [Elastomeric Closures for Injections](#) 381, and [Containers—Plastics](#) 661.

## DRUG CONTAINERS

### Biocompatibility of Plastic and Other Polymeric Drug Containers

Pharmaceutical containers consist of a container and a closure. Plastic containers may consist of polymers that upon extraction do not alter the stability of the contained product or do not exhibit toxicity. The biocompatibility testing requirements for drug containers are stated under [Injections](#) 1 and [Containers—Plastics](#) 661. As directed in these chapters, the plastic or other polymeric portions of these products are tested according to the procedures set forth under [Biological Reactivity Tests, In Vitro](#) 87. A plastic or other polymer that does not meet the requirements of [Biological Reactivity Tests, In Vitro](#) 87 is not a suitable material for a drug container. Materials that meet the in vitro requirements qualify as biocompatible materials without the need for further testing and may be used in the construction of a drug container. If a class designation (classes I–VI) for plastics or other polymers is desired, the appropriate testing procedures are performed as discussed in the section In Vivo Testing and Class Designation.

### Elastomeric Closures

Elastomeric closures are closures that can be pierced by a syringe and maintain their integrity because of their elastic properties. Elastomeric materials may be composed of several chemical entities including fillers, pigments, plasticizers, stabilizers, accelerators, vulcanizing agents, and a natural or a synthetic polymer. These materials are used for manufacturing a product with the desired elastomeric physical properties, and they frequently demonstrate biological reactivity—cellular degeneration and malformation—when tested with in vitro cell cultures.

The biocompatibility of an elastomeric material is evaluated according to the two-stage testing protocol specified in the Biological Test Procedures under [Elastomeric Closures for Injections](#) 381. Unlike plastics or other polymers, an elastomeric material that does not meet the requirements of the first-stage (in vitro) testing may qualify as a biocompatible material by passing the second-stage (in vivo) testing, which consists of the Systemic Injection Test and the Intracutaneous Test described under [Biological Reactivity Tests, In Vivo](#) 88. No class or type distinction is made between elastomeric materials that meet the requirements of the first stage of testing and those that qualify as biocompatible materials by meeting the second-stage requirements. Elastomeric materials are not assigned class I–VI designation.

## MEDICAL DEVICES AND IMPLANTS

Medical devices and implants, labeled nonpyrogenic, in direct or indirect contact with the cardiovascular system or other soft body tissues, meet the requirements described under [Transfusion and Infusion Assemblies and Similar Medical Devices](#) 161. The products listed in this chapter that meet the criteria are solution administration sets, extension sets, transfer sets, blood administration sets, intravenous catheters, dialyzers and dialysis tubing and accessories, transfusion and infusion assemblies, and intramuscular drug delivery catheters. The outlined criteria do not apply to medical products such as orthopedic products, latex gloves, and wound dressings.

The testing requirements described or referenced under [Transfusion and Infusion Assemblies and Similar Medical Devices](#) 161 include Sterility, Bacterial endotoxins, Pyrogen, and Other requirements. A procedure to evaluate the presence of bacterial endotoxins is set forth under [Bacterial Endotoxins Test](#) 85, and the limits are set in [Bacterial Endotoxins](#) under [Transfusion and Infusion Assemblies and Similar Medical Devices](#) 161. For devices that cannot be tested by the [Bacterial Endotoxins Test](#) 85 because of nonremovable inhibition or enhancement, the [Pyrogen Test](#) 151 is applied. The procedures for evaluating medical devices purported to contain sterile pathways are set forth in [Sterile Devices](#) under [Sterility Tests](#) 71. A procedure for evaluating the safety of medical devices is set forth in the Safety Test under [Biological Reactivity Tests, In Vivo](#) 88.

The plastic or other polymer components of medical devices meet the requirements specified for plastics and other polymers under [Containers—Plastics](#) 661; those made of elastomers meet the requirements under [Elastomeric Closures for Injections](#) 381. As directed in these chapters, the biocompatibility of the plastic, other polymeric, and elastomeric portions of these products are tested according to the procedures described under [Biological Reactivity Tests, In Vitro](#) 87. If a class designation for a plastic or other polymer is also required, the appropriate testing procedures described under [Biological Reactivity Tests, In Vivo](#) 88 are performed.

As required for elastomeric closures, elastomeric materials that do not meet the in vitro requirements may qualify as biocompatible materials and may be used in the construction of medical devices if they meet the requirements of the Systemic Injection Test and the Intracutaneous Test under [Biological Reactivity Tests, In Vivo](#) 88. As required for drug containers, plastics and other polymers that do not meet the in vitro testing requirements are not suitable materials for use in medical devices.

## IN VITRO TESTING, IN VIVO TESTING, AND CLASS DESIGNATION FOR PLASTICS AND OTHER POLYMERS

The testing requirements specified under [Biological Reactivity Tests, In Vitro](#) 87 and [Biological Reactivity Tests, In Vivo](#) 88 are designed to determine the biological reactivity of



mammalian cell cultures and the biological response of animals to elastomeric, plastic, and other polymer materials with direct or indirect patient contact. The biological reactivity of these materials may depend on both their surface characteristics and their extractable chemical components. The testing procedures set forth in these chapters can often be performed with the material or an extract of the material under test, unless otherwise specified.

#### Preparation of Extracts

Evaluation of the biocompatibility of a whole medical product is often not realistic; thus, the use of representative portions or extracts of selected materials may be the only practical alternative for performing the assays. When representative portions of the materials or extracts of the materials under test are used, it is important to consider that raw materials may undergo chemical changes during the manufacturing, processing, and sterilization of a medical product. Although *in vitro* testing of raw materials can serve as an important screening procedure, a final evaluation of the biocompatibility of a medical product is performed with portions of the finished and sterilized product.

The preparation of extracts is performed according to the procedures set forth under [Biological Reactivity Tests, In Vitro](#) (87) and under [Biological Reactivity Tests, In Vivo](#) (88). Extractions may be performed at various temperatures (121°, 70°, 50°, or 37°), for various time intervals (1 hour, 24 hours, or 72 hours), and in different extraction media. The choice of extraction medium for the procedures under [Biological Reactivity Tests, In Vitro](#) (87) includes Sodium Chloride Injection (0.9% NaCl) or tissue culture medium with or without serum. When medium with serum is used, the extraction temperature cannot exceed 37°. *In vivo* extraction medium includes the choices described under [Biological Reactivity Tests, In Vivo](#) (88) or the solvent to which the drug or medical device is exposed.

When choosing extraction conditions, select the temperature, solvent, and time variables that best mimic the "in use" conditions of the product. The performance of multiple tests at various conditions can be used to simulate variations in the "in use" conditions. Although careful selection of extraction conditions allows the simulation of manufacturing and processing conditions in the testing of raw materials, an evaluation of the biocompatibility of the product is performed with the finished and sterilized product.

#### In Vitro Testing

The procedures described under [Biological Reactivity Tests, In Vitro](#) (87) include an Agar Diffusion Test (indirect contact test), a Direct Contact Test, and an Elution Test (extraction test). The sample is biocompatible if the cell cultures do not exhibit greater than a mild reactivity (Grade 2) to the material under test, as described under [Biological Reactivity Tests, In Vitro](#) (87). The Agar Diffusion Test is designed to evaluate the biocompatibility of elastomeric materials. The material is placed on the agar overlay of the cell monolayer, which cushions the cells from physical damage by the material and allows leachable chemicals or materials to diffuse from the elastomer and contact the cell monolayer. Extracts of elastomeric materials are tested by placing the filter paper saturated with an extract of the elastomer on the solidified surface of the agar. The Direct Contact Test is designed for elastomeric or plastic materials that will not physically damage cells with which they are in direct contact. Any leachable chemicals diffuse from the material into the serum-supplemented growth medium and directly contact the cell monolayer. The Elution Test is designed to evaluate the extracts of polymeric materials. The material may be applied directly to the tissue culture media.

The performance of either the Agar Diffusion Test or the Direct Contact Test in combination with the Elution Test is the preferred testing protocol. Extraction of the product or materials for the Agar Diffusion Test or the Elution Test is performed as described in the Preparation of Extracts.

#### In Vivo Testing and Class Designation

According to the injection and implantation requirements specified in [Table 1](#) under [Biological Reactivity Tests, In Vivo](#) (88), plastics and other polymers are assigned a class designation between class I and class VI. To obtain a plastic or other polymer class designation, extracts of the test material are produced according to the specified procedures in various media. To evaluate biocompatibility, the extracts are injected systemically and intracutaneously into mice and rabbits. According to the specified injection requirements, a plastic or other polymer may initially be graded as class I, II, III, or V. If in addition to injection testing, implantation testing using the material itself is performed, the plastic or other polymer may be classified as class IV or class VI.

### BIOCOMPATIBILITY OF MEDICAL DEVICES AND IMPLANTS

In addition to evaluating medical products for compendial purposes according to the procedures specified under [Injections](#) (1), [Sterility](#) (71), [Biological Reactivity Tests, In Vitro](#) (87), [Biological Reactivity Tests, In Vivo](#) (88), [Transfusion and Infusion Assemblies and Similar Medical Devices](#) (161), [Elastomeric Closures for Injections](#) (381), and [Containers—Plastics](#) (661), medical devices and implants are evaluated for sensitization, subchronic toxicity, genotoxicity, hemocompatibility, chronic toxicity, carcinogenicity, reproductive or developmental toxicity, and biodegradation as required by the regulatory agencies.

The guidance provided by the regulatory agencies indicates that the extent of testing that is performed for a medical device or an implant depends on the following factors: (1) the similarity and uniqueness of the product relative to previously marketed ("predicate") products as considered in the Decision Flowchart; (2) the extent and duration of the contact between the product and the patient as described in the Categorization of Medical Devices; and (3) the material composition of the product as considered in the sections Decision Flowchart and In Vivo Testing and Class Designation.

#### Decision Flowchart

Guidance on comparing a medical device or an implant to previously marketed products is provided by the Biocompatibility Decision Flowchart (see [Figure 1](#)).

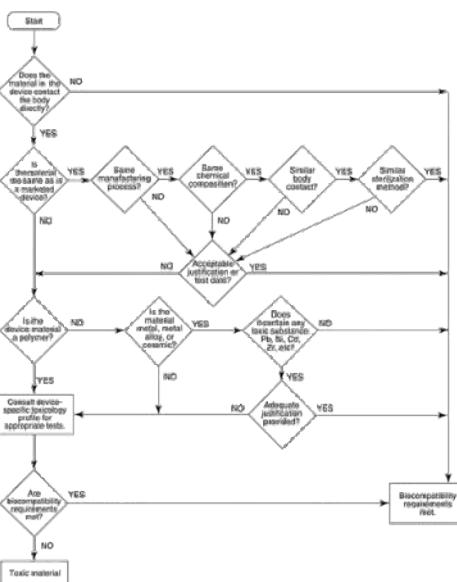


Fig. 1. Biocompatibility flowchart

as adapted from the FDA's Blue Book Memorandum #G95-1. The purpose of the flowchart is to determine whether the available data from previously marketed devices are sufficient to



...sure the safety of the device under consideration. As indicated by the flowchart, the material composition and the manufacturing techniques of a product are compared to those of the previously marketed products for the devices that come in direct contact with the body. In addition, the flowchart requires an evaluation of the toxicity of any unique material that has not been used in predicate devices. Responses to the questions posed in the flowchart lead to the conclusion that either the available data are sufficient or additional testing is required to ensure the safety of the product. When additional testing is required, guidance on the identification of appropriate testing procedures is provided in the section Test Selection Matrix.

#### Categorization of Medical Devices

To facilitate the identification of appropriate testing procedures, medical devices are divided and subdivided, as shown in [Table 2](#).

Table 2. Classification and Examples of Medical Devices

Device Category	Device Subcategory	Nature or Extent of Contact	Some Examples
	Skin	Devices that contact intact skin surfaces only	Electrodes, external prostheses, fixation tapes, compression bandages, and monitors of various types
Surface Devices	Mucosal Membrane	Devices communicating with intact mucosal membranes	Contact lenses, urinary catheters, intravaginal and intraintestinal devices (stomach tubes, sigmoidoscopes, colonoscopes, gas troscopes), endotracheal tubes, bronchoscopes, dental prostheses, orthodontic devices, and intrauterine devices
	Breached or Compromised Surfaces	Devices that contact breached or otherwise compromised body surfaces	Ulcer, burn, and granulation tissue dressings or healing devices and occlusive patches
	Blood Path, Indirect	Devices that contact the blood path at one point and serve as a conduit for entry into the vascular system	Solution administration sets, extension sets, transfer sets, and blood administration sets
External Communicating Devices	Tissue, Bone, or Dentin Communicating	Devices and materials communicating with tissue, bone, or pulp and dentin system	Laparoscopes, arthroscopes, draining systems, dental cements, dental filling materials, and skin staples
	Circulating blood	Devices that contact circulating blood	Intravascular catheters, temporary pacemaker electrodes, oxygenators, extracorporeal oxygenator tubing and accessories, dialyzers, dialysis tubing and accessories, hemoadsorbents, and immunoabsorbents
Implant Devices	Tissue or Bone	Devices principally contacting bone or principally contacting tissue and tissue fluid	Examples of the former are orthopedic pins, plates, replacement joints, bone prostheses, cements, and intraosseous devices; examples of the latter are pacemakers, drug supply devices, neuromuscular sensors and simulators, replacement tendons, breast implants, artificial larynxes, subperiosteal implants, and ligation clips
	Blood	Devices principally contacting blood	Pacemaker electrodes, artificial arteriovenous fistulae, heart valves, vascular grafts, internal drug delivery catheters, and ventricular-assist devices

according to the nature and extent of their contact with the body. Major categories of medical devices are surface devices, external communicating devices, and implant devices. These are then further subcategorized. Some examples of medical devices and implants belonging to each of the subcategories are also presented in [Table 2](#).

#### Test Selection Matrix

The matrix provides guidance on the identification of appropriate biological testing procedures for the three categories of medical devices: tests for Surface Devices (see [Table 3](#)), tests for External Communicating Devices (see [Table 4](#)), and tests for Implant Devices (see [Table 5](#)). Each category of devices is subcategorized and then even further subdivided according to the duration of the contact between the device and the body. The duration of contact is defined as (A) limited (less than 24 hours); (B) prolonged (24 hours to 30 days); or (C) permanent (more than 30 days). The biological effects that are included in the matrix are cytotoxicity, sensitization, irritation or intracutaneous reactivity, systemic toxicity, subchronic toxicity, genotoxicity, implantation, hemocompatibility, chronic toxicity, carcinogenicity, reproductive or developmental toxicity, and biodegradation. The general chapters that contain toxicity testing procedure for these biological effects are indicated in [Table 1](#).

Each subcategory in the matrix has an associated panel of testing requirements. Generally, the number of tests in the panel increases as the duration of the contact between the device and the body is extended and as the device or implant comes in closer contact with the circulatory system. Within several subcategories, the option of performing additional tests beyond those required should be considered on a case-by-case basis. Specific situations such as use of permanent implant devices or external communicating devices for pregnant women have to be taken into consideration in the manufacturer's decision to include reproductive or developmental testing. Guidance on the identification of possible additional testing procedures is provided in the matrix for each subcategory of medical devices.

#### GUIDANCE IN SELECTING THE PLASTIC OR OTHER POLYMER CLASS DESIGNATION FOR A MEDICAL DEVICE

To provide guidance on selecting the appropriate plastic or other polymer class designation for a medical device, each subcategory of Surface Devices (see [Figure 2](#))

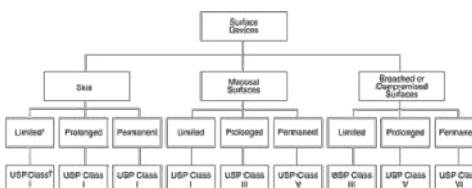
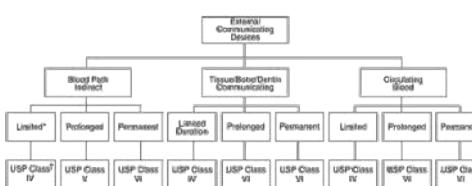


Fig. 2. USP plastic and other polymer class requirements for surface devices.\* Categorization based on duration of contact: limited—less than 24 hours; prolonged—24 hours to 30 days; permanent—more than 30 days.† USP Plastic Class designation.

and External Communicating Devices (see [Figure 3](#))





3. USP plastic and other polymer class requirements for external communicating devices. \* Categorization based on duration of contact: limited—less than 24 hours; prolonged—24 hours to 30 days; permanent—more than 30 days.† USP Plastic Class designation.

is assigned a USP Plastic Class designation (see [Biological Reactivity Tests, In Vivo](#) (88)). If the tests for each USP class designation are not sufficient for a specific device, the manufacturer or the practitioner must develop an appropriate set of tests. The indicated numerical class number increases relative to the duration (risk) of contact between the device and the body. In the category of Implant Devices, the exclusive use of class VI is mandatory. The assignment of USP Plastic Class designation is based on the test selection matrices illustrated in [Tables 3, 4, and 5](#).

The assignment of a plastic or other polymer class designation to a subcategory is not intended to restrict the use of higher classes of plastics or other polymers. Although the assigned class defines the lowest numerical class of plastic or other polymer that may be used in the corresponding device, the use of a numerically higher class of plastic is optional. When a device can be defined as belonging to more than one device category, the plastic or other polymer should meet the requirements of the highest numerical class.

Table 3. Test Selection Matrix for Surface Devices.<sup>a</sup>

Device Categories		Biological Effect <sup>b</sup>											
Body Contact		Contact Duration <sup>a</sup>	Cytotoxicity	Sensitization	Irritation or Intracutaneous Reactivity	Systemic Toxicity (Acute)	Subchronic Toxicity (Subacute)	Genotoxicity	Implantation	Hemocompatibility	Chronic Toxicity	Carcinogenicity	Reproductive or Development Toxicity
Surface Devices	Skin	A	X	X	X	—	—	—	—	—	—	—	—
		B	X	X	X	—	—	—	—	—	—	—	—
		C	X	X	X	—	—	—	—	—	—	—	—
	Mucosal Membrane	A	X	X	X	—	—	—	—	—	—	—	—
		B	X	X	X	O	O	—	O	—	—	—	—
		C	X	X	X	O	X	X	O	—	O	—	—
	Breached or Compromised	A	X	X	X	O	—	—	—	—	—	—	—
		B	X	X	X	O	O	—	O	—	—	—	—
		C	X	X	X	O	X	X	O	—	O	—	—

a Legend A—limited (less than 24 hours); B—prolonged (24 hours to 30 days); C—permanent (more than 30 days).

b Legend X—ISO evaluation tests for consideration; O—additional tests that may be applicable.

\* Adapted from the FDA's Blue Book Memorandum #G95-1 ([Table 1. Initial Evaluation Tests for Consideration](#) and [Table 2. Supplementary Evaluation Tests for Consideration](#)).

Table 4. Test Selection Matrix for External Communicating Devices.<sup>a</sup>

Device Categories		Biological Effect <sup>b</sup>											
Body Contact		Contact Duration <sup>a</sup>	Cytotoxicity	Sensitization	Irritation or Intracutaneous Reactivity	Systemic Toxicity (Acute)	Subchronic Toxicity (Subacute)	Genotoxicity	Implantation	Hemocompatibility	Chronic Toxicity	Carcinogenicity	Reproductive or Development Toxicity
External Communicating Devices	Blood Path, Indirect	A	X	X	X	X	—	—	—	X	—	—	—
		B	X	X	X	X	O	—	—	X	—	—	—
		C	X	X	O	X	X	X	O	X	X	X	—
	Tissue, Bone, or Dentin Communicating	A	X	X	X	O	—	—	—	—	—	—	—
		B	X	X	O	O	O	X	X	—	—	—	—
		C	X	X	O	O	O	X	X	—	X	X	—
	Circulating Blood	A	X	X	X	X	—	O	—	X	—	—	—
		B	X	X	X	X	O	X	O	X	—	—	—
		C	X	X	X	X	X	X	O	X	X	X	—

a Legend A—limited (less than 24 hours); B—prolonged (24 hours to 30 days); C—permanent (more than 30 days).

b Legend X—ISO evaluation tests for consideration; O—additional tests that may be applicable.

\* Adapted from the FDA's Blue Book Memorandum #G95-1 ([Table 1. Initial Evaluation Tests for Consideration](#) and [Table 2. Supplementary Evaluation Tests for Consideration](#)).

Table 5. Test Selection Matrix for Implant Devices.<sup>a</sup>

Device Categories		Biological Effect <sup>b</sup>											
Body Contact		Contact Duration <sup>a</sup>	Cytotoxicity	Sensitization	Irritation or Intracutaneous Reactivity	Systemic Toxicity (Acute)	Subchronic Toxicity (Subacute)	Genotoxicity	Implantation	Hemocompatibility	Chronic Toxicity	Carcinogenicity	Reproductive or Development Toxicity
Implant Devices	Tissue or Bone	A	X	X	X	O	—	—	—	—	—	—	—
		B	X	X	O	O	O	X	X	—	—	—	—
		C	X	X	O	O	O	X	X	—	X	X	—
	Blood	A	X	X	X	X	—	—	X	X	—	—	—
		B	X	X	X	X	O	X	X	X	—	—	—
		C	X	X	X	X	X	X	X	X	X	X	—

a Legend A—limited (less than 24 hours); B—prolonged (24 hours to 30 days); C—permanent (more than 30 days).

b Legend X—ISO evaluation tests for consideration; O—additional tests that may be applicable.

\* Adapted from the FDA's Blue Book Memorandum #G95-1 ([Table 1. Initial Evaluation Tests for Consideration](#) and [Table 2. Supplementary Evaluation Tests for Consideration](#)).

1 ISO document 10993-1:1997 entitled Biological Evaluation of Medical Devices—Part 1: Evaluation and Testing.

\* Adapted from the FDA Blue Book Memorandum #G95-1 ("Use of International Standard ISO-10993: Biological Evaluation of Medical Devices-Part 1: Evaluation and Testing.")

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Radhakrishna S Tirumalai, Ph.D.</a> Senior Scientist 1-301-816-8339	(GTMDB05) General Toxicology and Medical Device Biocompatibility

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### 1035 BIOLOGICAL INDICATORS FOR STERILIZATION

A biological indicator is broadly defined as a characterized preparation of a specific microorganism that provides a defined and stable resistance to a specific sterilization process. Microorganisms widely recognized as suitable for biological indicators are spore-forming bacteria, because, with the exception of ionizing radiation processes, these microorganisms are significantly more resistant than normal microflora. A biological indicator can be used to assist in the performance qualification of the sterilization equipment and in the development and establishment of a validated sterilization process for a particular article. Biological indicators are used in processes that render a product sterile in its final package or container, as well as for the sterilization of equipment, materials, and packaging components used in aseptic processing. Biological indicators may also be used to monitor established sterilization cycles and in periodic revalidation of sterilization processes. Biological indicators may also be used to evaluate the capability of processes used to decontaminate isolators or aseptic clean-room environments.

The principles and requirements for these applications are described under [Sterilization and Sterility Assurance of Compendial Articles](#) [1211](#).

#### TYPES OF BIOLOGICAL INDICATORS

There are at least three types of biological indicators. Each type of indicator incorporates a known species of a microorganism of known sterilization resistance to the sterilization mode. Some biological indicators may also contain two different species and concentrations of microorganisms.

One form of biological indicator includes spores that are added to a carrier (a disk or strip of filter paper, glass, plastic, or other materials) and packaged to maintain the integrity and viability of the inoculated carrier.

Carriers and primary packaging shall not contain any contamination (physical, chemical, or microbial) that would adversely affect the performance or the stability characteristics of the biological indicator. The carrier and primary packaging shall not be degraded by the specific sterilization process, which is used in a manner that will affect the performance of the biological indicator. The carrier should withstand transport in the primary and secondary packaging and handling at the point of use. The design of the carrier and primary packaging should minimize the loss of the original inoculum during transport, handling, and shelf life storage.

Another form of biological indicator is a spore suspension that is inoculated on or into representative units of the product to be sterilized. This represents an inoculated product; however, a simulated inoculated product may be used if it is not practical to inoculate the actual product. A simulated product is a preparation that differs in one or more ways from the actual product, but performs as the actual product using test conditions or during actual production sterilization processing. Spore suspensions with a known D value should be used to inoculate the actual or simulated product. If a simulated inoculated product is used, it must be demonstrated that it will not degrade the sterilization resistance of the biobinder. The physical design of actual or simulated product can affect the resistance of spore suspensions that are inoculated on or into the products. In the case of liquid inoculated products, it is often advisable to determine both the D value and z value of the specific biological indicator microorganism in the specific liquid product. The population, D value, z value where applicable, and endpoint kill time of the inoculated actual or simulated product should be determined.

A third form of biological indicator is a self-contained indicator. A self-contained biological indicator is designed so that the primary package, intended for incubation following sterilization processing, contains the growth medium for recovery of the process-exposed microorganisms. This form of biological indicator together with the self-contained growth medium can be considered a system. In the case of self-contained biological indicators, the entire system provides resistance to the sterilization process.

If the biological indicator is a paper strip or disk in a self-contained package that includes an available culture medium, the package design should be readily penetrable by the sterilizing agent. To allow for the time lag that may occur while the sterilizing agent reaches the contained microorganisms in the system, the D value, process endpoint kill time, and the survival time should be characterized for the system and not solely for the paper strip in the self-contained unit. Following the sterilizing treatment, the spore strip or disk is immersed in the self-contained medium by manipulation, which allows contact with the culture medium.

Self-contained biological indicators may also consist of a spore suspension in its own medium, and they often also contain a dye, which indicates positive or negative growth following incubation. Resistance of the self-contained system is dependent upon penetration of the sterilant into the package. Penetration may be controlled by the manufacturer through varying designs and composition of the self-contained biological indicator package, ampul, or container. Self-contained ampul biological indicators may be incubated directly following exposure to the sterilization process. The entire system is then incubated under the specified conditions. Growth or no growth of the treated spores is determined visually (either by observing a specified color change of an indicator incorporated in the medium or by turbidity) or by microscopic examination of the inoculated medium.

The self-contained system resistance characteristics must also comply with the labeling of the self-contained system and the relevant biological indicator monograph. The self-contained biological indicator system should withstand transport in the secondary packaging and handling at the point of use without breakage. The design of the self-contained system should be such to minimize the loss of the original inoculum of microorganisms during transport and handling. During or after the sterilization process, the materials used in the self-contained system shall not retain or release any substance that can inhibit the growth of low numbers of surviving indicator microorganism under culture conditions. Adequate steps must be taken to demonstrate that the recovery medium has retained its growth support characteristics after exposure to the sterilization process.

#### Preparation

All operations associated with the preparation of biological indicators are controlled by a documented quality system. Traceability is maintained for all materials and components incorporated in or coming into direct contact with the microorganism suspension, the inoculated carrier, or the biological indicator.

The preparation of stock spore suspensions of selected microorganisms used as biological indicators requires the development of appropriate procedures, including mass culturing, harvesting, purification, and maintenance of the spore suspensions. The stock suspension should contain predominantly dormant (nongerminating) spores that are held in a nonnutritive liquid.

The finished product (microbial suspension, inoculated carriers, or biological indicators) supplied by commercial manufacturers shall have no microorganisms, other than the test microorganism, present in sufficient numbers to adversely affect the product. The system to minimize the presence of microorganisms other than the biological indicator microorganism in the product will be validated, monitored, and recorded.

#### Selection for Specific Sterilization Processes

The selection of a biological indicator requires a knowledge of the resistance of the biological indicator system to the specific sterilization process. It must be established that the biological indicator system provides a challenge to the sterilization process that exceeds the challenge of the natural microbial burden in or on the product.

The effective use of biological indicators for the cycle development, process, and product validation, and routine production monitoring of a sterilization process requires a thorough knowledge of the product being sterilized, along with its component parts (materials and packaging). Only the widely recognized biological indicators specified in the particular biological indicator monograph should be used in the development or validation of a sterilization process. This will ensure that the biological indicator selected provides a greater challenge to the sterilization process than the bioburden in or on the product. Some users may require biological indicators with characteristics that differ from those widely available commercially. In such cases, users may grow their own spore cultures for the express purpose of preparing in-house biological indicators for their specific use. In such a case, the user is well advised to use organisms already described in the scientific literature as indicator organisms, and the user must have the capability of determining D and z values for in-house biological indicators. When biological indicators are prepared in-house, users must confirm the population, purity, and shelf life of the biological indicator to ensure the validity of any test conducted using the in-house biological indicator. When a bioburden-based sterilization process design is used, data comparing the resistance of the biological indicator to that of



bioburden are essential. Enumeration of the bioburden content of the articles being sterilized is also required. The process must result in a biologically verified lethality sufficient to achieve a probability of obtaining a nonsterile unit that is less than one in a million.

Alternatively, the overkill method may be used in the design of a sterilization process. In this case, specific assumptions are made regarding the resistance assumption used in establishing sterilization process lethality requirements. In general, all overkill processes are built upon the assumption that the bioburden is equal to one million organisms and that the organisms are highly resistant. Thus, to achieve the required probability of a nonsterile unit that is less than one in a million, a minimum 12 D process is required. A 12 D process is defined as a process that provides a lethality sufficient to result in a 12 log reduction, which is equivalent to 12 times a D value for organisms with sufficiently higher resistance than the

mean resistance of bioburden. Because the bioburden is assumed to be one million, an overkill process will result in a probability of nonsterility at much less than  $10^{-6}$  in actual practice. Overkill process design and evaluation may differ depending upon the sterilization process under test. The use of an overkill design and validation approach may minimize or obviate the need for bioburden enumeration and identification.

**Moist Heat**— For moist heat sterilization process, spores of suitable strains of *Bacillus stearothermophilus* are commercially available as biological indicators and frequently employed. Other heat-resistant spore-forming microorganisms such as *Clostridium sporogenes*, *Bacillus subtilis*, and *Bacillus coagulans* have also been used in the development and validation of moist heat sterilization processes.

**Dry Heat**— For dry heat sterilization, spores of *Bacillus subtilis* spp. are sometimes used to validate the process. During the validation of dry heat sterilization processes, endotoxin depyrogenation studies are frequently conducted in lieu of microbial inactivation studies during the establishment of sterilization cycles because the inactivation rate of endotoxin is slower than the inactivation rate of *Bacillus subtilis* spores. In practice the reduction of endotoxin titer by three or more logs will result in a process that also achieves a probability of nonsterility substantially lower than  $10^{-6}$ .

**Ionizing Radiation**— Spores of *Bacillus pumilus* have been used to monitor sterilization processes using ionizing radiation; however, this is a ceding practice. Radiation dose-setting methods that do not use biological indicators have been widely used to establish radiation processes. Furthermore, certain bioburden microorganisms can exhibit greater resistance to radiation than *Bacillus pumilus*.

**Ethylene Oxide**— For ethylene oxide sterilization, spores of a subspecies of *Bacillus subtilis* (*Bacillus subtilis* var. *niger*) are commonly used. The same biological indicator systems are generally used when 100% ethylene oxide or different ethylene oxide and carrier gas systems are used as sterilants.

**Vapor-Phase Hydrogen Peroxide (VPHP)**— This process has been shown to be an effective surface sterilant or decontaminant. VPHP is capable of achieving sterilization (probability of nonsterility of less than one in a million) when process conditions so dictate and if the target of sterilization is suitably configured. However, VPHP is also commonly used as a surface decontaminating agent in the treatment of sterility testing, biological and chemical containment, manufacturing isolators, and clean rooms.

Surface decontamination is a process that is distinct from sterilization of product contact materials, container-closure systems, or product. It is a process designed to render an environment free of detectable or recoverable microorganisms. Biological indicators are widely used to verify the efficacy of the decontamination process. However, in the case of decontamination, a spore log reduction value of three to four is adequate because the goal is decontamination rather than sterilization.

Table 1. Typical Characteristics for Commercially Supplied Biological Indicator Systems

Sterilization Mode	Example of a Typical D value (minutes)	Range of D values for Selecting a Suitable Biological Indicator (minutes)	Limits for a Suitable Resistance (depending on the particular D value [minutes])	
			Survival Time	Kill Time
Dry heat <sup>a</sup>	1.9	Min. 1.0	Min. 4.0	10.0
160°		Max. 3.0	Max. 14.0	32.0
Ethylene oxide <sup>b</sup>				
600 mg per liter	3.5	Min. 2.5	Min. 10.0	25.0
54°		Max. 5.8	Max. 27.0	68.0
60% relative humidity				
Moist Heat <sup>c</sup>	1.9	Min. 1.5	Min. 4.5	13.5
121°		Max. 3.0	Max. 14.0	32.0

a For  $1.0 \times 106$  to  $5.0 \times 106$  spores per carrier.

b For  $1.0 \times 106$  to  $5.0 \times 107$  spores per carrier.

c For  $1.0 \times 105$  to  $5.0 \times 106$  spores per carrier.

*Bacillus stearothermophilus* is the most prevalently used biological indicator for validating VPHP. Other microorganisms that may be useful as biological indicators in VPHP processes are spores of *Bacillus subtilis* and *Clostridium sporogenes*. Other microorganisms may be considered if their performance responses to VPHP are similar to those of the microorganisms cited above.

These spores may be inoculated on the surface of various gas-impermeable carrier systems having glass, metal, or plastic surfaces. Highly absorbent surfaces, such as fibrous substrates, or any other substrate that readily absorbs VPHP or moisture may adversely influence the VPHP concentration available for inactivation of inoculated microorganisms.

Paper substrates are not used because VPHP will degrade cellulose-based materials.

For representative characteristics of commercially supplied biological indicators, see Table 1.

The biological indicator may also be individually packaged in a suitable primary overwrap package that does not adversely affect the performance of the indicator, and is penetrable by VPHP. Spunbound polyolefin materials have proven to be well suited as an overwrap of biological indicators intended for use in evaluation of VPHP processes. The overwrap material may facilitate laboratory handling of the biological indicators following exposure to VPHP. Also, the use of an overwrap material to package VPHP biological indicators must be carefully assessed to ensure that, following VPHP exposure, residual hydrogen peroxide is not retained by the packaging material, possibly inducing bacteriostasis during the recovery steps. Microbial D values will be influenced by the presence of a biological indicator overwrap material relative to the rate of inactivation and the potential presence of residual VPHP. In cases where biological indicators (inoculated carriers) are being used without the primary package, stringent adherence to aseptic techniques is required.

#### PERFORMANCE EVALUATION

##### Manufacturer's Responsibility

The initial responsibility for determining and providing to the users the performance characteristics of a biological indicator<sup>1</sup> lot resides with the manufacturer of biological indicators. The manufacturer should provide with each lot of biological indicators a certificate of analysis that attests to the validity of biological indicator performance claims cited on the biological indicator package label or in the package insert of the label package. The manufacturer should define the sterilization process that the biological indicator will be used to evaluate. The characterization of each type of biological indicator, which provides the basis for label claims, should be performed initially by the manufacturer of the biological indicator using specialized and standardized apparatus under precisely defined conditions.<sup>1</sup> The manufacturer should also provide information concerning the D value, the method by which the D value was determined, and microbial count and resistance stability of the biological indicator throughout the labeled shelf life of the indicator. Optimum storage conditions should be provided by the manufacturer, including temperature, relative humidity, and any other requirements for controlled storage. The data obtained from the various required performance assays should be cited in a package insert or on the label of the biological indicator package. The manufacturer should provide directions for use, including the medium and conditions to be used for the recovery of microorganisms after exposure to the sterilization process. Disposal instructions should also be provided by the manufacturer of the biological indicator.



#### User's Responsibility

Commercial Product— When biological indicators are purchased from a commercial source, their suitability for use in a specific sterilization process should be established through developmental sterilization studies unless existing data are available to support their use in the process. The user should establish in-house acceptance standards for biological indicator lots and consider rejection in the event the biological indicator lot does not meet the established in-house performance standards. A Certificate of Performance should be obtained for each lot of indicators, and the user should routinely perform audits of the manufacturer's facilities and procedures. If certificates are not obtained and audits have not been performed, or if the biological indicators are to be used outside of the manufacturer's label claims, verification and documentation of performance under conditions of use must exist.

Upon initial receipt of the biological indicator from a commercial supplier, the user should verify the purity and morphology of the purchased biological indicator microorganisms.

Verification of at least the proper genus is desirable. Also, a microbial count to determine the mean count per biological indicator unit should be conducted. The manufacturer's comments relative to D value range, storage conditions, expiration dating, and stability of the biological indicator should be observed and noted. The user may consider conducting a D value assessment before acceptance of the lot. Laboratories that have the capability of performing D value assays could conduct a D value determination using one of the three methods cited in the general test chapter [Biological Indicators—Resistance Performance Tests](#) <sup>1</sup> and in the appropriate USP monographs for specific biological indicators.

Particularly important is the verification of the D value and count stability of the biological indicator system if long-term storage is employed.

In the event the spore crop is maintained for longer than 12 months under documented storage conditions, both spore count and resistance analysis must be conducted, unless performance of an original parent crop has been validated for a longer storage period. The result of spore count and resistance assays should be within the range of acceptability established during initial acceptance of the spore crop lot.

Noncommercial Product— A user of biological indicator systems may elect to propagate microorganisms for developing in-house biological indicators to develop or validate sterilization processes. In the event a user becomes a "manufacturer" of biological indicators, biological indicator performance requirements must be met. If the biological indicator system is used for the development of new sterilization processes or validation of existing processes, the same performance criteria described for commercial manufacturers of biological indicators must be followed.

#### Spore Crop Preparation

Because most biological indicators use microbial spores, accurate records of spore crop identification must be maintained by commercial and noncommercial biological indicator manufacturers. These records should include records pertaining to the source of the initial culture, identification, traceability to the parent spore crop, subculture frequency, media used for sporulation, changes in media preparation, any observation of crop contamination, and pre- and post-heat shock data. Records of usage of the spore crop and resistance to sterilization (namely, D values and z values where applicable) should also be maintained.

#### Instrumentation

The instrumentation used to evaluate the sterilization resistance of spore crops must be consistent with existing standards<sup>2</sup> related to the performance evaluation of biological indicator systems.

Equipment for the determination of D values of microorganisms exposed to VPHP should be able to closely control equipment operating parameters as described for other biological indicator systems under [Biological Indicators—Resistance Performance Tests](#) <sup>1</sup>. Particularly important is the assurance of a consistently reproducible VPHP concentration, delivered within a finite time, and maintained within a specified concentration range or VPHP pressure range for a defined increment of time. Introduction of biological indicators into a stabilized concentration of VPHP conditions should be via a system that permits rapid entry and removal of the test units from the chamber. Also, the design of the test chamber should allow for the attainment of steady-state VPHP concentrations and pressure, or the use of a defined amount of cubic feet of free flowing VPHP at a standardized pressure and temperature. Currently, VPHP concentration measurement devices may not be widely used. Therefore, exposure conditions may need to be based on the maintenance of steady-state VPHP pressures or flow rates resulting from a known initial weight of hydrogen peroxide, admitted to the chamber in a defined unit of time. Using this information, together with the known fixed volume of the chamber environment, a calculation of the approximate VPHP concentration can be made. If conditions are maintained constant throughout each D value assessment run, comparisons of relative resistance among different biological indicator lots may be readily determined.

#### USE FOR IN-PROCESS VALIDATION

Regardless of the mode of sterilization, the amount of the initial population of the microorganisms, its resistance to sterilization, and the site of inoculation on or in the product can all influence the rate of biological indicator inactivation.

During product microbial challenges, various areas of the product should be inoculated with biological indicators. If, for example, a container with a closure system is sterilized, both the product solution and the closure should be challenged to ensure that sterilization equivalent to a 10<sup>-6</sup> (one in a million probability of a nonsterile unit) sterilization assurance level (SAL) will be obtained in the solution as well as at the closure site.

One may need to determine through laboratory studies whether product components are more difficult to sterilize than, for example, a solution or drug within the product. Depending on the locations of the product components most difficult to sterilize, different process parameters may be involved in assuring microbial inactivation to an SAL of 10<sup>-6</sup>. The product performance qualification phase should identify the most important process parameters for inactivation of microorganisms at the sites most difficult to sterilize. Once these critical processing parameters are determined, during sterilization in-process validation of the product, they should be operated at conditions less than the conditions stated in the sterilization process specifications. Biological indicator survival is predicated upon both resistance and population. Therefore, a 106 biological indicator population is not always required to demonstrate a 10<sup>-6</sup> SAL. The appropriate use for biological indicators is to employ them to confirm that the developed process parameters result in the desired SAL. In moist heat sterilization, the biological indicator is used to establish that physically measured lethality can be verified biologically. Biological indicators with substantive D values and populations substantially less than 106 are adequate to validate many sterilization and decontamination processes. It is important that the users be able to scientifically justify their selection of a biological indicator.

1 See Apparatus under [Biological Indicators—Resistance Performance Tests](#) <sup>1</sup>. These apparatuses have been designed to provide consistent physical conditions applicable to the characterization of biological indicators. The required performance characteristics are also indicated.

2 BIER/Steam Vessels, American National Standards, ANSI/AAMI ST45:1992.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

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1041 BIOLOGICS

Products such as antitoxins, antivenins, blood, blood derivatives, immune serums, immunologic diagnostic aids, toxoids, vaccines, and related articles that are produced under license in accordance with the terms of the federal Public Health Service Act (58 Stat. 682) approved July 1, 1944, as amended, have long been known as "biologics." However, in Table III, Part F, of the Act, the term "biological products" is applied to the group of licensed products as a whole. For Pharmacopeial purposes, the term "biologics" refers to those products that must be licensed under the Act and comply with Food and Drug Regulations—Code of Federal Regulations, Title 21 Parts 600-680, pertaining to federal control of these products (other than certain diagnostic aids), as administered by the Center for Biologics Evaluation and Research or, in the case of the relevant diagnostic aids, by the Center for Devices and Radiological Health of the federal Food and Drug Administration.



Each lot of a licensed biologic is approved for distribution when it has been determined that the lot meets the specific control requirements for that product as set forth by the Office. Licensing includes approval of a specific series of production steps and in-process control tests as well as end-product specifications that must be met on a lot-by-lot basis. These can be altered only upon approval by the Center for Biologics Evaluation and Research and with the support of appropriate data demonstrating that the change will yield a final product having equal or superior safety, purity, potency, and efficacy. No lot of any licensed biological product is to be distributed by the manufacturer prior to the completion of the specified tests. Provisions generally applicable to biologic products include tests for potency, general safety, sterility, purity, water (residual moisture), pyrogens, identity, and constituent materials (Sections 610.10 to 610.15 and see Safety Tests—Biologics under [Biological Reactivity Tests, In Vivo](#) (88), [Sterility Tests](#) (71), [Water Determination](#) (921), and [Pyrogen Test](#) (151), as well as [Bacterial Endotoxins Test](#) (85)). Constituent materials include ingredients, preservatives, diluents and adjuvants (which generally should meet compendial standards), extraneous protein in cell-culture produced vaccines (which, if other than serum-originating, is excluded) and antibiotics other than penicillin added to the production substrate of viral vaccines (for which compendial monographs on antibiotics and antibiotic substances are available). Additional specific safety tests are also required to be performed on live vaccines and certain other items. Where standard preparations are made available by the Center for Biologics Evaluation and Research (Section 610.20), such preparations are specified for comparison in potency or virulence testing. The U.S. Opacity Standard is used in estimating the bacterial concentration of certain bacterial vaccines and/or evaluating challenge cultures used in tests of them. (See also Units of Potency in the General Notices.)

The Pharmacopeial monographs conform to the Food and Drug Regulations in covering those aspects of identity, quality, purity, potency, and packaging and storage that are of particular interest to pharmacists and physicians responsible for the purchase, storage, and use of biologics. Revisions of the federal requirements affecting the USP monographs will be made the subjects of USP Supplements as promptly as practicable.

**Vehicles and Added Substances**— Vehicles and added substances suitable for biologics are those named in the Food and Drug Regulations.

**Containers for Injections**— Containers for biologics intended to be administered by injection meet the requirements for Containers for Injections under [Injections](#) (1).

**Volume in Container**— The volumes in containers of biologics intended to be administered by injection meet the requirements for Volume in Container under [Injections](#) (1).

**Labeling**— Biologics intended to be administered by injection comply with the requirements for Labeling under [Injections](#) (1). In addition, the label on the final container for each biologic states the following: the title or proper name (the name under which the product is licensed under the Public Health Service Act); the name, address, and license number of the manufacturer; the lot number; the expiration date; and the recommended individual dose for multiple-dose containers. The package label includes all of the above, with the addition of the following: the preservative used and its amount; the number of containers, if more than one; the amount of product in the container; the recommended storage temperature; a statement, if necessary, that freezing is to be avoided; and such other information as the Food and Drug regulations may require.

**Packaging and Storage**— The labeling gives the recommended storage temperature (see General Notices). Precautions should be taken where products labeled to be stored at a temperature between 2° and 8° are stored in a refrigerator, in order to assure that they will not be frozen. Diluents packaged with biologics should not be frozen. Some products (as defined in Section 600.15) are to be maintained during shipment at specified temperatures.

**Expiration Date**— For compendial articles the expiration date identifies the time during which the article may be expected to meet the requirements of the Pharmacopeial monograph, provided it is kept under the prescribed storage conditions. This date limits the time during which the product may be dispensed or used (see General Notices, page 1). However, for biological products, the stated date on each lot determines the dating period, which begins on the date of manufacture (Section 610.50) and beyond which the product cannot be expected beyond reasonable doubt to yield its specific results and to retain the required safety, purity, and potency (Section 300.3 (1) and (m)). Such a dating period may comprise an in-house storage period during which it is permitted to be held under prescribed conditions in the manufacturer's storage, followed by a period after issue therefrom. The individual monographs usually indicate both the latter period and (in parentheses) the permissible in-house storage period. If the product is held in the manufacturer's storage for a longer period than that indicated (in parentheses), the expiration date is set so as to reduce the dating period after issue from the manufacturer's storage by a corresponding amount.

**Auxiliary Information**— Please [check for your question in the FAQs](#) before contacting USP.

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## 1043 ANCILLARY MATERIALS FOR CELL, GENE, AND TISSUE-ENGINEERED PRODUCTS

### INTRODUCTION

A wide variety of reagents and materials, many of which are unique or complex, are required for the manufacture of cell, gene, and tissue-engineered products. These materials include plasma- or serum-derived products, biological extracts, antibiotics, cytokines, culture media, antibodies, polymeric matrices, separation devices, density gradient media, toxins, conditioned media supplied by "feeder cell layers", fine chemicals, enzymes, and processing buffers. Many of these items are used to ensure the survival and promote the growth of certain cell populations, although their mechanism of action may not be entirely understood. Examples include fetal bovine serum (FBS) and various media supplements. Other items, such as highly purified cholera toxin, are introduced into the processing stream during manufacturing to exert a specific biochemical effect and are immediately washed out in subsequent processing steps to avoid unwanted toxicity at a later point. The finished biological products produced in such processes are often complex mixtures that, in some cases, cannot be completely characterized. Careful scrutiny of the materials used in manufacturing is necessary to prevent the introduction of adventitious agents or toxic impurities, as well as to ensure the ultimate safety, effectiveness, and consistency of the final product.

In cell, gene, and tissue-engineered product manufacturing, these reagents and materials are collectively called ancillary materials (AMs). AMs have also been referred to as ancillary products, ancillary reagents, processing aids, and process reagents. AMs were first discussed under the synonym ancillary products in the U.S. Food and Drug Administration Notice, "Application of Current Statutory Authorities to Human Somatic Cell Therapy Products and Gene Therapy Products" (Federal Register 58(197), October 14, 1993, pp. 53248–53251). This document established the FDA's authority to regulate human somatic cell therapy products and gene therapy products. AMs are also synonymous with "processing materials" that were defined in 21 CFR Part 1271, "Current Good Tissue Practice for Manufacturers of Human Cellular and Tissue-Based Products; Inspection and Enforcement; Proposed Rule" (Federal Register 66(5), January 8, 2001, pp. 1508–1559). AMs can be analogous to "components", and in some cases, "containers" as described in the current good manufacturing practice (cGMP) regulations for finished pharmaceuticals as outlined in 21 CFR 211.80 through 211.94 and 211.101(b) and (c).

The defining property of AMs is that they are not intended to be present in the final product. They are materials used as processing and purification aids or agents that exert their effect on the therapeutic substance. Materials or components that are intended to be in the final product dosage form (e.g., genetic materials, biopolymeric supports, physiological buffers) are not AMs. Cell banks and virus banks are also not considered AMs; there are a number of guidances that describe requirements for their certification. However "helper" viruses and "helper" plasmids may be considered AMs when they are not intended to be part of the final product.

The quality of an AM can affect the stability, safety, potency, and purity of a cell, gene, or tissue-engineered product. For example, the mechanism by which an AM exerts its effect may not be known, and the impact of normal variation of the AM on the quality and safety of the therapeutic product may not be understood. Alternatively, AMs of human or animal origin may present an infectious disease transmission risk. Other AMs, if administered to humans, may cause an immune reaction. Finally, an AM with toxic properties that is introduced into a manufacturing process and is not adequately removed in subsequent processing steps will expose the patient to a toxic substance and may impair the effectiveness of the



therapeutic entity. These risks to the quality and safety of the therapeutic product are often heightened with cell, gene, and tissue-engineered products, due to the limited ability to conduct extensive in-process and release tests. For example, lack of in-process holding steps or limited shelf life may create the need to administer the cell, gene, or tissue-engineered products before in-process or final-release testing results are available. In other cases, the scarcity of suitable donor tissue or the complex logistics in the transport of biological materials may limit the amount of material available for testing. To minimize these risks, whenever possible, it is necessary to implement rigorous material qualification and prudent application of manufacturing process controls.

Frequently, these novel therapeutic products are created using complicated biological processes. The AMs employed in these procedures may be selected primarily for their unique functional contributions or biological effects. Whenever possible, it is preferable to source AMs that are approved or licensed therapeutic products because they are well characterized, have an established toxicological profile, and are manufactured according to controlled and documented procedures. Conversely, the AM may be intended "for research use" and may, therefore, lack the level of qualification necessary for use in the production of a therapeutic product. In either case, the manufacturer of the cell, gene, or tissue-engineered product should develop comprehensive and scientifically sound qualification plans to ensure the traceability, consistency, suitability, purity, and safety of the AM. In cases where AMs are products approved for use for therapeutic purposes, the level of qualification will probably be less extensive than that for a material intended for research purposes. However, their suitability in the manufacturing process will still need to be established when the AM is being used beyond the scope of its intended use or labeling. The purpose of this chapter is to provide guidance in developing appropriate qualification programs for AMs employed in cell, gene, and tissue-engineered product manufacturing.

#### QUALIFICATION OF ANCILLARY MATERIALS

Qualification is the process of acquiring and evaluating data to establish the source, identity, purity, biological safety, and overall suitability of a specific AM. The responsibility for AM qualification resides with the developer or manufacturer of the cell, gene, or tissue-engineered product. This section outlines the basis by which a manufacturer can establish rational and scientifically sound programs for qualifying AMs, although the broad nature of the cell, gene, and tissue-engineered products and of the AM used to produce these products make it difficult to recommend specific tests or protocols for a qualification program. Thorough documentation is the cornerstone of any qualification program.

A well-designed qualification program becomes more comprehensive as product development progresses. In the early stages of product development, safety is the primary focus. In the later stages, AM production and qualification activities should be comprehensively developed to support eventual licensure of the cell, gene, and tissue-engineered product. On some occasions, complex or unique substances that have been shown to be essential for process control or production may not be available from suppliers that produce them in compliance with cGMP. In these situations, the manufacturer will have to develop a scientifically sound strategy for qualification. A qualification program for AMs used in cell, gene, and tissue-engineered product manufacturing should address each of the following areas: (1) identification, (2) selection and suitability for use in manufacturing, (3) characterization, (4) vendor qualification, and (5) quality assurance and control.

##### Identification

The first step in any qualification program is the listing of all of the AMs used in a given product manufacturing and where in the manufacturing process they are to be employed. The source and intended use for each material should be established, and the necessary quantity or concentration of each material should be determined. Also, alternate sources for each material should be identified.

##### Selection and Suitability for Use

Developers of cell, gene, and tissue-engineered products should establish and document selection criteria for AMs and qualification criteria for each vendor early in the design phase of product development. Selection criteria should include assessments of microbiological and chemical purity, identity, and biological activity pertinent to the specific manufacturing process. It is important to address these issues early in product development because certain AMs that are initially considered necessary may be impossible or prohibitively expensive to qualify, thereby justifying the investigation of alternatives or replacements. Examples include some animal- or human-derived materials that in some cases have alternate (i.e., plant or chemically synthesized) sources.

AMs of animal or human origin should be selected cautiously due to the potential infectious or zoonotic disease risks associated with these materials. Vendors should be selected that can supply documentation regarding the country of origin for animal-derived AMs to address concerns regarding transmissible spongiform encephalopathies and other diseases of agricultural concern, like tuberculosis and brucellosis. In many cases, the chain of custody for animal-derived AMs (i.e., abattoir → intermediate processing center → final processing center) will need to be documented. Vendors of human-derived AMs should be able to supply documentation regarding material traceability. For instance, human plasma-derived AMs should be sourced from licensed facilities that control the donor pool and appropriately screen the individual donors for relevant human infectious diseases. In some cases, vendors of animal- and human-derived AMs supply different grades of materials, some of which will be more suitable for use in cell, gene, and tissue-engineered product manufacturing than other grades. For example, FBS can be obtained that has been processed to reduce the risk of bovine viral contamination by subjecting it to validated irradiation and nanofiltration processes. Also, many animal and human plasma-derived components are subjected to chemical (detergent or solvent treatment) or physical (heat exposure for extended periods of time) treatments that have been shown through validation studies to significantly reduce the risk of adventitious microbial or viral contamination associated with starting AMs. Such AMs are preferred for use in cell, gene, and tissue-engineered product manufacturing processes because they significantly reduce the risks associated with the original material.

The complexity of risk assessment can be reduced by employing one of a number of quantitative or semiquantitative approaches, such as failure mode effects analysis (FMEA), quality function deployment (QFD), or hazard analysis and critical control point (HACCP). These programs typically assign a point value to each risk parameter for an AM that results in cumulative scores that make it easier to prioritize effort and resources for decreasing the risks associated with AMs. For example, an AM that has a strong safety profile and is used in minimal amounts in upstream steps of the manufacturing process and is thoroughly washed from the system would accumulate a low point score. Conversely, an AM that is known to be toxic and is employed in downstream processing would, therefore, possess a higher potential for appearing as a residual in the final product and would be assigned a higher point value. One can also assign points based on the risk classification (see Risk Classification).

##### Characterization

Specific quality control characterization tests need to be developed or adopted and implemented for each AM. The set of tests for each AM should assess a variety of quality attributes, including identity, purity, functionality, and freedom from microbial or viral contamination. The appropriate level of testing for each AM is derived from its risk assessment profile and the knowledge gained during development. Test specifications should be developed for each AM to ensure consistency and performance of the manufacturing process. Acceptance criteria should be established and justified on the basis of the data obtained from lots used in preclinical and early clinical studies, lots used for demonstration of manufacturing consistency, and relevant development data, such as those arising from analytical procedure development and stability studies.

Some AMs that are biological in nature may be difficult to fully characterize. Because these materials exert their effects through complex biological activities, and biochemical testing may not be predictive of the AM's process performance, functional or performance testing may be needed. Performance variability of such materials may have a detrimental impact on the potency and consistency of the final therapeutic product. Examples of complex functionality testing for AMs include growth promotion testing of individual lots of FBS on the cell line used in manufacturing, performance testing of digestive enzyme preparations, and in vitro tissue culture cytotoxicity assays. (see aspects of Performance Testing).

##### Vendor Qualification

Vendors supplying AMs should be qualified at the earliest opportunity. An early audit of the vendor's manufacturing facility, including their GMP and AM testing program, are basic elements of a vendor qualification program. A review of the vendor's processing procedures and documentation program is essential in establishing confidence in the vendor as a reliable supplier. Additionally, vendors that have been certified through an ISO inspection program or audited by other governmental agencies tend to have robust quality systems in place. Reports of past audits of U.S. suppliers obtained through the Freedom of Information (FOI) Act may augment the qualification process.

It is important to develop a good working relationship with a vendor. In some cases, the vendor may provide higher manufacturing standards, custom formulation services, or replacement of substandard components upon request, with or without additional costs. A good rapport is essential if further investigation into AM suppliers is warranted. It is also critical to ensure that the vendor takes appropriate steps to prevent cross contamination between its products during manufacture. Vendors should be familiar with the principles of validation, especially cleaning validation, as well as viral inactivation and sterilization validation. Finally, systems should be established where vendors supply written certification of processing or sourcing changes to customers, well in advance of the implementation of the changes so that customers can evaluate the potential impact of such changes.

##### Quality Control and Quality Assurance



Because the components of the qualification program are multifaceted and need to be in compliance with cGMP, they should be monitored by a quality assurance/quality control unit (QAU). Typical QAU activities include the following systems or programs: (1) incoming receipt, segregation, inspection, and release of materials prior to use in manufacturing, (2) vendor auditing and certification, (3) certificate of analysis verification testing, (4) formal procedures and policies for out-of-specification materials, (5) stability testing, and (6) archival sample storage.

#### RISK CLASSIFICATION

A scientifically sound and rational qualification program should be designed for each AM and should take into account the source and processes employed in its manufacture. Whenever available, AMs that are approved or licensed therapeutic products are preferable because they are well-characterized with an established toxicological profile and are manufactured according to controlled and documented procedures. Licensed biologics, approved drugs, and approved or cleared medical devices or implantable materials that have been incorporated into cell, gene, or tissue-engineered product manufacturing processes present a known or more favorable safety profile for the patient than nonapproved or nonlicensed versions. Qualification programs for these AMs should reflect the extensive scrutiny that these items were subjected to in their development and manufacture. Consequently, greater emphasis should be placed on the investigation of the impact of inherent variability of these AMs on final product function. For instance, a manufacturer may utilize human serum albumin, intended for human administration, as a supplement to a cell cultivation medium for a cell-based product. Because the cell-based product is marketed as a licensed biological, one need not repeat all the testing already performed by the supplier as part of material qualification. In contrast, the impact of lot-to-lot variability on cell growth rate or maintenance of an important differentiated cellular property may be a prudent area of investigation. Alternatively, the stability of this material at the concentration employed in processing or its potential for interaction with other processing components may also be areas worthy of investigation. Such approaches to AM qualification therefore focus on the AM as a potential source of variability that may influence final product potency and safety. Qualification programs for these AMs should be comprehensive to minimize consumer risk and ensure that unacceptable lots or adulteration will be detected.

The qualification program must also take into account the quantity of the AM employed in manufacturing as well as its point of introduction in the manufacturing process. A relevant example is the use of FBS as a supplement to a tissue culture medium used to expand a stem cell population from a specific tissue for eventual administration to a patient (see Manufacturing Overview under [Cell and Gene Therapy Products \(1046\)](#)). A qualification program for such an AM would include (a) assurance that the serum was sourced from a country or region known to be free of bovine spongiform encephalopathy (BSE); (b) assurance that the source herds are monitored and test negative for specific diseases relevant in agricultural settings (e.g., tuberculosis, brucellosis, foot and mouth disease); (c) testing of the serum for sterility, mycoplasma, endotoxin content, and adventitious bovine viruses known to be associated with the material; <sup>1</sup> (d) the review and archiving of the supplier's certificate of analysis; (e) lot-to-lot assessment of the ability of the serum to consistently expand a representative cell population using a standardized cell culture quality control assay; and (f) on-site audit of the supplier to ensure that the material is sourced and processed in a manner deemed acceptable by a responsible QA unit.

To aid manufacturers and developers in the design of their qualification programs for a variety of AMs, tiers of sample risk categories are presented in [Tables 1–4](#) and are provided as a guide. Risk is also dependent on the amount and the stage at which the AM is used in the manufacturing process. [Tables 1–4](#) do not address the impact of quantity or stage of use.

**Tier 1**—These AMs are low-risk, highly qualified materials that are well-suited for use in manufacturing. The AM is either a licensed biologic, an approved drug, an approved or cleared medical device, or it is intended for use as an implantable biomaterial. Generally these components or materials are obtained as a sterile packaging system or dosage form intended for their label use, but are instead utilized "off label" in the manufacturing process for the cell, gene, or tissue-engineered product.

**Tier 2**—These AMs are low-risk, well-characterized material that are well-suited for use in manufacturing. Their intended use is for drug, biologic, or medical device manufacture, including cell, gene, and tissue-engineered products as AMs, and they are produced under relevant cGMPs. Most animal-derived materials are excluded from this category.

**Tier 3**—These AMs are a moderate risk material that will require a higher level of qualification than previous tier materials. Frequently, these materials are produced for in vitro diagnostic use and are not intended for use in the production of cell, gene, or tissue-engineered products. In some cases, upgrade of AM manufacturing processes may be necessary in order to employ the AM in manufacturing of these products (e.g., modification of the production process for a diagnostic grade monoclonal antibody to include robust viral removal steps in purification).

**Tier 4**—This is the highest risk level for AMs. Extensive qualification is necessary prior to use in manufacturing. The material is not produced in compliance with cGMPs. AMs are not intended for use in the production of cell, gene, or tissue-engineered products. This risk level includes highly toxic substances with known biological mechanisms of action, and also includes most complex, animal-derived fluid materials not subjected to adventitious viral removal or inactivation procedures. These materials may require (a) an upgrade of AM manufacturing processes; (b) treatment of AMs to inactivate or remove adventitious agents, disease-causing substances, or specific contaminants (e.g., animal viruses, prions); (c) testing of each lot of material to ensure that it is free of adventitious agents, disease-causing substances, or specific contaminants; (d) validation of the manufacturing process of the cell, gene, or tissue-engineered product to assess consistency of removal of a known toxic substance or lot-release testing to demonstrate reduction levels considered to be safe; or (e) validation of the manufacturing process of the cell, gene, or tissue-engineered product to assess consistency of removal or inactivation of adventitious agents, disease-causing substances, or specific contaminants associated with the material. Developers in the early stages of development should evaluate the necessity of these materials and explore alternative substances or sources.

Table 1. AM Risk Tier 1

Low-Risk, Highly Qualified Materials with Intended Use as Therapeutic Drug or Biologic, Medical Device, or Implantable Material

Example	Typical Use in Cell, Gene, or Tissue-Engineered Product Manufacturing	Qualification or Risk Reduction Activities
Recombinant insulin for injection	Cell culture medium additive	<ul style="list-style-type: none"> <li>• DMF cross reference (when possible or practical)</li> <li>• Certificate of analysis</li> <li>• Assess lot-to-lot effect on process performance<sup>1</sup></li> <li>• Assess removal from final product</li> <li>• Stability assessment on AM as stored for use in manufacturing<sup>2</sup></li> </ul>
Organ preservation fluid	Process biological fluid employed in tissue transport or processing	
Human serum albumin for injection	Cell culture medium	
Sterile fluids for injection	Process biological fluid employed in tissue transport, cell processing, purification	
Implantable biomaterials (formed collagen, silicone, polyurethane constructs intended for surgical implantation)	Scaffolds, matrices for immobilized cellular cultivation	
Recombinant deoxyribonuclease for inhalation or injection	Process enzyme employed in viral vector manufacturing, stem cell processing	
Antibiotics for injection <sup>3</sup>	Cell culture medium and biopsy transport fluid additive to reduce risk of bacterial contamination	
Injectable monoclonal antibodies	Immunologically targeting specific cell populations for selection or removal	
Injectable cytokines	Cell culture medium	
Vitamins for injection; defined nutrients, chemicals, or excipients intended for injection	Cell culture medium additive employed in cell expansion, controlled cellular differentiation/activation step, or manufacture of a viral vector	
IV bags, transfer sets and tubing, cryopreservation bags, syringes, needles	Storage vessels or container closure systems, closed aseptic transfer systems	

<sup>1</sup> See Performance Testing.

<sup>2</sup> Often AMs are aliquoted or stored at different concentrations, in different buffers, or under conditions that are different from those stated on the label or previously validated. Data



should be generated that demonstrate the stability and preservation of activity of the AM under the conditions that are specific to the manufacturing application.

3 Beta lactam antibiotics should not be used as AMs due to the risk of patient hypersensitivity.

Table 2. AM Risk Tier 2  
Low-Risk, Well Characterized Materials with Intended Use as AMs, Produced in Compliance with GMPs

Example	Typical Use in Cell, Gene, or Tissue-Engineered Product Manufacturing	Qualification or Risk Reduction Activities
Recombinant growth factors, cytokines <sup>1</sup>	Cell culture medium additive	
Immunomagnetic beads	Immunomagnetic separation of cells	
Human AB serum	Cell culture medium additive	
Progesterone, estrogen, vitamins, purified chemicals (USP-grade)	Cell culture medium additives, induction agents, buffer components	
Sterile process buffers	Process biological fluid employed in tissue transport, cell processing, purification	
Biocompatible polymers, scaffolds, hydrogels	Scaffolds, matrices for immobilized cellular cultivation	
Proteolytic enzymes	Process enzyme	
Tissue culture media	Cell culture medium additive	
Monoclonal antibodies	Immunologically targeting specific cell populations for selection or removal	
Density gradient media	Cell separation via centrifugation	

1 These AMs should be produced from nonmammalian, recombinant sources (i.e., microbially grown in the absence of animal-derived growth medium components).

2 See Performance Testing.

3 Often AMs are aliquoted or stored at different concentrations, in different buffers, or under conditions that are different from those stated on the label or previously validated. Data should be generated that demonstrates the stability and preservation or activity of the AMs under the conditions that are specific to the manufacturing application.

Table 3. AM Risk Tier 3  
Moderate-Risk Materials Not Intended for Use as AMs  
(frequently produced for in vitro diagnostic use or reagent grade materials)

Example	Typical Use in Cell, Gene, or Tissue-Engineered Product Manufacturing	Qualification or Risk Reduction Activities
Recombinant growth factors, cytokines	Cell culture medium additive	
Tissue culture media	Cell culture medium additive	
Monoclonal antibodies (diagnostic-grade produced in cell culture)	Immunologically targeting specific cell populations for selection or removal	
Process buffers	Process biological fluid employed in tissue transport, cell processing, purification	
Novel polymers, scaffolds, hydrogels	Scaffolds, matrices for immobilized cellular cultivation	
Proteolytic enzymes	Process enzyme	
Purified chemicals (reagent-grade)	Culture medium additives, induction agents, buffer components	

1 See Performance Testing.

2 Often AMs are aliquoted or stored at different concentrations, in different buffers, or under conditions that are different from those stated on the label or previously validated. Data should be generated that demonstrates the stability and preservation or activity of the AM under the conditions that are specific to the manufacturing application.

Table 4. AM Risk Tier 4  
High-Risk Materials

Example	Typical Use in Cell, Gene, or Tissue-Engineered Product	Qualification or Risk Reduction Activities
FBS	Cell culture medium additive	
Animal-derived (including human) extracts	Cell culture medium additive	
Animal-derived polymers, scaffolds, hydrogels	Scaffolds, matrices for immobilized cellular cultivation	
Purified enzymes	Process enzyme	
Ascites-derived antibodies or proteins	Immunologically targeting specific cell populations for selection or removal	
Animal or human cells used as feeder layers	Cell culture substratum or source of medium components	
Chemical entities with known toxicities (i.e. methotrexate, cholera toxin, Staphylococcus aureus pore-forming hemolysin, Staphylococcus enterotoxins A and B, toxic shock syndrome toxin)	Selection agents used in cell culture to improve or maintain transgene expression, enhance cellular proliferation, improve cell survival upon cryopreservation, superantigens for the activation of T cells	<ul style="list-style-type: none"> <li>Same as in <a href="#">Table 3</a>, plus</li> <li>Verify traceability to country of origin</li> <li>Assure country of origin is qualified as safe with respect to source-relevant animal diseases, including TSE</li> <li>Adventitious agent testing for animal source-relevant viruses</li> </ul>

#### PERFORMANCE TESTING

In cases where AMs are chosen for their ability to provide a particular biological function in producing the therapeutic product, performance testing becomes an essential component of their overall qualification. This is especially true when the AM plays a critical role in modulating a complex biochemical effect and has a large impact on product manufacturing yield, purity, or final product potency. These AMs tend to be complex substances or mixtures, are frequently biologically sourced, and can exhibit significant lot-to-lot variability. As a result, these AMs usually have no simple identity test, nor can they be easily characterized by physical or chemical tests. The development of well-defined performance assays for complex

AMs will not only ensure process reproducibility and final product quality, but in many cases will satisfy the identity testing criteria in accordance with 21 CFR 211.84(d).

In some cases, the initial qualification of an AM for use in manufacturing should be the investigation of the effect of the amount of the AM on the desired response (increased yield,



purity, or potency of the therapeutic product). The amount of the AM used in manufacturing should be chosen to consistently yield the desired effect while minimizing issues by removing the AM in subsequent processing steps. Such testing frequently assesses the important functional attribute expected of the AM in a scaled-down or simulated manufacturing process. Some examples follow:

- If an AM is added to the culture media because it promotes cellular proliferation or the secretion of a critical therapeutic agent, the assay could demonstrate that each lot of AMs produces the expected rate and amount of cellular proliferation or the expected level of secreted therapeutic agent.
- If a monoclonal antibody is used to purify a particular cell type, the new lot of monoclonal antibody could be shown to purify the cell population with the expected recovery and purity for the desired cell type.
  - If a deoxyribonuclease is used to degrade cellular DNA, new lots could be tested for the ability of the deoxyribonuclease to degrade DNA.
- If a particular type of density gradient material is used to purify a vector or cell, new lots of the material used to make the gradient could be shown to purify the vector or cell to an acceptable level.
- If a plasmid or viral vector is used in the production of a gene therapy vector (e.g., helper function), new lots of the helper vector could be shown to produce the expected amounts of the gene therapy vector.
- If a cell therapy is produced in a hollow-fiber bioreactor, new lots of the bioreactor could be shown to produce the anticipated amount of cell product.

The actual assay used may well evolve as the manufacturing process is developed further and the critical relationships of the AM and the final product are better understood.

Because most performance testing yields relative results, it is often helpful to assay a new lot of AMs side by side with an approved lot of AMs or an official reference standard, if available. This simultaneous comparison helps to reduce the variability due to different lots of cells or vectors and will help discern variability associated with the different lots of AMs. If performance testing involves assays to demonstrate that the new lot of AMs does not affect the impurity profile of the final therapeutic product, either by generating new impurities or by increasing the level of existing impurities, it is helpful to assay both for the total level of impurities, as well as look for the presence of new impurities. An immunologically-based binding assay can typically assess only the total level of impurities. For example, a Western blot of the gene therapy product that is probed both with antibodies to the product and antibodies to host cell proteins is useful for detecting new protein species and significant increases in the levels of host cell impurities. This initial qualification is enhanced by a performance assay that has a quantitative readout with a clear change in the signal when a significant change in the amount of AMs is introduced into the assay (e.g., dose response). A threshold-type response (i.e., there are two levels of response to the AM and neither large changes in an AM below a certain dose nor above a certain dose change the response) can make it more difficult to select a concentration of AM that consistently results in the desired effect and minimizes the residual levels of the AM in the final therapeutic product.

#### ANCILLARY MATERIALS RESIDUAL LEVEL ASSESSMENT AND REMOVAL

AMs are not intended to be present in the final dosage form in cell, gene, and tissue-engineered products. Their presence in the final product could lead to undesired effects in the recipient or have a detrimental effect on product potency. Undesired effects in humans include direct toxicity of the AM or an unwanted immunogenic response. Some examples include the following:

- In the generation of a tumor vaccine using a patient's tumor biopsy as the starting material, a chemical entity is introduced to denature the cell surface proteins and tumor antigens to enhance their antigenicity. The chemical entity is known to be highly toxic.
- Antibiotics may be added to a transport solution for human cells to address microbial contamination issues associated with the procurement procedure. Residual levels of the antibiotic may affect the proliferative capacity of the final engineered cellular product. Residual antibiotics could also cause an anaphylactic response in some individuals.
  - FBS, employed in the cultivation of an engineered human skin graft, may cause the development of a humoral antibody response directed against bovine proteins.
- Aggregated mouse immunoglobulin, a trace impurity in a purified preparation of mouse monoclonal antibody used to target a cell population for immunoselection, may be immunogenic.
  - A cytokine, employed as an immunomodulator in the generation of a gene-modified autologous tumor vaccine product, may elicit a severe reaction in the recipient.
  - Cholera toxin, employed as part of a cell culture medium for a cell therapy product intended for intravenous administration, will be highly toxic to the recipient if it is not removed during processing.

These risks can be mitigated through the design of processes to include steps to adequately remove the AM through dilution, separation, or inactivation, as well as the development of analytical detection assays to assess the AM levels during processing and in the final therapeutic product. Assessment and removal strategies for residual AMs should be considered in the early phases of process development. There are two different approaches for assessing residual AM levels in the final therapeutic product: (1) Validation studies can demonstrate that the process is capable of removing more of the AM than would be present in a worst-case scenario. (2) The residual levels of an AM can be measured for each lot at an appropriate step in the manufacturing process.

Validation of an AM removal is often best performed by spiking the impure product with "worst case" or higher levels of the AM and showing the purification process is capable of removing the AM to "undetectable levels". Clearance factors can then be generated for each purification step in a manner analogous to that done in viral clearance studies. When designing the validation studies, the following three considerations should be included: (1) The assay should be able to accurately quantitate the AM in each sample matrix. (2) If the validation is conducted at a scale smaller than that used for routine lot production, the comparability of this smaller scale process to the full scale process needs to be demonstrated. This usually means that the smaller scale process is operated using the same critical parameters as the full scale process with the product generated at each step having a similar purity and yield. (3) As with any spiking study, one has to demonstrate that the additional, higher level of AM has not affected the purification process. If the second approach of measuring residual levels of the AM in each lot is used, the specification for the maximum amount of AM in the final therapeutic product is based on the amount of the AM in the lots used in toxicological or clinical studies or known toxicological data.

The development of sensitive and reproducible analytical assays for AMs is another important component of a risk reduction approach. Two types of assays are useful in assessing the levels of residual AM impurity: a limit test and a quantitative test. Either test should be accurate, precise, robust, and have a low limit of detection. Assays for residual AMs may be performed on the product before it is formulated (e.g., on the drug substance) to avoid any interference of the components used in the formulation with the assay for residual AMs or in the final drug product. Spike-recovery controls are often included in such assays to demonstrate that the sample matrix does not inhibit the detection of the AM. Preferably, assays should be designed to detect all forms of AMs including aggregates, fragments, or conjugates. Aggregated protein has been shown to be particularly immunogenic.

Immunoassays such as ELISA are most commonly used to assess residual levels of AMs. An ELISA for bovine serum albumin (BSA) has been used to assess residual levels of FBS. Polymerase chain reaction (PCR) technology has been employed to assess residual levels of host cell DNA. Labeling cells with 3H thymidine or performing PCR for a feeder cell-specific gene sequence are two ways to assess for residual levels of feeder cells. If "wash out" of the AM is achieved by exhaustive dilution associated with further processing activities, it may be useful to calculate the dilution factor for the AM during this processing. In some cases, this is sufficient to ensure that the AM has been reduced to safe levels for early clinical development. Data should be obtained later in clinical development to confirm the wash out of the AM at the expected step(s). This approach is particularly useful when there is pre-existing knowledge of the therapeutic levels and toxicity of the AM. In other cases, information regarding the safety and tolerability of the AM should be collected (in preclinical toxicology studies or later with human clinical studies) in order to determine the safe or nontoxic levels that must be achieved. These data may be needed even for an AM that is approved for use for therapeutic purposes if it is being used in a manner inconsistent with its intended use or labeling or if the route of administration or dosage level of the AM may present risks not previously encountered or considered.

#### CONCLUSION

While many types of AMs are used during the manufacture of cell, gene, and tissue-engineered products, they have received less emphasis than the final products. However, the importance of AM quality to the quality of the final product cannot be overstated. Good quality AMs should perform as intended in a consistent manner, batch-to-batch, if they are carefully selected and appropriately used. AMs of insufficient quality will affect the quality and the effectiveness of the final product and endanger the health of patients. Thus, implementing an AM qualification program that addresses the risks associated with the AM, the stage of manufacture at which it is used, and the amount of the AM used during manufacture will ensure the safety and effectiveness of the final product.



AMs used in cell, gene, and tissue-engineered products will be regulated in the context of the manufacturing process of the cell, gene, and tissue-engineered products. Certain AMs may already be approved for uses other than for cell, gene, and tissue-engineered product manufacture. It is preferable to source AMs that are approved therapeutic products when they are available because they are well-characterized with an established toxicological profile and are manufactured according to controlled and documented procedures. The following list of documents should provide relevant regulatory guidance and a description of best practices in product and process development, manufacturing, quality control, and quality assurance:

- [Biological Reactivity Tests, In Vitro](#) 87
- [Biological Reactivity Tests, In Vivo](#) 88
- [Biotechnology-Derived Articles](#) 1045
- [Cell and Gene Therapy Products](#) 1046
- [Biotechnology-Derived Articles—Amino Acid Analysis](#) 1052
- [Biotechnology-Derived Articles—Capillary Electrophoresis](#) 1053
- [Biotechnology-Derived Articles—Isoelectric Focusing](#) 1054
- [Biotechnology-Derived Articles—Peptide Mapping](#) 1055
- [Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis](#) 1056
- [Biotechnology-Derived Articles—Total Protein Assay](#) 1057
- 21 CFR 211 Subpart E, 211.80 through 211.94 and 211.101
  - 21 CFR 312
  - 21 CFR 314
  - 21 CFR 801.109 (b) (1)
- 21 CFR 807.81 through 21 CFR 807.97
  - 21 CFR 812
  - 21 CFR 814
- FDA Center for Biologics Evaluation (CBER) "Draft Guidance for Monoclonal Antibodies Used as Reagents in Drug Manufacturing" (1999)
- FDA Center for Biologics Evaluation (CBER) "Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals" (1993)
- FDA Center for Devices and Radiological Health (CDRH) "Class II Special Controls Guidance Document: Tissue Culture Media for Human ex vivo Tissue and Cell Culture Processing Applications; Final Guidance for Industry and FDA Reviewers" (May 16, 2001)
  - CDRH Blue Book Memorandum G95-1
- ISO 10993-1: 1997 "Biological Evaluation of Medical Devices—Part 1: Evaluation and Testing"
- International Conference on Harmonization (ICH) Q5A "Guidance for Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human and Animal Origin"
- International Conference on Harmonization (ICH) Q5D "Guidance on Quality of Biotechnological/Biological Products: Derivation and Characterisation of Cell Substrates Used for Production of Biotechnological/Biological Products"
- Public Health Service Guideline on Infectious Diseases Issues in Xenotransplantation (October 18, 2000)

1 Most suppliers test for adventitious agents according to 9 CFR 113, which was developed by the Center for Veterinary Biologics, Animal and Plant Health Inspection Service, United States Department of Agriculture. These tests may differ from those used to test products developed for human use (e.g., mycoplasma).

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Fouad Atouf, Ph.D.</a> Senior Scientific Associate 1-301-816-8365	(BBCGT05) Biologics and Biotechnology - Cell Gene and Tissue Therapies

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#### 1045 BIOTECHNOLOGY-DERIVED ARTICLES

Macromolecular substances can be obtained by a number of methods including extraction from natural sources, modification of naturally occurring protein, mammalian cell culture in vitro, mammalian cell culture in vivo, production by microorganisms, and chemical syntheses. From a compendial perspective, macromolecular articles derived from biotechnology processes—or more specifically from recombinant-DNA (rDNA) technology, hybridoma technology, and transformed continuous cell lines—are those articles for which official names have been established. These articles have official public standards for identity, strength (potency), quality, and purity. Advances in genetics and the applications of genetic engineering have made the production of new and existing macromolecular articles technologically and economically feasible.

The technologies involved in producing a protein by biotechnological processes have been widely documented and general guidelines have been established by the federal government. The products of biotechnology may be regulated as drugs, biologics, or diagnostics, depending on their source, composition, and intended use. The novel approaches permitted by biotechnology can make it difficult to apply classic definitions of these categories and FDA has advised manufacturers to seek clarification in the early stages of development for how a product will be regulated when classification is not obvious.<sup>1</sup> The overall regulatory scheme for biotechnology-derived products is the same as for products in the same category produced by traditional manufacturing methods, with the addition of specific requirements suited to the biotechnology-derived product. The general requirements are described primarily in the applicable parts of the Code of Federal Regulations, Title 21. NIH has published a guideline for rDNA research that is mandatory for both public and private NIH-supported research. This guideline has wide acceptance and voluntary compliance is common by institutions and corporations not specifically governed by it.<sup>2</sup> Laboratory safety practices, particularly protection from potentially infectious materials, are a concern.<sup>3</sup> Producing macromolecular articles by biotechnological processes involves initially the cloning of a specific gene in the laboratory, or the construction of a synthetic gene, with subsequent insertion into a host cell and subcloning in a microorganism or cell culture; then a process development on a pilot scale to optimize yield and quality; and finally large-scale fermentation or cell culture processes. The next step, which is the most relevant to the development of compendial monographs, is the purification of the macromolecular proteins. This is followed by animal testing, clinical testing, regulatory approval, and marketing.

Development of relevant public standards for these macromolecular articles is generally closely linked to the processing technology used and the physicochemical and biological characteristics of a specific drug. Characterizations of these articles to ensure safety, purity, and activity should incorporate classical techniques as well as methods specific to the technology. There is always the possibility that these articles may cause some untoward effects in patients using them due to immunological sensitization as a result of a single (or multiple) molecular modification. Such a possibility requires precise characterization of these substances. Although it is theoretically possible to develop public standards for a macromolecular article, it is not possible to develop specific standards that incorporate all prospective methods of production. The compendial perspective is to develop public standards that can be applied to a final product without comprehensive knowledge of production details but which can ensure maintenance of safety, identity, strength, quality, and purity.

Testing for identity, purity, and activity generally requires the use of USP Reference Standards. It will be necessary to consider what USP Reference Standards might be required and how relevant they might be to the method of production as it relates to a final product's characteristics. Such decisions will be made on a product-by-product basis. Favorable consideration will be given to the use of USP Reference Standards that are representative of the specific products that have undergone clinical testing and are fully characterized.

Although early adoption in USP of general methods of analysis of macromolecular drugs could be conducive to early standardization of methods, the technology and analytical procedures are evolving very rapidly. Analytical procedures—chemical, physical, microbiological, and immunological—will be included in the specific product monographs.



## SCOPE OF BIOTECHNOLOGY IN THE DEVELOPMENT OF PHARMACOPEIAL ARTICLES

## Definition of Biotechnology—Historical Perspective

In its broadest definition, biotechnology refers to the use of living organisms, including isolated mammalian cells, in the production of products having beneficial use. This definition would place alcohol, antibiotic production, and dairy processing, for example, within the scope of biotechnology. However, the current interest in biotechnology is primarily a result of two major advances. The first advance was the development of rDNA technology, which allowed the genes of one species to be transplanted into another species. Thus, gene coding for the expression of a desired protein (usually human) could be inserted into a host prokaryotic or eukaryotic cell in such a manner that the host cell would then express usable quantities of the desired protein. The second major advance was the development of techniques for producing large quantities of monoclonal antibodies (i.e., antibodies arising from a single lymphocyte).

Biotechnology within the pharmaceutical industry generally refers either to the production of protein products using rDNA techniques or to the production of monoclonal antibodies. Other technologies, such as transgenic animals and plants, gene therapy, and antisense DNA, may have potential implications for the pharmaceutical industry in the future but are not within the scope of this chapter.

## rDNA Technology

The major steps in the application of rDNA technology for production of a desired protein are outlined in this section. The critical first step is identification of the protein that is to be produced, followed by the isolation of the gene of interest (i.e., the DNA sequence coding for the desired protein). Once this gene is isolated and fully characterized, it is inserted into a suitable vector such as a plasmid, which is an extrachromosomal segment of DNA usually found in certain bacteria. The plasmid is then inserted into the host cell. Clones of the transformed host cell line are isolated, and those that produce the protein of interest in the desired quantities are preserved under suitable conditions as a cell bank. As manufacturing needs arise, the cloned cells can be scaled up in a fermentation or cell culture process to produce the protein product.

Although the rDNA process is more fully described elsewhere in this chapter, the following important points should be recognized. The vector (plasmid) generally contains a selectable marker that can be used to identify cells that contain this gene. This is in addition to the gene coding for the protein of interest and the regulatory nucleotide sequences necessary for plasmid replication and messenger RNA (mRNA) transcription (the first step in protein synthesis). Selection of the desired cells is simplified because only properly transformed cells containing the selectable marker gene will survive under the growth conditions used to identify and propagate the transformed cells. Typically, the bacterial and eukaryotic selectable markers may include both antibiotic resistance or genes that complement an auxotrophic host mutation. There are numerous examples of both types of markers in each system.

Significant differences exist in the rDNA production process between prokaryotic and eukaryotic cells. In general, bacterial cells express greater concentrations of protein product and require relatively simple media components. However, prokaryotic cells do not perform many important post-translational modifications such as glycosylation and, historically, it was not possible to express large proteins in *E. coli*. These limitations necessitate the use of eukaryotic cells in many cases. The production differences between eukaryotic and prokaryotic host cells have significant impacts that are reflected in the requirements for process validation, purification, and analytical methodology. These requirements are addressed later in this chapter.

## Monoclonal Antibodies

Antibodies are proteins produced by differentiated B lymphocytes. Each lymphocyte produces an antibody of defined specificity (i.e., the antibody molecule recognizes a specific site or epitope on the antigen). Antibodies that are produced in immunized animals are formed from many different clones of B lymphocytes; hence, the name polyclonal antibodies. Because the harvest of blood from these animals, by definition, results in polyclonal antibody mixtures, the antisera have multiple epitope recognition sites with a wide variety of binding constants (avidity) and therefore vary from lot to lot. Antibodies that are produced by immortalized cell lines (hybridomas) derived from single B cells are referred to as monoclonal antibodies. The harvest of these cultures leads to an antibody of specific epitope recognition with a homogeneous binding constant.

B lymphocytes have a finite life span in culture and have to be immortalized to enable continuous monoclonal antibody production. At present, the most common procedure is through chemically-induced fusion of a mouse spleen cell with a mouse myeloma cell. The resultant mouse-mouse hybridoma cell inherits from the myeloma cell the ability to replicate continuously in culture and inherits from the spleen cell the ability to produce the desired monoclonal antibody. Cell banks of the hybridoma cell line can be used to produce a continuous supply of the monoclonal antibody, either *in vivo* (i.e., by injection into mice and subsequent collection of the ascites fluid), or *in vitro* (i.e., by conventional cell culture techniques). It should be mentioned that recent advances in molecular genetics have led to the development of transfecomas and *E. coli*- and bacteriophage-based production schemes that may offer advantages for future production of monoclonal antibodies.

Process validation, purification, and analytical considerations for monoclonal antibodies are conceptually similar to those for rDNA products. This is because both types of products are proteins and therefore require similar handling and assay procedures. Because monoclonal antibodies are the products of immortalized cell lines, there is concern that potential viral nucleic acid contaminants be effectively excluded or inactivated by the manufacturing processes, just as for recombinant products of continuous cell lines.

Commercial applications of monoclonal antibodies include both diagnostic and therapeutic uses. In some cases, the monoclonal antibody is coupled to another substance (e.g., an oncolytic agent, radionuclide, toxin), with the resultant antibody conjugate being the final product of interest. In this case, both the antibody intermediate and the final product require extensive process development and analytical characterization.

For the purposes of this chapter, the scope of biotechnology will be confined to rDNA and monoclonal antibody pharmaceutical products.

## CHARACTERISTIC PRODUCTION PROCESSES

The major difference between biotechnology-derived products and other pharmaceutical products is the means of production used to generate the product. Biotechnology makes use of genetically modified living organisms to produce protein or peptidyl products. This statement is true for both rDNA-derived products as well as monoclonal antibody products. Biotechnology-derived products are therefore readily differentiated from proteins or peptides that have been obtained by isolation from natural source materials such as plasma, serum, or tissue, or by chemical synthesis.

Biotechnology-derived products are not significantly different from other protein pharmaceuticals after the protein purification process. Thus, the basic requirements for process validation, environmental control, aseptic manufacturing, and quality control/quality assurance systems are fundamentally the same for all pharmaceutical products. However, the complexity of these systems is often greater for biotechnology-derived products because the production of such bio-molecules generally requires highly developed cell propagation processes, complicated purification methods, and analytical control to ensure their homogeneity, lot-to-lot consistency, and safety.

This section describes in some detail only those significant factors that are unique to the processing of biotechnology-derived products. This includes descriptions of the various biological production systems now in use, and a discussion of purification issues.

## rDNA Production

rDNA products are presently produced in prokaryotic (bacteria) or eukaryotic systems (e.g., yeast, mammalian cell culture). The choice of the production organism is generally a direct function of the molecular complexity of the protein that is to be produced as well as the economics and efficiency of the fermentation or cell culture process. The earliest biotechnology-derived products were produced in *E. coli* based on the high degree of understanding of its molecular biology. Within the last few years, however, the use of large-scale eukaryotic cell culture has become relatively commonplace.

## prokaryotic (bacterial) production

Bacterial production of biotechnology-derived products offers a number of distinct advantages as well as certain disadvantages. As previously stated, the biology of bacteria is quite well understood and the safe and effective use of *E. coli* as the host organism for production has been well documented. Thus, the expression of a new protein in *E. coli*, if possible, is often easier to accomplish than in other, more theoretically suitable, expression systems. This may be offset, however, by the fact that *E. coli* produces proteins usually in a chemically reduced state. For proper folding, such proteins require the production of intramolecular disulfide bonds by oxidation. A second disadvantage is that all *E. coli* proteins begin their sequence with an N-formyl methionine residue that may not always be removed by *E. coli* proteolytic systems, thus possibly yielding a methionyl derivative of the desired natural protein. A third disadvantage of expression in *E. coli* is the potential for product degradation because of trace protease impurities. A fourth disadvantage is the requirement for endotoxin removal during purification. These limitations aside, the ease of use of *E. coli* and their generally high-expression yields for most proteins often have resulted in the continued



#### preferential use of these bacteria, where feasible.

As previously described, the key element in rDNA technology is the recombinant plasmid, which contains the gene that codes for the protein of interest. Plasmids are simple and small circular extrachromosomal segments of bacterial DNA that are isolated from a bacterium and are self-replicating. The basic technology involves the specific enzymatic cleavage of a plasmid using endonucleases followed by the insertion of a new piece of DNA that contains the gene of interest. The resultant recombinant plasmid is considered the key raw material of rDNA technology. The recombinant plasmid is introduced into the host organism through a process called transformation, where it passes on its new genetic information and results in the production of the protein product. The large-scale growth of recombinant organisms can be conducted in commercial fermenters at scales in excess of 100,000 L, making these types of production systems extremely economical. There are, however, a number of issues that complicate *E. coli* fermentation systems. In some cases, the expressed protein product may cause cellular toxicity, and/or be extremely difficult to recover or purify because it may be sequestered into bacterial inclusion bodies as large semisoluble aggregates. Recent advances in *E. coli* molecular biology have led to the ability to express proteins into the periplasmic space, allowing for the removal of unwanted N-terminal methionine groups and leading to more readily purified proteins.

#### eukaryotic (mammalian cell and yeast) production

The development of eukaryotic cell culture for the production of vaccines has long been established in the pharmaceutical industry and an extensive database has been developed to ensure the suitability of such protein products in humans. The extension of this technology to rDNA products was primarily a response to the limitations in the use of *E. coli*. Particularly with respect to large proteins or glycoproteins, eukaryotic cell expression is an attractive alternative to a bacterial system because eukaryotic cells can secrete proteins that are properly folded and identical in primary, secondary, and tertiary structure to the natural human protein. Concerns about the economics of this production system originally hindered its development. Recent advances, however, in improved expression levels, in large-scale cell culture using Chinese Hamster Ovary (CHO) cells, and in the formulation of more highly defined growth media have combined to dramatically improve the economic feasibility of eukaryotic cell substrates. The number of cell passages required for cloning, selection, amplification, and cell banking prior to production generally necessitates the use of immortal cell lines because nonimmortalized strains (i.e., diploid cultures) cannot be propagated long enough to provide an economically useful time in the production stage. Initial questions regarding the safety of such immortal cell lines were based on concerns over potential oncogenes and potential viral and retroviral contamination. These concerns have been minimized by the exhaustive analysis and characterization of master cell banks for adventitious (accidentally introduced) agents, by effective process validation studies, and by the safety data gathered to date for products produced by this method. The resultant thoroughly characterized master cell bank is used for full-scale production. Other eukaryotic cell lines, such as those derived from insect cells, may be useful in achieving many of the conformational and post-translational advantages that have been described for mammalian cell culture.

The use of yeast strains such as *Saccharomyces cerevisiae* for production has been extensively explored. The production of proteins in yeast offers many theoretical advantages over *E. coli* while raising certain new concerns. Like *E. coli*, yeast can maintain stable plasmids extrachromosomally; however, unlike *E. coli*, yeast possesses the ability to produce glycoproteins.

#### monoclonal antibody production

Monoclonal antibodies can be produced in two major ways, depending on whether they are of human or murine (mouse) origin. For antibodies of murine origin, appropriate lymphocytes are selected from the spleens of previously inoculated mice or rats. The cell is then fused with a transformed cell line such as a myeloma cell line, producing a hybridoma cell. The hybridoma cells are then clonally selected and used to produce the monoclonal antibody products. For antibodies of human origin, human B lymphocytes can be clonally selected for the hapten binding specificity of their product antibodies; these selected cells can then be immortalized by infection with a virus. The resultant fused or transformed cell can proliferate indefinitely in a bioreactor/cell culture environment or can be injected into mice from whose ascites fluid the protein can be obtained. Antibody is produced as directed by the chromosomal information that resides in the cell or was acquired during fusion and is secreted into the medium from which it can be readily purified. The hybridoma cells must be thoroughly analyzed and characterized in the same general way as an rDNA cell bank. The resultant cell bank is used for production of product either by large-scale cell culture or by harvesting ascites fluid from mice inoculated with transformed cells.

#### Control of Fermentation and Cell Culture Processes

Because the production process using a living system is the fundamental cornerstone of biotechnology, the issues that relate directly to the control of biotechnology processes need to be examined. Concerns over the production of proteins in bacteria, for example, primarily involve systems for ensuring genetic stability, consistent product yield, and evidence of the lack of contamination by adventitious organisms. These same concerns apply to large-scale eukaryotic cell culture, where, as stated above, there are also significant issues relating to the use of immortalized cell lines such as the putative presence of oncogenic DNA/RNA and impurities from media proteins.

#### fermentations (bacteria and yeast)

A considerable amount of knowledge has been obtained for the production of recombinant proteins in bacteria and yeast; therefore, the major fermentation issues typically are resolved by the demonstration of consistency in fermentation conditions. Fermentations with bacteria and yeast usually are performed over short, well-defined time periods to monitor and control growth rate and product expression conditions. The presence of contaminating foreign organisms may be detected by effects on growth rate, culture purity, fatty acid profile, etc., and is cause for termination of the fermentation. The genetic stability of the production plasmid for bacteria may be addressed by isolation and nucleotide sequence analysis or by DNA restriction mapping. These results may be confirmed by peptide mapping of the expressed protein for each product lot manufactured. It is very important to optimize the fermentation conditions so that the amount of proteolytic processing of the target protein that may occur can be either limited or avoided completely. Proteolytic processing is often a problem in *E. coli* fermentations and may lead to recovery difficulties and low product yields. Finally, the conformation of the protein and its effects on potency must be addressed by the fermentation process.

#### eukaryotic cell cultures

The origin of large-scale cell culture techniques for the production of biotechnology-derived products can be traced back to the vaccine industry. Developments such as large-scale cell suspension cultures using recombinant organisms that secrete the desired protein into the media have had a significant impact on biotechnology. Large glycosylated proteins in quantities sufficient for the marketplace can now be produced. The use of eukaryotic cell cultures, however, is complicated by issues such as genetic stability, protein folding, and culture conditions, including cell viability and growth rates. For example, the genetic stability of cell cultures cannot be addressed as readily as *E. coli* fermentations by techniques such as plasmid sequence analysis because the gene that codes for the product is incorporated into the cell genome and is not easily recovered. One alternative is peptide mapping of the expressed protein, which requires a resolution and sensitivity adequate to detect subtle mutations.

The absence of adventitious organisms in cell cultures is critical. In addition to demonstrating that bacteria, yeast, and molds are not present in cell cultures, the manufacturer must provide for each culture evidence that mycoplasmas and adventitious viruses are not present. It is important to recognize that certain hybridomas used for monoclonal antibody production may contain endogenous retroviruses. However, it must be demonstrated that any viruses present in the culture are removed from the final product. This requires the development of suitable analytical techniques to ensure the absence of contamination by mycoplasmas or human and animal adventitious viruses.

The degree and type of glycosylation may be important in the design of cell culture conditions for the production of glycosylated proteins. The degree of glycosylation present may affect the half-life of the product *in vivo* as well as its potency and antigenicity. Although the glycosylation status of a cell culture product is difficult to determine, it can be verified to be consistent if the culture conditions are highly reproducible.

#### Process for Recovery and Purification

The recovery of protein products obtained from either fermentation or cell culture is generally based on efficient protein separation techniques such as those listed in [Table 1](#). The recovery process begins with isolation of the desired protein from the fermentation or cell culture medium, often in a very impure form. The advantage of cell culture and yeast derived products is that many of these proteins are secreted directly into the medium, thus requiring only cell separation to obtain a significant purification. For *E. coli*-derived products, lysis of the bacteria is often necessary to recover the desired protein. It is important in each case to achieve rapid purification of the desired protein because proteases released by the lysed organisms may cleave the desired product. Such trace proteases are a major concern in the purification of biotechnology-derived products because they can be very difficult to remove, may complicate the recovery process, and can significantly affect final product stability.

Table 1. Chromatographic Purification Methods Used for Biotechnology-derived Products



Chromatofocusing
Reversed-phase chromatography
Hydrophobic interaction chromatography
Charge-transfer chromatography
Size-exclusion chromatography (molecular sizing)
Ion-exchange chromatography
Anion
Cation
Affinity chromatography
Chemical
Monoclonal antibodies
Cellular receptors
Dye/Ligand
Metal chelate

The recovery process is usually designed to purify the final product to a high level. The purity requirement for a product depends on many factors, although chronic use products may be required to have much higher purity than those intended for single-use purposes. Biotechnology products contain certain impurities that the recovery processes are specifically designed to eliminate or minimize. These impurities include trace amounts of DNA, growth factors, residual host proteins, endotoxins, and residual cellular proteins from the media. The most common impurities of concern and suitable assay methods to detect them are presented in [Table 2](#).

Table 2. Potential Impurities and Contaminants in Biotechnology-derived Products

Impurities or Contaminants	Detection Method
Impurities	
Endotoxin	<a href="#">Bacterial Endotoxins Test</a> (85), <a href="#">Pyrogen Test</a> (151)
Host cell proteins	SDS-PAGE <sup>a</sup> , Immunoassays
Other protein impurities (media)	SDS-PAGE, HPLC <sup>b</sup> , Immunoassays
DNA	DNA hybridization, UV spectrophotometry, Protein binding
Protein mutants	Peptide mapping, HPLC, IEF <sup>c</sup> , MS <sup>d</sup>
Formyl methionine	Peptide mapping, HPLC, MS
Oxidized methionines	Peptide mapping, amino acid analysis, HPLC, Edman degradation analysis, MS
Proteolytic Cleavage	IEF, SDS-PAGE (reduced), HPLC, Edman degradation analysis
Aggregated proteins	SDS-PAGE, HPSEC <sup>e</sup>
Deamidation	IEF, HPLC, MS, Edman degradation analysis
Monoclonal antibodies	SDS-PAGE, immunoassays
Amino acid substitutions	Amino acid analysis, peptide mapping, MS, Edman degradation analysis
Contaminants	
Microbial (bacteria, yeast, fungi)	<a href="#">Microbial Enumeration Tests</a> (61), <a href="#">Tests for Specified Microorganisms</a> (62), <a href="#">Sterility Tests</a> (71), microbiological testing
Mycoplasma	Modified 21 CFR Method <sup>f</sup> , DNAF <sup>g</sup>
Viruses (endogenous and adventitious)	CPE <sup>h</sup> and HAD <sup>i</sup> (exogenous virus only), reverse transcriptase activity, MAP <sup>j</sup>

a Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

b High-performance liquid chromatography.

c Isoelectric focusing.

d Mass spectrometry.

e High-performance size-exclusion chromatography.

f Draft guidelines relating to Code of Federal Regulations, Title 21.

g DNA-binding fluorochrome.

h Cytopathic effect.

i Hemadsorption.

j Murine antibody production.

Chromatofocusing and reversed-phase chromatography are purification methods that use chemicals, either in the stationary (bonded) phase or in the mobile phase, that may become impurities in the final product. As in any new technology, the burden of validation (i.e., demonstrating removal of potentially harmful chemicals) is incumbent on the manufacturer. Validation is necessary when isolating end product monoclonal antibodies or using a technique that contains a monoclonal antibody purification step. The process must demonstrate removal of leaching antibody or antibody fragments. It is necessary to ensure the absence of adventitious agents such as viruses and mycoplasmas in the cell line that is the source of the monoclonal antibodies. The main concern is the possibility of contamination of the product with an antigenic substance whose administration could be detrimental to patients. Continuous monitoring of the process is necessary to avoid or limit such contamination. The problem of antigenicity related to the active as well as host proteins is one that is unique to biotechnology-derived products in contrast to traditional pharmaceuticals. Manufacturing methods that use certain solvents should be monitored if these solvents are able to cause chemical rearrangements that could alter the antigenic profile of the drug substance. The manufacturer is also obligated to produce evidence regarding performance consistency of novel chromatographic columns. Considerations for single-use products such as vaccines may differ because they are not administered continuously and, in this case, antigenicity is desirable. On the other hand, validating the removal of ligand or extraneous protein contamination is necessary. Unlike drugs derived from natural sources, manufacturers of biotechnology-derived products have been required to provide validation of the removal of nucleic acids during purification. Vaccines may again be different in this regard because of the accumulated clinical history on these products.

#### QUALITY CONTROL

In general, quality control systems for biotechnology-derived products are very similar to those quality control systems routinely employed for traditional pharmaceutical products in such areas as raw material testing and release, manufacturing and process control documentation, and aseptic processing. Quality control systems of biotechnology-derived products incorporate some of the same philosophies applied to the analysis of low molecular weight pharmaceutical products. These include the use of chemical reference standards and



...ated methods to evaluate a broad spectrum of known and/or potential product impurities and potential breakdown products. The quality control systems for biotechnology-derived products are generally analogous to those established for traditional biologicals with respect to determining product sterility, product safety in experimental animals, and product potency. For example, refer to [Injections](#) (1), [pH](#) (791), [Particulate Matter in Injections](#) (788), [Bacterial Endotoxins Test](#) (85), and [Impurities in Official Articles](#) (1086).

The fundamental difference between quality control systems for biotechnology-derived products and traditional pharmaceuticals is in the types of methods that are used to determine product identity, consistency, purity, and impurity profiling. Furthermore, in biotechnology quality control, it is frequently necessary to use a combination of final product and validated in-process testing and process validation to ensure the removal of undesired real or potential impurities to the levels suggested by regulatory agencies. Biotechnology-derived products generally require a detailed characterization of the production organism (cell), a complete assessment of the means of cell growth/propagation, and explicit analysis of the final product recovery process.

The complexity of the quality control systems for biotechnology-derived products is related to both the size and structural characteristics of the product and manufacturing process. In general, the quality control systems required for products produced in prokaryotic cells are less complex than the systems required for products produced in eukaryotic cells. The quality control systems for prokaryotic production organisms usually entail documentation of the origin of the producer strain and encompass traditional testing for adventitious organisms, karyology, phenotyping, and antibiotic resistance. In addition, newer techniques such as DNA restriction mapping, DNA sequence analysis, and routine monitoring that may include measurement of mRNA and/or plasmid DNA levels may be useful. The quality control of the master cell bank and working cell bank for eukaryotic production organisms generally includes testing for adventitious organisms, karyology, identity, and stability monitoring. All eukaryotic cell lines (except yeast) are generally tested for the presence of retroviruses, retroviral activity markers, and tumorigenicity, although many of these tests may be of limited value.

#### PRODUCT FORMULATION

The products of biotechnology are proteins and peptides that are relatively unstable molecules compared to most organic pharmaceuticals. Most biotechnology processes involve the transfer of proteins from one stabilizing or solubilizing buffer to another during the purification process. Ultimately, the protein is exchanged into its final solution dosage form where long-term stability is achieved. In addition, these products often require lyophilization to achieve long-term stability because of the potential for degradation by a variety of mechanisms, including deamidation, aggregation, oxidation, and possible proteolysis by trace levels of host cell proteases. The final dosage form of the protein usually contains stabilizing compounds that result in the optimal pH and solution conditions necessary for long-term product stability and/or the desired properties for administration of the product (tonicity). These compounds include proteins, polyhydric alcohols, amino acids, carbohydrates, bulking agents, inorganic salts, and nonionic surfactants. In addition, these excipients may be required for stable lyophilized cake formation. There are special requirements for lyophilized products, such as the control of moisture levels, that generally are defined in the individual USP monograph and that may be important to product stability. Significantly, the assessment of protein stability usually requires the use of multiple analytical methods, each of which may be used to assess a specific mode of protein degradation. Many of these assays are described in the following section. The use of accelerated stability studies to predict the shelf life of protein formulations is often complicated by the effects of temperature on protein conformation, resulting in non-Arrhenius behavior. Thus, reliance on real-time, recommended storage condition stability studies is often required for establishing the expiration dating of biotechnology-derived products.

#### ANALYTICAL METHODOLOGY

The analysis of biotechnology-derived products relies heavily on the use of sophisticated analytical methods for demonstrating the structural identity and homogeneity of proteins and for evaluating the shelf life or stability of these products. This section discusses accuracy, precision, informational content, and general applicability of the most commonly used methods. Some methods, such as host cell impurity assays and residual DNA procedures, may be both highly process- and product-specific and thus should be included in the individual monographs.

##### Reference Standard Considerations

The use of suitable reference standards and/or reference materials is extremely important in the analysis of biotechnology-derived products. These standards may be either natural materials or proteins produced by genetic engineering. Many biotechnology-derived products require the availability of accurately characterized reference standards from internationally recognized sources such as the USP (see [USP Reference Standards](#) (11)), WHO, NIH, and FDA. Currently, reference standards with defined activity units are available from these sources for some biologicals. These standards are used by the manufacturers in testing or to calibrate secondary standards using many of the assays described in this section. The potency value of the reference standard is obtained through collaborative studies that, when statistically evaluated, are used to determine the ultimate potency value assigned to the reference standard. The secondary standard can be used to determine the labeled amount of drug substance or potency defined on a product label. Thus, reference standards/reference materials for biotechnology-derived products that are used for the analytical purposes described in specific USP monographs will be approved and made available from USP. Ideally, these reference standards should be in use worldwide and should always be calibrated against the U.S. standard that is deposited by the manufacturer at FDA for those products licensed by FDA. This ensures the accurate and consistent determination of the activity, strength, and purity of these products. Because of a number of issues unique to biotechnology-derived products, such as process and product specificity, separate reference standards for similar products may be required. In addition, thorough development and recalibration of reference standards to replace depleted or expired standards will be conducted by USP to ensure that the label claims of the drug products do not change. One caveat in the assignment of the potency of the primary standard through collaborative studies is that units of activity so defined are only meaningful when compared in a single assay that is both suitably accurate and well described. Attempts to compare activity values from even subtly different assays can be expected to yield widely varying results.

##### Typical Methodology

There are a number of specific analytical methods that pertain to biotechnology-derived products. Many of the assays and tests described may be performed in different ways and, because some of these may be product specific as well, there is a need for clear guidelines on the application of specific methods to particular situations. See the chapters [Design and Analysis of Biological Assays](#) (111) and [Validation of Compendial Procedures](#) (1225) for some general information on methodology.

##### protein content

Protein content assays are used to quantitatively determine the amount of protein in a given biotechnology-derived product. The determination of protein content is often one of the most difficult measurements that needs to be made and often requires independent confirmation by alternate methods. Where applicable, methods such as UV spectrophotometry with a valid absorptivity and Kjeldahl nitrogen analysis can be used to determine absolute amounts of protein independent of reference standards. However, methods such as Lowry protein, biuret, and quantitative amino acid analysis, which require reference standards, also yield accurate values. Protein content assays are among the most important of all the methods used for these products because the results of other types of assays, such as potency, are also dependent on them.

There are several assays for the determination of protein content that are commonly used. These assays may be used at different points in the production process of a given biotechnology-derived product. For highly pure proteins, the simplest protein content method is based on the determination of the UV absorbance of a protein solution by spectrophotometry. The absorbance at the absorption maximum is determined and the protein concentration is calculated with the use of an empirically determined absorptivity. This technique is applicable to proteins containing the aromatic amino acid residues tryptophan, tyrosine, and/or phenylalanine. The absorption wavelength often used is 280 nm. The extinction coefficient, or molar absorptivity, should be determined in the same solvent that is used for the sample to be measured. If necessary, the product may be diluted prior to analysis to obtain solutions with absorbance values in the linear range of detection. Higher molecular weight aggregates and particulates may give rise to light-scattering effects, which provide artificially high absorbance values. Excipient components that have significant absorbance at 280 nm will also interfere with this test. UV spectrophotometry is unique among the protein content methods in that it is an absolute measure of concentration of a specific protein requiring no calibration with standards.

A commonly used general protein content method is the Lowry assay. This is based on the biuret reaction of proteins with copper (II) in a basic solution and the Folin-Ciocalteu phosphomolybdate-phosphotungstic acid reduction to heteropolyphosphomolybdate blue by the copper-catalyzed oxidation of the aromatic amino acids tyrosine, tryptophan, and phenylalanine in the protein. The reaction products are blue and are quantitated spectrophotometrically in the visible region between 540 and 560 nm. This reaction is linear at microgram protein levels. The assay, however, is prone to interferences from a number of substances such as alcohols, sugars, and detergents. In some cases, interfering substances or product may be removed prior to analysis, e.g., by precipitation. Also, the preparation of controls containing interfering substances that are in the drug product may correct for their presence. Although bovine serum albumin historically has been used to prepare the standard curve, different proteins are known to react with differing intensity, so that a reference material of the same product should be used for calibration. The bicinchoninic acid (BCA) assay is a useful alternative to the Lowry assay because it is less sensitive to interfering substances. The working reagent is a BCA-copper (II) solution. The copper (II) complex is reduced to copper (I) in the presence of protein, and the purple color may be quantitated

spectrophotometrically at approximately 560 nm.

Other colorimetric assays can also be used. The Bradford method, for example, employs the binding of the dye Coomassie Brilliant Blue to the protein in an acidic environment. The concentration of the protein in solution is then determined by comparing the absorbance at 595 nm with a standard curve of a reference material.

Fluorescent methods used are normally based on either fluorescamine or o-phthaldialdehyde (OPA). The main advantage of these assays is increased sensitivity. Another advantage is their use with hydrophobic proteins. Fluorescamine and OPA react with primary amines both at the N-terminus of the polypeptide and with amino acid side chains, such as lysine.

The Kjeldahl nitrogen method, [Nitrogen Determination](#) (461), provides an accurate and precise determination of protein concentration and is often used in the determination of UV protein absorptivities. The assay is performed in two stages. The sample is first decomposed with sulfuric acid to produce ammonium sulfate, carbon dioxide, and water. The decomposition is performed at the boiling point of sulfuric acid in long-necked, pear-shaped flasks. These flasks serve to condense water vapor and prevent the loss of material.

Depending on the efficiency of decomposition, various salts such as potassium sulfate may be added to increase the boiling point of the sulfuric acid solution. Oxidizing agents such as perchloric acid or potassium permanganate have also been used to improve the decomposition. The second stage of the assay involves the direct determination of ammonia. In most macrodeterminations, ammonia is steam distilled from the mixture after basification with sodium hydroxide. The ammonia can typically be quantitatively distilled out of the mixture in 5 to 20 minutes and absorbed quantitatively into a standardized acidic solution of known volume and normality. The excess acid is then back-titrated with standardized base. For crude determinations of protein, the ammonia value (and therefore the nitrogen content), is multiplied by a factor of 6.25 mg of protein per mg of nitrogen, which corresponds to a nitrogen content of 16%. The protein value so obtained is generally valid for most proteins. If a more accurate value is required, as for an absorptivity determination, then the conversion factor must be calculated for the nitrogen content of the individual pure protein from the known amino acid composition. For glycoproteins that contain amino sugars, the calculated value is biased high unless a correction is applied.

Amino acid analysis is used in the determination of the appropriate absorptivity of the protein and may also be used quantitatively for the determination of protein content. This procedure, although more complicated than those described above, can also yield accurate results.

#### amino acid analysis

Amino acid analysis is a classical protein chemistry method for the determination of the amino acid composition of proteins and peptides. The method consists of the complete hydrolysis of a protein or peptide to its component amino acids, which are then chromatographically separated and quantitated. Amino acid analysis, therefore, can be used to determine both the amino acid composition of a product (i.e., identity) and the total amount of protein present. The method has some inherent difficulties, such as complete or partial destruction of some amino acids, that can be circumvented by appropriate analytical methodology. The amino acid tryptophan is destroyed by 6 N hydrochloric acid hydrolysis and thus requires the use of alternate hydrolysis conditions. The amino acids serine and threonine may be partially destroyed, whereas peptide bonds between bulky hydrophobic residues such as valine and isoleucine may be more resistant to hydrolysis, in both cases yielding values lower than actual. Accordingly, analysis of time-course hydrolysis samples may be used to compensate for these factors. Cysteine and methionine may require preoxidation to cysteic acid and methionine sulfone, respectively, for accurate quantitation. Each specific protein may require a procedure of optimized hydrolysis conditions for its amino acid analysis to obtain the optimal results.

Amino acid analysis is performed in two stages. The first stage involves the hydrolysis of the protein into its component amino acids. This hydrolysis is normally performed with 6 N hydrochloric acid at about 110° for 24 hours. Some proteins may require longer or more stringent hydrolysis conditions. The second stage is the separation and quantitation of the individual amino acids by some form of chromatography that can be performed with either precolumn or postcolumn derivatization. A number of precolumn derivative procedures are available, such as with OPA, phenylisothiocyanate (PITC), and fluorenylmethoxycarbonyl (FMOC). These derivatives are then separated by reversed-phase (RP) high-performance liquid chromatography (HPLC) and quantitated following UV or fluorescence detection. Postcolumn derivative methods involve separation of the component amino acids by high-performance ion-exchange chromatography (HPIEC) followed by postcolumn reaction with a chromophore, such as ninhydrin, and quantitation following UV/visible detection. All of these methods are suitable for performing amino acid analyses and each has its inherent advantages and disadvantages. OPA derivatives are very simple to prepare and are sensitive, requiring only a small amount of sample, but they are unstable and have to be chromatographed immediately upon preparation. Phenylthiocarbamyl (PTC) derivatives, on the other hand, are relatively more stable. Postcolumn derivatization with ninhydrin is often performed in the low-pressure mode and has the advantage of stability of the amino acid hydrolysate. Its disadvantage is the need for dual detection at 440 and 570 nm and for post-column apparatus.

#### PROTEIN SEQUENCING

Protein sequencing is useful in the control of quality of protein biologicals because it can provide primary structure information, i.e., amino terminal and/or carboxy terminal structure. For rDNA-derived biologicals, this methodology has the additional purpose of confirming the complementary DNA (cDNA)-predicted amino acid sequence, protein homogeneity, and the potential extent of proteolytic clips. For monoclonal antibodies, this technique is used for determining protein homogeneity. Protein sequencing is divided into amino-terminal and carboxy-terminal sequencing applications and procedures.

**Amino-Terminal Sequencing**— Amino-terminal sequence analysis is a classical protein chemistry technique that yields significant information about primary structure (sequence), homogeneity, and the presence of known or unknown cleavages in the polypeptide. N-terminal sequence analysis is performed with a number of commercially available automatic peptide sequencers. The method is based on the coupling reaction of the amino terminal residue of a protein or peptide with PITC. The resulting PTC-amino acid derivative is cleaved from the protein by a perfluorinated organic acid (generally trifluoroacetic or heptafluorobutyric acid), which exposes the adjacent amino acid. This next amino acid serves as a new N-terminus and is derivatized in the subsequent coupling and cleavage cycle. This process is repeated until an appropriate number, normally 8 to 10, of the amino acids are removed. The modified amino acid residue resulting from the cleavage cycle (anilinothiazolinone [ATZ]) is generally converted in the presence of acid and heat to a phenylthiohydantoin-amino acid (PTH-AA). The PTH-AA may then be determined following RP-HPLC analysis. Any intrachain cleavages as well as heterogeneity of the N-termini (e.g., N-terminal methionine) on the polypeptide will also be sequenced at the same time. These result in smaller peaks in the chromatogram and may enable both the relative quantitation of the amount of the N-termini and the identification of the location of the cleavage site on the polypeptide. This procedure for protein sequence analysis may also be performed manually. The limitations of the PITC sequencing method are that the method is only semiquantitative (i.e., the amount of the N-termini can only be estimated) and the PTH derivatives of serine and threonine may be severely degraded, making their determination difficult. Cysteine residues in order to be determined, must first be modified, for example by alkylation. In addition, the amino acids glycine and proline are slow to rearrange, resulting in minor difficulty in their determination.

**Carboxy-Terminal Sequencing**— Sequencing of the protein from the carboxy terminus also yields valuable primary structure information as well as possible C-terminal cleavages. The sequential degradation of a protein from the C-terminus can be performed by either chemical or enzymatic methods. The reaction of hydrazine, ammonium thiocyanate, or cyanogen bromide with a protein can be used to sequentially degrade the protein at or near the C-terminus. The ammonium thiocyanate reaction has been extended for use on proteins coupled to solid supports. The C-terminal amino acids can be sequentially cleaved enzymatically with exopeptidases such as carboxypeptidases. Limitations of the carboxypeptidase approach are the potential contamination with endopeptidase and the inherent difficulty and unpredictable nature of the sequencing. Mass spectrometry can be used either directly on protein digests or in conjunction with HPLC peptide mapping to identify the C-terminus of the protein. However, these methods are only semiquantitative.

#### peptide mapping by hplc

For pharmaceutical proteins, peptide mapping has two primary purposes: it is a highly specific identity method and, in the case of biotechnology-derived products, may serve as a confirmation of genetic stability. Peptide mapping is used to compare the protein structure of a specific lot of material to that of a suitable reference material/reference standard or to those structures of previous lots to confirm correctness of the primary structure and to confirm lot-to-lot consistency of primary structure (within the limits of this technique). The amino- and carboxy-terminal peptides and carbohydrate-containing peptides often can be separated and identified. The latter are valuable in the peptide maps of glycosylated proteins such as monoclonal antibodies. Peptide mapping may be used to determine the presence of single or multiple incorrect amino acids that may result from such events as a single point mutation or mistranslation of the cDNA sequence.

The procedure involves the selective fragmentation of the protein into discrete peptides that are resolved by some chromatographic technique. The fragmentation is accomplished with endoproteases, such as trypsin, chymotrypsin, thermolysin, or V8 protease, or by selective chemical degradation with cyanogen bromide, which cleaves at specific sites on the molecule. Selection of the appropriate endoprotease to be used is directed by the primary sequence of the protein. Trypsin cleaves on the C-terminal side of the basic residues lysine and arginine; chymotrypsin cleaves after the aromatic residues phenylalanine, tyrosine, and tryptophan; thermolysin cleaves after the hydrophobic residues leucine, isoleucine, and valine; V8 protease cleaves after the acidic residues glutamic acid and aspartic acid; and cyanogen bromide cleaves at methionyl residues. Other enzymes, such as clostrin (arginine) and endoproteinase lys-C (lysine), and chemical methods, such as 2-nitro-5-thiocyanobenzoic acid (cysteine), may also be used. Each of these methods has its own set of



antages and disadvantages. One common disadvantage to all these techniques is that nonspecific cleavages occur to some degree. It is important that the peptides generated from the digestion are large enough to provide structural information about the protein, and yet small enough to allow their analysis and separation by a technique such as RP-HPLC. For this reason and the fact that cleavage with this enzyme is almost quantitative, trypsin is the enzyme with the most general applicability for most proteins. For large proteins of greater than 60,000 daltons (about 520 amino acids), cleavage with trypsin may result in too many fragments, so another endoprotease may be chosen. I-TPCK (tosyl-l-phenylalanine chloromethyl ketone)-treated trypsin normally is used because TPCK inhibits the action of chymotrypsin, a contaminant present in many trypsin preparations. Although reaction with cyanogen bromide cleaves at methionyl residues, proteins do not contain many of these residues. As a result, relatively few peptides are obtained and these may be too large or hydrophobic for HPLC separation.

Once the digestion is complete, the peptides are generally separated by either RP-HPLC and/or HPIEC. Selection of the appropriate column is empirically based and will vary for different proteins. For RP-HPLC, both 100-Å and 300-Å pore size supports work well, and the selection of the silica support may be an important criterion for optimal separation. For the smaller peptides generated by these digestions, C8 and C18 stationary phases generally have been found to be more efficient than C4 supports. The most common solvents used

for reversed-phase separations are water and acetonitrile containing a constant (~0.1%) amount of trifluoroacetic acid. Buffered mobile phases containing phosphate also offer excellent selectivity depending on the pH. Screening the effect of pH in the 3.0 to 5.0 range causes a shift of peptides containing the acidic residues, glutamic acid and aspartic acid.

For ion-exchange separations, less information is available, but both silica and polymeric supports with both weak and strong ion-exchange stationary support can be used successfully. Because many of the peptides are somewhat hydrophobic, the addition of small amounts of organic solvents in the mobile phase, such as 5% to 10% methanol or acetonitrile, may be necessary. A potential disadvantage of HPIEC analysis of peptide mixtures is that sometimes neutral peptides or peptides that have the same charge as the support may not be retained on the column and thus may not be separated or identified by this method.

#### immunoassays

Immunoassays are used either as active drug substance methods to identify and quantitate the protein of interest or as impurity profile methods to detect and quantitate known host cell protein impurities. Because these protein impurities may represent a large number of potential impurities at trace levels rather than a single impurity, the immunoassays must be sensitive and selective to detect as many of these impurities as possible. Immunoassays that can measure these impurities to very low levels have been developed for E. coli proteins (ECPs) and CHO proteins. Immunoassays additionally may serve as potency assays for monoclonal antibodies using an appropriate antigen.

Immunoassays consist of a large group of assays that depend on specific high-affinity antibody:antigen interactions. These assays include the radioimmunoassays (RIAs) and the enzyme-linked immunosorbent assays (ELISAs). RIAs are performed in a liquid or solid phase using an unlabeled antibody directed against the radiolabeled protein of interest. The principle of the RIA is that the inhibition of binding of labeled antigen to unlabeled antibody by samples is compared to the inhibition by known standards, thus allowing quantitation of the protein of interest.

Immunoradiometric assays (IRMAs) or sandwich RIAs employ two antibody preparations that are used to sandwich the protein of interest. The first antibody is unlabeled and is directed against the protein, and the second antibody is radiolabeled and may be directed against the protein or the first antibody. The entire antibody:antigen complex is isolated and the amount of radioactivity, and, therefore, the protein of interest, is determined. The development of an RIA or IRMA for a biotechnology-derived product requires careful attention to production of the antiserum, preparation of the labeled tracer, preparation of a suitable reference standard, and methods for the separation of free antigen from bound antigen.

The most commonly used ELISA format of trace impurity analysis is the sandwich ELISA that utilizes two antibody preparations like the IRMA, but without radiolabeling. The first antibody is unlabeled and the second antibody has an enzyme such as horseradish peroxidase (HRP) or alkaline phosphatase attached. Basically, the ELISA method consists of applying a layer of purified antibodies to the host cell proteins onto microtiter plates, followed by the protein product. The enzyme antibody conjugate is added and allowed to bind to the antibody-bound host cell proteins. An appropriate substrate is added for color development, which is analyzed with a spectrophotometric plate reader. Such a multiantigen ELISA requires a representative reference standard preparation of appropriate host cell protein impurities to serve as the immunogen for preparation of the antibodies used for the assay. This reference standard preparation is usually prepared from a manufacturing production run yielding all of the expected host cell proteins except the product protein. The total absence of the product protein is necessary in this preparation to avoid the production of antibodies to the product itself when the reference standard is used as an immunogen. Because of varying affinities of polyclonal antibodies to multiantigen preparations, the absolute accuracy of the multiantigen methods and the ability to detect every potential antigen cannot be guaranteed.

#### electrophoresis

Electrophoretic assays are among the most common and powerful of the assays used to evaluate protein purity and homogeneity. They are valuable not only for the initial evaluation and release of biotechnology-derived products but also as stability-indicating methods for detecting molecular or chemical changes in the molecule as a result of denaturation, aggregation, oxidation, deamidation, etc. The use of these methods is facilitated by their simplicity and their requirement of only microgram quantities of sample. The two types of electrophoretic assays most often used for biotechnology-derived products are sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF).

The SDS-PAGE method separates proteins primarily by their molecular weight because, in the presence of the anionic detergent SDS, a net negatively charged protein SDS complex is formed. The sample is first denatured in the detergent, which disrupts the noncovalent intramolecular and intermolecular bonds that hold proteins together, and then it is electrophoresed through a polyacrylamide gel support. Protein migration through the gel is proportional to size so that smaller proteins migrate faster through the gel than larger ones. Samples are often electrophoresed under both reduced and nonreduced conditions to determine if impurities of the same molecular weight or if intramolecular proteolytic cleavages of the protein of interest are present. Although nonreducing SDS-PAGE is commonly used to estimate the state of aggregation and/or oligomerization of the protein of interest, this method will only permit observation of aggregates or oligomers that are stable in the presence of SDS and the conditions used for sample preparation and electrophoresis. Proteins consisting of multiple chains held together by disulfide bonds are broken down and separated into their individual polypeptide chains. Sample detection following electrophoresis can be quantitative with densitometric analysis of Coomassie Brilliant Blue stain or qualitative, but with increased sensitivity in the nanogram range, with silver stain. Silver stain SDS-PAGE may also be performed quantitatively under suitable conditions. With proper validation, Coomassie Brilliant Blue staining and densitometry may also be used to give quantitative determination of polypeptides in the nanogram range. SDS-PAGE coupled with Coomassie Brilliant Blue stain is used to quantitatively determine the purity of the sample with regard to dimer and larger covalent aggregates and fragments. When the method is combined with the silver stain technique, an assessment of low/trace levels of a new impurity can be made by directly comparing the electrophoresed sample to the electrophoresed reference material or standard under reduced and nonreduced conditions. Generally, silver staining is used qualitatively because of potential major variations in binding of silver from protein to protein, and relatively inconsistent background on a routine basis. An estimation of the quantity of an impurity can be obtained by electrophoresis of a known amount of an internal standard such as bovine serum albumin or lesser dilutions of the protein of interest on other lanes of the same gel. The SDS-PAGE separation of a protein can be combined with an immunological method such as immunoblotting. The resulting Western blot is used to determine the identity of an electrophoretic band (i.e., product related or host cell protein impurity). After electrophoresis, the separated proteins are transferred onto a nitrocellulose or polyvinylidene difluoride (PVDF) membrane and reacted with the antibody of interest. Visualization of the complex is done with an enzymatically or radiolabeled antibody.

The IEF method separates proteins on the basis of their charge in an electrical field. The charges on a protein originate from various sources within its amino acid composition, such as protonated amino groups, unprotonated carboxyl groups, deprotonated sulfhydryl groups or tyrosine residues, oxidized cysteine residues, and deamidated residues. However, for each protein there is a pH at which the protein is isoelectric and these charges cancel each other, with the net charge being effectively zero. IEF is performed in the native state in a support of loose-pore polyacrylamide or agarose gel containing ampholytes (amphoteric low molecular weight ions) that set up a pH gradient because of their migration within the support matrix when an electrical field is applied. Simultaneously, in the presence of the electrical field, positively charged proteins migrate towards the cathode and negatively charged proteins migrate towards the anode. Migration stops when each protein reaches the pH value in the support gradient where its net charge is zero. This is the apparent pI or isoelectric point of the protein. Because the migration of a protein is dependent on its amino acid composition, altered forms of the protein and other proteins will migrate to different points on the support. IEF gels may be stained for protein visualization with either Coomassie Brilliant Blue or silver stains. IEF is employed as an identity tool or to ensure the homogeneity of a protein (e.g., monoclonal antibodies) as demonstrated by a banding pattern with the correct pI range. The method can also be used to evaluate the stability of a biological product. Protein deamidation (i.e., glutamine or asparagine residue deamidation) over time leading to the production of a new carboxylic acid group results in molecules with a more acidic pI. IEF can provide information on the state of glycosylation of glycoproteins such as monoclonal antibodies, which may appear as many bands because of changes in the apparent charge on the protein molecule as a result of the sialic acid residues. IEF gel patterns are usually more complicated to interpret than those of SDS-PAGE and interpretation may require many assumptions or subjective judgment.

High-performance capillary electrophoresis (HPCE), which offers the potential advantages of very high protein resolution, is being thoroughly investigated because of recent advances in the technology.



## chromatographic methods

Chromatographic methods have long been used in the determination of the purity of small organic molecules and proteins such as insulin (see [Chromatography](#) 621) and in the determination of the active ingredient and/or excipient concentration of pharmaceutical products. Chromatographic methods are also very effective in the determination of the purity of recombinant pharmaceuticals. However, the chromatography of proteins is far more difficult because of multiple modes of interaction with the chromatographic support as a result of the size and/or shape, charge, and hydrophobicity of the proteins. The most common chromatographic methods used to profile recombinant proteins are RP-HPLC, HPIEC, size-exclusion chromatography (HPSEC), and hydrophobic interaction chromatography (HIC). These methods involve the separation of proteins and are used to determine the purity of drug substances as well as the levels of known impurities or degradation products. A complication with all column chromatographic methods is determining the mass balance between column load and column eluate. Nevertheless, HPLC techniques are valuable for determining the purity and strength of protein pharmaceuticals.

The most common RP-HPLC analyses are performed on columns containing a C4 or C8 stationary phase on a silica-based or polymeric support. C18 stationary phases are also used but more often with smaller peptides in an application such as peptide mapping. Supports with pore sizes of at least 300 Å are preferred for proteins of molecular weight greater than about 10,000. For most RP-HPLC analyses, the proteins are eluted with aqueous acetonitrile gradients and the trifluoroacetic acid is kept constant at 0.1%. Other buffers such as phosphate or tris(hydroxymethyl)aminomethane (Tris) are also used, where the pH may be adjusted for added selectivity to achieve optimal separations.

HPIEC is an important method for purity determination. These separations are based on changes in the charge of the molecule and are useful for identifying and quantitating in protein pharmaceuticals common impurities such as oxidized (primarily oxidized methionine) and deamidated forms (glutamine and asparagine) and clipped or truncated forms. Both strong and weak ion-exchange stationary phases on either silica or polymeric supports can be used. Cation-exchange chromatography may be performed on sulfopropyl-type resins and are effective in distinguishing oxidation and deamidation products. Proteins are typically loaded onto a column equilibrated with water or a weak buffer, and eluted with a salt gradient, such as 0 to 1 M sodium chloride.

HPSEC is a technique that may provide information on the levels of aggregation and fragmentation in a protein pharmaceutical. Depending on the information needed, the mobile phase may be native, containing an aqueous buffer such as 100 mM phosphate, pH 7, or it may be denaturing, containing a low level of a chaotrope or detergent such as 0.1% SDS. The analyses are performed isocratically, with detection typically between 210 and 220 nm depending on the buffer used. Detection at 280 nm may also be used but is less sensitive. Classical size-exclusion chromatography was performed on soft polymeric supports such as cross-linked dextrans, polyacrylamide, or agarose. These, however, are better suited for low-pressure applications. As a result, a number of supports with increased mechanical strength have been developed. Commercially available silica-based and cross-linked agarose supports are now commonly used. HPSEC is also useful for the determination of clipped forms of proteins. Clipped chains often remain attached through the disulfide bonds of cysteine residues. Treatment of the sample with a reducing agent such as dithiothreitol or mercaptoethanol will cleave the disulfide bond and separate the chains. The clipped chains may then be resolved from unclipped forms by HPSEC.

HIC provides separation of proteins based on differences in their hydrophobicity under mild adsorption and elution conditions that generally prevent denaturation and subsequent loss of biological activity. A stationary phase that is weakly hydrophobic is used with a buffered aqueous mobile phase and an initial high-salt concentration to adsorb the protein, which is then selectively eluted using a decreasing salt gradient. Interactions occur between nonpolar amino acid residues that are exposed on the surface of the protein and hydrophobic groups that are present on the chromatographic matrix. A number of silica-based and polymeric supports combined with weakly hydrophobic ligands, such as polyethers, phenyl ethers, or short alkyl chains, have been developed for use in HIC. This technique can be used in the analysis, purification, and characterization of more labile hydrophobic proteins. Protein retention and selectivity can be modified by control of variables such as salt type and concentration, pH and selective-ion effects, temperature and gradient design, as well as by careful selection of the stationary phase.

## quantitative assays

Biomimetic assays (assays that mimic the biological effect of the product) are of major significance in the discussion of assays for biotechnology-derived products. These assays measure the activity of the product and ensure that it is efficacious. Essentially, there are three major types of quantitative assays: animal model assays, cell culture-based assays and in-vitro (physicochemical) assays. Each of these assays has application in the control of biological products. Regardless of the type of quantitative assay employed, it is desirable and, in some cases, necessary, to use a biomimetic assay.

**Animal Model Assays**—Biomimetic assays in animal models have been developed for routine use. Although these assays have a relatively long history of use, they have several major disadvantages such as the large number of animals and appropriate animal facilities and handlers required, the high cost of analysis, the long analysis time (i.e., several days to weeks), and poor reproducibility of results. They are, however, in use mainly because a cell culture-based or in-vitro assay has not been developed and demonstrated to be of equal or greater value. An example of such an assay is that used for the determination of the activity of human growth hormone (somatrem and somatropin). The potency of human growth hormone is determined with a rat weight gain bioassay. Hypophysectomized female rats are monitored for weight gain over an 11-day period after daily injections with human growth hormone. The relative potency of the test sample is obtained by statistical comparison of the activity of the sample to that of a reference material/reference standard. Animal models can be used as bioidentity tests if and when appropriate in-vitro biological and/or physicochemical assays are developed for the measurement of potency of products.

**Cell Culture-Based Bioassays**—This group of assays is comparatively easier to perform, gives results faster (1 to 3 days), and is considerably less expensive and less wasteful of resources than the animal model assays. Cell culture-based bioassays provide information on the effect of the biological product in a living system, but they are imprecise as a consequence of the variances of living cells but not as imprecise as an animal model assay. However, they can be automated and therefore can be repeated sufficiently to provide relatively reproducible and accurate results. An example of this type of assay is the measurement of antiviral activity of human  $\alpha$ -interferon in a human diploid foreskin cell line or in a human lung carcinoma cell line (A549). This assay is performed in microtitration plates by incubation of cells with  $\alpha$ -interferon and subsequent challenge with encephalomyocarditis virus. The cells that survive are detected by dye binding and the dilution of  $\alpha$ -interferon where 50% protection of the monolayer occurs is calculated.

**In Vitro (Physicochemical) Assays**—This group of assays does not rely on a living model, but is usually based on the chemical action of a biological product. These methods are comparatively simple, fast, precise, and accurate. The activity of tissue-type plasminogen activator (alteplase), for example, can be determined with an in vitro clot lysis assay that can be automated and can provide the required results within hours. A synthetic fibrin clot is formed in the presence of plasminogen as a result of the action of the enzyme thrombin on fibrinogen. When alteplase is added, the plasminogen is converted to the active enzyme plasmin, which then lyses the synthetic clot. The assay endpoint is followed spectrophotometrically or visually by noting the release of entrapped air bubbles. Another advantage of this type of assay, because of its precision and accuracy, is that it can be used to provide reliable estimates of the stability of the product. Examples of antibody:antigen and protein:ligand (receptor)-based in-vitro bioassays have also been developed for specific applications. These types of assays offer many advantages in their application to determine the potency of monoclonal antibodies or other highly ligand-specific proteins whose reactivity includes a binding step.

## dna determination

Residual host cell DNA is a potential process-specific impurity in a biotechnology-derived product. The residual DNA is unique for each product because it is dependent on the host organism and the process recovery procedure used to manufacture the product. Although adverse health effects have not been reported from biologicals because of their DNA content, regulatory agencies have requested manufacturers to ensure that the DNA level in biotechnology-derived products is reduced to low levels.

The technique of DNA hybridization (dot blot analysis) is the most sensitive, routine DNA assay available to determine the DNA content of products. It is valuable as a purification process assay to demonstrate that a low level of DNA has been attained early in the manufacturing process. The method relies on the hybridization of cellular DNA from the sample with either specific 32P-labeled or chemically modified DNA probes obtained from the DNA of the host cell. The analysis is performed by first isolating any residual DNA in the sample by a procedure that may include hydrolysis of the protein, chromatography, organic extractions, and alcohol precipitation. The isolated DNA is denatured and then applied to a nitrocellulose or nylon membrane along with a set of serially-diluted host DNA standards. Positive and negative DNA controls are also applied and the membrane may be baked at approximately 80° or placed under UV light to complete binding of the DNA to the membrane. A DNA probe is then prepared either by nick translation, random primer synthesis, or chemical modification of a DNA extract of the host cell. The DNA probe is purified and thermally denatured at 95°. It is then added to the baked or UV-treated membrane and allowed to hybridize with the DNA of the samples at approximately 42° in the presence of formamide or at higher temperatures without formamide for 24 to 48 hours. The membrane is subsequently placed between two X-ray films and exposed to produce an autoradiograph or is developed by immunochemical means using an enzyme conjugate/substrate system similar to ELISA and/or Western blot. The DNA of the sample is estimated by visual comparison of the dot intensity of the sample to those of the diluted DNA standards. The autoradiogram can also be scanned by optical densitometry. The sensitivity of the assay, i.e., 10 to 250 pg, is determined by the limit of visual detection above background of the



serially-diluted DNA standards.

Other methods for DNA determination have been developed using biosensor technology. This methodology currently determines total DNA/nucleic acid impurities rather than specific host cell DNA. This technology may become quite valuable in the future, especially when more specific DNA binding methods are developed. Finally, the recently developed polymerase chain reaction (PCR) technology, which involves DNA amplification, may prove useful in detection and identification of contaminant DNA. Quantitative use of this technology, however, will require further development.

#### carbohydrate determination

One of the possible post-translational modifications that occurs on proteins is the covalent attachment of oligosaccharide chains. Glycosylation is a characteristic of recombinant proteins that are expressed from eukaryotic cell lines. Although the polypeptide chain of a glycoprotein is synthesized under the direct control of the genetic code, oligosaccharides are not primary gene products, but are synthesized by enzymes known as glycosyltransferases. This synthesis results in microheterogeneity of the carbohydrate chains. Also, glycosylation is cell-line dependent, so glycoproteins with identical polypeptide chains made in different cell lines may have considerably different carbohydrate structures. The sugars commonly found in glycoproteins include neutral sugars (d-galactose, d-mannose, and L-fucose), amino sugars (N-acetylglucosamine and N-acetylgalactosamine), and the acidic sugar, sialic acid.

Two main approaches can be taken to determine the sugars covalently attached to the glycoprotein. Both are based on the understanding that microheterogeneity is a common phenomenon among glycoproteins, and that the information represents either average composition or representative structures.

The first approach is the determination of the composition of sugars in a glycoprotein, which can be performed by several methods. Neutral sugars and sialic acid may be determined by simple colorimetric tests. Total neutral sugars can be determined following reaction with phenol and sulfuric acid and measuring the absorbance of the solution at about 490 nm compared to a standard curve. Following mild acid hydrolysis and periodate oxidation, free sialic acid content can be determined with thiobarbituric acid and the absorbance of the solution at about 550 nm compared to a standard curve. Individual neutral sugars can be determined following acid hydrolysis by several methods. Underivatized, they can be separated by HPIEC at high pH and quantitated by pulsed amperometric detection. They may also be converted to the alditol peracetates with acetic anhydride or to the aldononitrile acetates with hydroxylamine hydrochloride and pyridine prior to peracetylation, and the derivatives separated by gas chromatography.

The second approach in determining the carbohydrate composition is to release and separate individual oligosaccharide structures covalently attached to the glycoprotein. This requires an understanding of the types of structures attached. The attachment of sugars to proteins can occur in two major ways: through an O-glycosidic bond involving the hydroxy group of serine, threonine, or modified amino acids such as hydroxylysine or hydroxyproline, or through the N-glycosidic bond of asparagine. O-linked oligosaccharides can be released from the protein following beta-elimination under alkaline conditions and reduction of the reducing end sugar with sodium borohydride. N-linked oligosaccharides can be released chemically by hydrazinolysis or enzymatically by one of a variety of specific glycosidases, such as endo H, endo F, or peptid-N-glycanase. The oligosaccharides can then be separated by HPIEC at high pH and quantitated with pulsed amperometric detection. This results in an oligosaccharide or carbohydrate map analogous to the peptide map for the protein.

#### adventitious and endogenous agent detection

Specific assays pertaining to biotechnology focus on the detection of bacteria, fungi, mycoplasma, and viruses. These reflect the possible contaminants that may occur in both bacterial fermentation and mammalian cell culture. Control is exerted in a variety of ways including characterization of the master seed bank and the working cell banks to ensure freedom from these contaminants, evaluation of raw materials, the design and operation of closed manufacturing systems, testing of production lots, and validation of specific manufacturing processes to ensure that contaminants would be inactivated or removed if present.

Freedom of final sterile dosage forms from bacteria and fungi is usually evaluated by tests for sterility as described in [Sterility Tests](#) (71). Mycoplasma assays are performed by standard cultivation methods employing aerobic and anaerobic incubation of solid medium in plates and semisolid broth in tubes and must comply with the code of federal regulations (21 CFR 610.12). In addition, noncultivable mycoplasma are detected microscopically by using the Hoechst bisbenzimide staining method.

Various methods that are used for the detection of adventitious virus contamination in cell lines include inoculation of indicator cell lines selected for their ability to support the replication of a broad range of viruses and monitoring these for markers of virus infection such as cytopathology, hemadsorption, hemagglutination, and immunofluorescence; inoculation of intact animals and monitoring for illness and death; inoculation of animals and, after four weeks, collection and evaluation of serum for antibodies to specific viruses of concern; and specific immunologic assays or genetic probes for some viruses of concern that cannot be detected by the other methods listed.

The expression of endogenous retrovirus genes is highly variable among different mammalian cells and cell lines. The unpredictable nature of their expression and the diversity of their biochemical and biological properties preclude the use of a single test and instead require an integrated testing strategy. Test methods generally used include transmission electron microscopy of cells from the master seed bank and ultracentrifuged pellets of cell-free, cell culture harvests; various assays for infectious retroviruses that use retrovirus-susceptible indicator cell lines; reverse transcriptase activity; and induction of retroviruses in cells of the master cell bank with chemicals known to induce retroviruses. In addition to classical virological methods, newer techniques such as molecular probe hybridization are also beginning to be used for these evaluations.

1 A series of documents entitled Points to Consider are available from the Director, FDA Center for Biologics Evaluation and Research, HFB-1, 8800 Rockville Pike, Bethesda, MD 20892.

2 This guideline was originally published in the Federal Register, Guidelines for Research Involving Recombinant DNA Molecules 1986; 51 (88): 16957-16985. Copies may be obtained from the Office of Recombinant DNA Activities, 12441 Parklawn Drive, Suite 58, Rockville, MD 20852.

3 A comprehensive guideline, Biosafety in Microbiological and Biomedical Laboratories, is available from the Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402, stock #107-040-000508-3.

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Topic/Question	Contact	Expert Committee
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## 1046 CELL AND GENE THERAPY PRODUCTS

### INTRODUCTION

#### General Definitions

Recent advances in biotechnology have resulted in the development of two new categories of products—cell therapy products and gene therapy products. Cell therapy products contain living mammalian cells as one of their active ingredients, while gene therapy products contain pieces of nucleic acid, usually deoxyribonucleic acid (DNA), as one of their active ingredients. Some products combine both categories, resulting in a therapy that uses cells that express a new gene product. Both cell and gene therapy products may be combined with synthetic or natural biomaterials to form tissue constructs.

For the purpose of this chapter, cell and gene therapy products include any product that has live cells or pieces of nucleic acid, however formulated. The following are excluded at this time: (1) tissue-based products in which the cells are removed or killed, (2) enhanced reproductive methods such as in vitro fertilization, (3) non-mammalian cell-based products, (4) traditional vaccines such as live attenuated virus, and (5) noncell, nongene products manufactured by using cells or recombinant-DNA (rDNA) technology, which are discussed under

[Biotechnology-Derived Articles](#) (1045).

Cell Therapy Products



Cell therapy products are products with live cells that replace, augment, or modify the function of a patient's cells that are diseased, dysfunctional, or missing. Transplantation of bone marrow to replace marrow that has been destroyed by chemotherapy and radiation is an example of a cell-therapy product. These products are also referred to as somatic cell therapy products because nongerm-line cells are used in the product. In addition, cells may be combined with biomaterials. For example, dermal or epidermal cells can be grown on a collagen substrate to produce a sheet of cells for wound and burn therapy. Examples of cell therapy products are shown in [Table 1](#).

Table 1. Examples of Cell Therapy Products

Indication	Product
Bone marrow transplantation	Devices and reagents to propagate stem and progenitor cells, to select stem and progenitor cells, or to remove diseased (cancerous) cells
Cancer	T cells, dendritic cells, or macrophages exposed to cancer-specific peptides to elicit an immune response
	Autologous or allogeneic cancer cells injected with a cytokine and irradiated to elicit an immune response
Pain	Cells secreting endorphins or catecholamines (encapsulated in a hollow fiber)
Diabetes	Encapsulated $\beta$ -islet cells secreting insulin in response to glucose levels
Wound healing	Sheet of autologous keratinocytes or allogeneic dermal fibroblasts on a biocompatible matrix
	Sheet of allogeneic keratinocytes layered on a sheet of dermal fibroblasts
Tissue repair	
Focal defects in knee cartilage	Autologous chondrocytes
Cartilage-derived structures	Autologous or allogeneic chondrocytes in a biocompatible matrix
Bone repair	Mesenchymal stem cells in a biocompatible matrix
Neurodegenerative diseases	Allogeneic or xenogeneic neuronal cells
Liver assist (temporary; for bridging until liver transplant or recovery)	Allogeneic or xenogeneic hepatocytes in an extracorporeal hollow fiber system
Infectious disease	Activated T cells

There are three sources of donor cells for cell therapy products: (1) the patient's own cells (autologous cell products), (2) the cells from another human being (allogeneic cell products), and (3) the cells derived from animals such as pigs, primates, or cows (xenogeneic cell products). Autologous cells are not rejected by the patient, but they are not available for many treatments because they are missing, dysfunctional, or diseased. In those situations allogeneic and xenogeneic cells can be used. The advantage of allogeneic cells is that they do not trigger a rejection reaction as strong as that caused by xenogeneic cells. Xenogeneic cells are used when human cells with the desired characteristics are not available or the supply of human donors is too limited. Cell therapy products are sometimes encapsulated in a device that prevents the patient's cells and antibodies from killing the xenogeneic cells. However, the use of xenogeneic cells has the potential to cause zoonoses in humans. Much research is focused on identifying and propagating stem cells, regardless of the source, because stem cells can be manipulated to differentiate either during manufacturing or after administration. Cell lines are preferable to freshly isolated cells because they can be tested extensively for viruses, tumorigenicity, and other features. They also ensure a constant and reproducible product by minimizing donor-to-donor variability.

Cell therapy products can be modified by treatment with DNA or another nucleic acid so that the pattern of gene expression is changed. This new product, a combination of gene therapy and cell therapy, is referred to as an *ex vivo* gene therapy product. Typically, cells are taken from the patient and modified outside of the body before they are returned to the patient.

Cell therapy products face several unique manufacturing challenges that are addressed in other sections of this chapter. First, cells cannot be terminally sterilized or filtered so removal or inactivation of microorganisms or viruses without killing the cells is problematic. Second, every raw material used in manufacturing has the potential of remaining associated with the cells. Qualification and sourcing of all raw materials is critical to producing a safe and effective product. Third, storage of the cell therapy products may present a challenge. Freezing is the main mode of long-term storage, and some cell therapy products cannot be frozen without changing their basic characteristics, especially those for differentiated functions. These types of products may have to be administered to patients within hours, or days at the most, after completion of the manufacturing process. Fourth, there is often an urgent clinical need to administer a product as soon as possible. Fifth, some products consist of a batch size equivalent to one dose, very often in a small volume. For these last three challenges, traditional analytical methods, especially those for sterility, mycoplasma, and potency, are not always applicable because these methods are not rapid or they are not amenable to small volumes. Even when these traditional methods are performed, the results are not available in time for products requiring rapid release. These products are often released on the basis of the results of new, very rapid or small-volume methods. Currently there are no compendial standards for such methods, although, as stated in the General Notices and 21 CFR 610.9, alternative methods to compendial tests are permissible, provided they are shown to be equivalent. As such new methodologies become properly validated, they will be included in the compendia.

#### Gene Therapy Products

Gene therapy products are products in which nucleic acids are used to modify the genetic material of cells. A retroviral vector used to deliver the gene for factor IX to cells of patients with hemophilia B is an example of a gene therapy product. Gene therapy products can be broadly classified on the basis of their delivery system. Means for delivering gene therapy products include viral vectors (viruses with the genes of interest but usually without the mechanism to self-replicate *in vivo*), nucleic acids in a simple formulation (naked DNA), or nucleic acids formulated with agents, such as liposomes, that enhance their ability to penetrate the cell. Some types of gene therapy block the expression of a gene by the administration of antisense oligonucleotides, which are complementary to a naturally occurring ribonucleic acid (RNA) and block its expression. Most of the initial clinical work has been done using viral vectors. The choice of a gene vector is complex (see Design Considerations for Gene Vectors under Manufacturing of Gene Therapy Products). The most common viruses used to date include murine retroviruses, human adenovirus, and human adeno-associated viruses (AAVs). Antisense-oligonucleotide products are in clinical development and on the market. Examples of gene therapy products are shown in [Table 2](#).

Table 2. Examples of Gene Therapy Products

Categories or Strategies	Indication: Administered Product
Gene replacement	
Short term	Cardiovascular disease: growth factor vector on a biocompatible scaffold <sup>1</sup>
Long term	Cystic fibrosis: transmembrane conductance regulatory vector
	Hemophilia: factor VIII or IX vector
Immunotherapy	Cancer or arthritis: autologous tumor cells or lymphocytes, respectively, transduced with cytokine genes
Conditionally lethal genes <sup>2</sup>	Cancer (solid tumor): thymidine kinase (TK) or cytosine deaminase (CD) vector into tumor cells
	Graft versus host disease (GVHD): TK or CD vector transduced into donor T cells
Antisense	Cancer: anti-oncogene vector
	Cytomegalovirus retinitis: antiviral vector
Ribozyme	Human immunodeficiency virus (HIV): antiviral ribozyme vector into autologous lymphocytes
Intrabodies	Cancer or HIV: single-chain antibody to a tumor protein or a viral protein, respectively

1. This product promotes formation of new blood vessels.

2. Cells with conditionally lethal genes as well as their neighboring cells are killed after the administration of a second drug *in vivo*. For TK, the drug is gancyclovir. For CD, the drug is



fluorcytosine.

Although manufacturing of vectors or nucleic acids can be analogous to that used for rDNA products or vaccines, there are some unique challenges. Analytical methodologies for vectors (see Analytical Methodologies) are still being developed. Methods for quantitating viral vector particles and determining the number of particles that are active (potent) are important areas that are rapidly evolving. Traditional assays for viral dose, such as the plaque assay or the tissue culture infectious dose assay, detect a fraction of the active vector particles. The precision of such assays is about a factor of three (half a log). Further, manufacture of large batches of viral vectors with no, or minimal amounts, of replication-competent viruses (RCVs) is challenging. Detecting a small number of RCV particles in the presence of large amounts of replication-defective vector is difficult. As in cell therapy products, sourcing of raw materials is critical. Removal of adventitious agents or other process contaminants from viral vectors can be impossible. Even defining purity is an issue for enveloped viral vectors, such as retroviruses or herpes viruses, as they incorporate cellular proteins in their envelope when they bud from the cell. This makes it difficult to determine if contaminating extracellular cellular proteins have been adequately removed.

For gene therapy vectors administered directly to patients, there are safety concerns related to the fate of their nucleic acids. For example, alteration of germ-line DNA is undesirable. Integration of gene therapy products into somatic cell DNA carries a theoretical risk of insertional mutation, which could result in modified gene expression and deregulation of the cell. For viral gene therapies, patients may need to be monitored for the presence of RCV. To address the risks associated with specific products, preclinical studies, quality control (QC) testing, and patient monitoring strategies need to be developed in accordance with the applicable regulations and guidance documents. The methodologies used to support these activities, including polymerase chain reaction (PCR) methods, are amenable to compendial standardization.

#### Chapter Purpose and Organization

Clinical uses for cell and gene therapy products, their manufacturing processes, and analytical schemes for determining identity, dose, potency, purity, and safety are rapidly evolving and are as diverse as the products themselves. This informational chapter summarizes the issues and best current practices in the manufacturing, testing, and administration of cell and gene therapy products. Usually informational chapters focus on materials that are already commercially available. This chapter, however, not only discusses products for commercial applications, but it also addresses the production of clinical trial materials and the other unregulated uses, such as bone marrow transplantation. When different approaches can be used for clinical trial material as compared to those used for commercial product, it is so stated.

The traditional compendial perspective is to develop public standards that can be applied to a particular final product without expressly defining production details. Efforts have been made in this chapter to specify when traditional methodologies or standards can be adapted. Novel methodologies applicable to cell and gene therapies are also highlighted. As these new methodologies become properly validated, they will be included in subsequent publications.

This chapter is extensive because of the diverse nature of the products and the special considerations that they require. Manufacturing has been divided into three sections. The first section, Manufacturing Overview, discusses general aspects of manufacturing and process development. The other two manufacturing sections are Manufacturing of Cell Therapy Products and Manufacturing of Gene Therapy Products. The latter section includes a subsection on designing gene vectors. On-Site Preparation and Administration follows the manufacturing sections because the handling of these products at the clinic often requires facilities and expertise not found in a typical hospital. Storage, shipping, and labeling issues are addressed under Storage and Shipping and under Labeling. Regulations, Standards, and New Methodologies summarizes existing guidelines and highlights the need for the development and validation of new methodologies to assess product quality. The final sections of this chapter, Definition of Terms and Abbreviations, list and define the terms and abbreviations referred to in this chapter and those commonly employed in this field.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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## MANUFACTURING OVERVIEW

### Introduction

The manufacturing of cell and gene therapy products has been divided into three sections. This section, Manufacturing Overview, discusses five topics that apply to manufacturing of all cell and gene therapy products: (1) raw materials, (2) characterization of banked materials, (3) in-process controls, (4) specifications, and (5) validation considerations. Manufacturing of Cell Therapy Products addresses the manufacturing of cell therapy products including cell products into which genetic material has been introduced. Manufacturing of Gene Therapy Products addresses the manufacturing of gene therapy vectors, both viral and nonviral, discussing the design of gene vectors in detail.

All the general principles of current good manufacturing practice (CGMP) as outlined by the FDA in 21 CFR 210, 21 CFR 211, 21 CFR 600s (especially 21 CFR 610), 21 CFR 820, and discussed in USP informational chapters, such as [Biotechnology-Derived Articles](#) (1045), apply to the manufacturing of cell and gene therapy products. The manufacturing facility, equipment and process, raw materials, quality systems, and trained personnel are some of the key elements of CGMP. CGMPs apply throughout the clinical development to both the manufacturing process and facility. The extent of control increases as clinical development progresses, with full CGMP compliance expected by initiation of Phase III of the pivotal clinical trial(s). The facility should be carefully designed, built, and validated to accommodate the unique features of the product's manufacturing process. Equipment should be robust, provide consistent product, and allow periodic calibration. Critical equipment, such as incubators and freezers, needs to be fitted with alarm systems that can remotely signal failure. Quality control (QC) and quality assurance (QA) programs should exert control over the manufacturing facilities, the manufacturing process, the validation efforts, and all testing of the raw materials, in-process material, bulk product, and final formulated product. Training and certification programs are central to maintaining a technically competent manufacturing staff. A documentation program should be implemented to support all manufacturing, training, and quality operations. Changes to processes and procedures should follow a formal program based on well-established CGMP and ISO change control principles.

### Raw Materials

#### types of raw materials

A wide variety of raw materials may be used in manufacturing. They may include relatively simple materials or complex substances, such as cells, tissues, biological fluids, polymeric matrices, mechanical supports, hydrogels, culture media, buffers, growth factors, cytokines, cultivation and processing components, monoclonal antibodies, and cell separation devices. These materials may remain in the final therapeutic product as active substances or as excipients. They may also be used in the manufacturing process as ancillary products. Ancillary products are components or substances that exert an effect on a therapeutic substance (for example, a cytokine may activate a population of cells). However, the mode of action of the ancillary product is limited to the interaction with the therapeutic entity, and the ancillary product is not intended to be present in the final therapeutic product. "Feeder cells," which are used to provide nutrients or growth factors for the product cell, are an example of an ancillary product. The quality of raw materials used in the production of a cell or gene therapy product can affect the safety, potency, and purity of the product. Therefore, qualification of raw materials is necessary to ensure the consistency and quality of all cell and gene therapy products.

#### qualification

It is the responsibility of the manufacturer of the product to ensure that all raw materials used in manufacturing are appropriately qualified. Qualification is the process of acquiring and evaluating data to establish the source, identity, purity, biological safety, and overall suitability of a specific raw material so as to ensure the quality of all raw materials used in the manufacturing process. The broad natures of the cell and gene therapy products and of the materials used to produce these products make it difficult to recommend specific tests or protocols for a qualification program. Therefore, rational and scientifically sound programs must be developed for each raw material.

Activities involved with raw material qualification will change as products move through various stages from clinical trials to licensure and commercialization. A well-designed qualification program becomes more comprehensive as product development progresses. In the early stages of product development, safety concerns are a focus in a raw material



lification plan. In the later stages, raw material qualification activities should be completely developed and should comply with CGMP. Ultimately, each raw material employed in the manufacture of a cell or gene therapy product should be produced under conditions that are in compliance with CGMP. On rare occasions, complex or unique substances that have been shown to be essential for process control or production may not be available from suppliers that produce them in compliance with CGMP. In these situations, the cell or gene therapy product manufacturer will have to develop a scientifically sound strategy for qualifying the raw material.

A qualification program for raw materials used in cell and gene therapy manufacturing should address each of the following areas: (1) identification and selection, (2) suitability for use in manufacturing, (3) characterization, (4) fetal bovine serum, and (5) quality assurance.

**Identification and Selection**— In the early stages of product development, important decisions regarding the types of raw materials to be employed in the manufacture must be made. As manufacturing progresses and products mature, certain materials that are deemed necessary at this point may turn out to be impossible or prohibitively expensive to qualify. Attention must be paid to issues such as suitability, toxicity, availability, consistency, contamination, and traceability. Raw materials that could be difficult to qualify may have to be investigated and identified in the early stages of product development.

Every material employed in the manufacturing process should be accounted for. The source and intended use for each material should be established, and the necessary quantity or concentration of each material used should be determined. Primary sources, and when possible secondary sources, for each material should be identified. In all cases, suppliers should provide information regarding the traceability of each material, especially for human- and animal-derived raw materials. For instance, human serum albumin and processed allogeneic non-A,B human serum require donor infectious disease status information prior to use, and a material such as fetal bovine serum (FBS) requires herd qualification and country of origin certification before being used in a manufacturing process (see Fetal Bovine Serum).

**Suitability**— An assessment of the suitability of each raw material used in manufacturing is necessary in order to ascertain the risk that the raw material may pose to the safety, potency, and purity of the final therapeutic product. Knowing the source and the processes employed in the manufacture of each raw material will help determine the relative level of risk for each item. The quantity of the material and its point of introduction in the manufacturing process also affect the risk profile of a raw material. Materials that may have toxic effects or raise biological safety concerns receive special attention. Such materials should be subjected to extensive testing prior to use or should be monitored in the final product. Validation studies that demonstrate that such materials are effectively and consistently removed or rendered inactive in the course of manufacturing will also be necessary for eventual licensure of each product. The biocompatibility of natural or synthetic biomaterials used in cell therapy products may be assessed by subjecting the material to the testing protocols outlined in the FDA's Blue Book Memorandum (May 1, 1995), which is a modification of the ISO document 10993-1:1997 entitled "Biological Evaluation of Medical Devices—Part 1: Evaluation and Testing". USP chapters [Biological Reactivity Tests, In Vitro](#) (87) and [Biological Reactivity Tests, In Vivo](#) (88) should also be consulted.

**Characterization**— Once the suitability of each raw material employed in manufacture has been assessed, specific QC characterization tests need to be developed or implemented for each material. The test panel for each raw material should assess a variety of quality attributes, including identity, purity, functionality, freedom from adventitious or microbial contaminants, and suitability for intended use. The level of testing for each component is a product of its risk assessment profile and the knowledge gained about each component during development. Test specifications should be developed for each raw material to ensure consistency and performance of the manufacturing process. Acceptance criteria should be established and justified on the basis of the data obtained from lots used in preclinical and early clinical studies, lots used for demonstration of manufacturing consistency, and relevant development data, such as those arising from analytical procedures and stability studies.

Many raw materials, such as cells isolated from blood, serum-derived fluids and extracts, or growth factors, are biological in nature; therefore, they are highly complex and difficult to fully characterize. Tests for sterility, pyrogenicity, mycoplasma, and adventitious or infectious viral agents, including RCV, should be performed on these materials. The specific adventitious viral agent test panel is dependent on the source of the component and how that component is manufactured or prepared. Because these materials exert their effects through complex biological activities—and biochemical testing may not be predictive of process performance—functional or performance testing may be necessary. Performance variability of such materials may have a detrimental impact on the eventual potency of the final therapeutic product. Examples of complex functionality testing for raw materials are growth-promotion testing of individual lots of FBS on the cell line used in manufacturing, performance testing of digestive enzyme preparations, and *in vitro* tissue culture cytotoxicity assays.

**Fetal Bovine Serum**— One commonly employed animal-derived material in manufacturing is fetal bovine serum (FBS). FBS is often added to the cultivation medium to promote cellular proliferation of a wide variety of cell types, including cell cultures that are derived from primary tissue explants and biopsy specimens. Growth factors, hormones, and other nutritive components present in FBS, many of which are undefined or present in trace quantities, provide the necessary components required by many cells to survive and undergo cellular division *in vitro*. The production of high-titer gene therapy vectors from cell lines can also require rich culture medium that includes FBS at levels between 10% and 15%. Defined, serum-free media have been developed for a number of cell types. Although some cell lines may be gradually adapted to serum-free or low-serum culture conditions, this may not be possible for certain fastidious cells, thereby necessitating FBS use.

Although FBS use may be required, development and assessment of serum-free or reduced-serum culture media should be considered. A number of bovine-derived bacteria, mycoplasma, and viruses are known to be associated with FBS. These organisms could potentially enter the process stream and contaminate the final product. The potential risk of BSE, the bovine form of TSE (transmissible spongiform encephalopathy), transmission with this material has been the subject of international discussion. Although FBS has been categorized as a low-risk material, adequate testing and sourcing of FBS lots must be managed appropriately in a qualification program. Reduction or elimination of FBS in the manufacturing process can reduce the risks associated with contamination by adventitious agents.

FBS must be obtained from herds that are monitored for specific diseases relevant in agricultural settings (for example, tuberculosis or brucellosis) and that are from regions known to be free from BSE. Each lot must meet established guidelines for sterility and endotoxin content as well as freedom from specific bovine viruses. To increase the level of safety assurance, consideration should be given to employing methods, such as irradiation or nanofiltration, that remove or inactivate viral entities known to be associated with FBS.

It should be noted that defined media formulations typically include components, such as albumin and transferrin, that are purified from animal or human plasma. The purification, processing, and extensive testing of such components further minimize, but do not eliminate, the risk of viral or microbial contamination. Apart from the risks associated with adventitious agents, residual FBS in the final product may trigger an immune response in patients. The level of residual FBS in the final product does not necessarily correlate with the starting amount of FBS, and it can depend upon the nature of the product and the purification process. Even if FBS is not included in the manufacture of the product, residual amounts of other components used in the manufacturing process, including recombinant proteins or other defined media components, may be potentially antigenic.

**Quality Assurance**— The components of this part of the qualification program are multifaceted and should reflect those found in a typical manufacturing environment for a pharmaceutical product produced in compliance with CGMP. These activities should include the following systems or programs: (1) incoming receipt, segregation, inspection, and release of materials prior to use in manufacturing, (2) vendor auditing and certification, (3) certificate of analysis verification testing, (4) formal procedures and policies for out-of-specification materials, (5) stability testing, and (6) archival sample storage.

#### Characterization of Cell and Virus Banks

**Cell Banks**— A cell bank is a collection of vials containing cells stored under defined conditions, with uniform composition, and obtained from pooled cells derived from a single cell clone. The cell bank system usually consists of a master cell bank (MCB) and a working cell bank (WCB), although more tiers are possible. The MCB is produced in accordance with CGMP and preferably obtained from a qualified repository source (source free from adventitious agents) whose history is known and documented. The WCB is produced or derived by expanding one or more vials of the MCB. The WCB, or MCB in early trials, becomes the source of cells for every batch produced for human use. Cell bank systems contribute greatly to consistency of production of clinical or licensed product batches, because the starting cell material is always the same. Mammalian and bacterial cell sources are used for establishing cell bank systems.

**Virus Banks**— The master virus bank (MVB) is similar to the MCB in that it is derived from a single production run and is uniform in composition. The working virus bank (WVB) is derived directly from the MVB. As with the cell banks, the focus of virus bank usage is to have a consistent source of virus, shown to be free of adventitious agents, for use in production of clinical or product batches. In keeping with CGMP guidelines, testing of the cell bank to be used for production of the virus banks, including quality assurance testing, should be completed prior to the use of this cell bank for production of virus banks.

**Qualification**— Cell and viral bank characterization is an important step toward obtaining a uniform final product with lot-to-lot consistency and freedom from adventitious agents. Testing to qualify the MCB or MVB is performed once and can be done on an aliquot of the banked material or on cell cultures derived from the cell bank. Specifications for qualification of the MCB or MVB should be established. It is important to document the MCB and MVB history, the methods and reagents used to produce the bank, and the storage conditions. All the raw materials required for production of the banks, namely, media, sera, trypsin, and the like, must also be tested for adventitious agents.



**Qualifying Master Cell Bank**— Testing to qualify the MCB includes the following: (1) testing to demonstrate freedom from adventitious agents and endogenous viruses and (2) identity testing. The testing for adventitious agents may include tests for nonhost microbes, mycoplasma, bacteriophage, and viruses. Freedom from adventitious viruses should be demonstrated using both *in vitro* and *in vivo* virus tests, and appropriate species-specific tests such as the mouse antibody production (MAP) test. Identity testing of the cell bank should establish the properties of the cells and the stability of these properties during manufacture. Cell banks should be characterized with respect to cellular isoenzyme expression and cellular phenotype and genotype, which could include expression of a gene insert or presence of a gene-transfer vector. Suitable techniques, including restriction endonuclease mapping or nucleic acid sequencing, should be used to analyze the cell bank for vector copy number and the physical state of the vector (vector integrity and integration). The cell bank should also be characterized for the quality and quantity of the gene product produced.

**Qualifying Master Virus Bank**— Testing of the MVB is similar to that of the MCB and should include testing for freedom from adventitious agents in general (such as, bacteria, fungi, mycoplasma, or viruses) and for organisms specific to the production cell line, including RCV. Identity testing of the MVB should establish the properties of the virus and the stability of these properties during manufacture.

**Qualifying Working Cell or Virus Bank**— Characterization of the WCB or WVB is generally less extensive, requiring the following: (1) testing for freedom from adventitious agents that may have been introduced from the culture medium, (2) testing for RCV, if relevant, (3) routine identity tests to check for cell line cross-contamination, and (4) demonstration that aliquots can consistently be used for final product production.

#### In-Process Control

Manufacturing processes should have well-defined go–no go decision point criteria that are applied to key in-process intermediates and are used to pool material that has been processed through a step in several sublots. Quality must be built into the product, rather than tested during batch release. In-process controls are the assays or tests that are performed to ensure that the in-process intermediate is of sufficient quality and quantity to ensure manufacture of a quality final product. Examples of in-process controls are listed in [Table 3](#). The main reason for performing the in-process control is to ensure that the correct product with anticipated quality and yield is obtained. Intermediate in-process material that fails to satisfy the in-process control criteria should not be used for further manufacturing. This material may be reprocessed if there are procedures in place for such activities. The reprocessed material must satisfy the original in-process specifications before it can undergo further manufacturing. If several sublots are to be pooled for further processing, sublots that fail to satisfy the criteria should not be included in the pool, even if the pool containing these failed sublots would pass the in-process assay criteria.

Table 3. Examples of In-Process Control Applications

Type of Product	Attribute to Control
Cell therapy	Quantity and viability of cells after a key processing step
	Cell phenotype after a culture step
Viral gene therapy	Quantity and viability of cells during bioreactor culture
	Quantity of virus after virus culture
	Specific activity of virus in fractions after column chromatography
	Quantity of host-cell DNA in fractions after column chromatography
Nonviral gene therapy	Optical density or change in oxygen consumption during culture
	Amount and form of plasmid prior to culture harvesting
	Amount and form of plasmid after extraction steps
	Amount of pyrogen or endotoxin after extraction steps in plasmid pool
Antisense-oligonucleotide therapy	Purity from strand side-products after key extension steps
	Quantity in fractions after chromatography

During clinical development, assays for product quality and yield should be performed after most processing steps to determine which steps are critical and which assays are most sensitive to deviations in the process. The information from these runs is also used to set the criteria for the selected assays. In-process controls are performed for fully validated processes to ensure that the process continues to be under control. The results of these assays should be trended and actions should be taken to correct problems as they arise.

#### Specifications

Specifications for cell and gene therapy products should be chosen to confirm the quality of the product by testing to ensure the safety and efficacy of the product. Selected tests should be product-specific and should have appropriate acceptance criteria established to ensure that the product exhibits consistent quality parameters within acceptable levels of biological variation, loss of activity, physicochemical changes, or degradation throughout the product's shelf life. The development and setting of specifications for cell and gene products should follow the principles outlined in the International Conference on Harmonization (ICH) guidance entitled "Q6B Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products".

Establishing specifications for a drug product is part of an overall manufacturing control strategy that includes control of raw materials, excipients, and cell and virus banks; in-process testing; process evaluation and validation; stability testing; and testing for consistency of lots. When combined, these elements provide assurance that the appropriate quality is maintained throughout the manufacture of the product.

Appropriate specifications are established on the basis of thorough characterization of the product during the development phase and an understanding of the process and its capability. Characterization should include measurements of the physicochemical properties, safety, purity, process and product-related impurities, potency, viability, sterility, and quantity. Specifications for each product should be developed from this information by applying appropriate statistical methods. The data should include lots used in preclinical and clinical studies and should also include assay and process validation data that can be correlated to safety and efficacy assessments. Specifications should allow for the inherent variabilities exhibited by the production process and by the assay. The traditional lot-release specifications that apply to biologics may have to be re-examined for these product types. For example, the general safety test stated in 21 CFR 610.11 is a lot-release requirement that has been deleted for cell therapies, because it exhibits little relevance for these products.

Specifications for the product are anchored by an appropriate reference standard for the product. The reference standard for the product ensures that the process, as measured by the release assays, does not change significantly over time. The reference standard is made from a lot that is produced under CGMP and passes all in-process and final release testing. In addition, this reference standard is subjected to an additional level of characterization that includes tests not normally performed for product release. The reference standard need not be stored at the same dose, formulation, or temperature as the product. However, the stability of this reference standard needs to be determined. The reference standard verifies that a test produces acceptable results (passes its system suitability tests). Alternatively, a specific assay standard (working standard) can be used. If so, in the test it should behave similarly to the reference standard. Changing to a new reference standard (lot) should include many tests, all of which are run side by side with the existing reference standard. The impact of any change in the properties of the new reference standard should be carefully evaluated before it is adopted. One option for a reference standard for a cell product with a short shelf life or for a patient-specific application can be a bank of normal donor cells of the appropriate cell type. This cell bank can be used to ensure that the manufacturing process is capable of making a consistent product.

Production of a safe and efficacious product involves establishing not only lot-release specifications but also specifications designed to maintain control of the manufacturing process and the final product. This includes in-process specifications (see In-Process Controls), raw material and excipient specifications (see Raw Materials), product-release specifications, and shelf-life specifications. Specifications should be established for acceptance of raw materials and excipients used in the final formulation of the product. In addition, tests should be performed at critical decision steps during manufacture or at points where data serve to confirm consistency of the process. In-process release specifications should be established for each control step. Heterogeneity can result from the manufacturing process or storage of the product. Therefore, the manufacturer should define the pattern of heterogeneity within the product and establish limits that will maintain the therapeutic efficacy and safety of the product.

In some cases, specifications may be established for lot release as well as for shelf life. As discussed in ICH guideline Q5C, presented under [Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products](#) (1049), the use of different specifications should be supported by sufficient data to demonstrate that the clinical performance



not affected. Acceptance criteria should be established and justified on the basis of data obtained from lots used in preclinical and clinical studies and lots used for demonstration, manufacturing consistency and on the basis of relevant development data, such as those arising from validated analytical procedures and stability studies. Acceptance criteria should also be correlated with safety and efficacy assessments.

Once specifications have been established, test results should be trended. Results that are out of specification (OOS), or even those that are out of trend, need to be investigated prior to dispositioning of the material. The purpose of an investigation is to determine the cause of the discordant result. The FDA's Draft Guidance for Industry: Investigating Out of Specification (OOS) Test Results for Pharmaceutical Production provides a systematic approach for conducting an investigation. An assay result can be rejected if it can be confirmed that an error, such as an analyst error, calculation error, or equipment failure, has taken place. If the investigation concludes that the product is not within the specification, the lot should be rejected. In unique situations, a product that does not meet all specifications may have to be administered to a patient. However, procedures must be in place to govern the communication of the OOS results to the physician or to the person responsible for making the decision to use the product and to provide instruction for any follow-up testing, patient monitoring, and communication of those results.

#### Considerations for Validation

The potential for wide biological variation in cell and gene therapy products, particularly for patient-specific treatments, affects the validation effort. Nevertheless, the basic principles of process validation for any biological product, including those recommended by the ICH and FDA guidance documents and recommended under [Validation of Compendial Procedures](#)

([1225](#)) and [Validation of Microbial Recovery from Pharmacopeial Articles](#) ([1227](#)), apply to the validation of most cell and gene therapy products. Guidelines for validating viral vaccines can be relevant to gene therapy processes that produce viral vectors. The hold steps in a manufacturing process should be validated to ensure that in-process intermediates are within specification and that the final product can be formulated successfully. Any assay used during the process validation must itself be validated before the process validation is commenced.

Process validation for patient-specific products, such as autologous cell therapy products or custom gene therapy products, presents some unique issues. First, the starting materials for patient-specific products typically arise from patient-derived materials, such as biopsy material or apheresis cell products. The process should be designed to accept a wide range in the quality and quantity of starting material. Sometimes use of alternative procedures with additional steps are required when the starting material is of poor quality or below specified amounts. Validation should confirm that these alternative procedures still result in a final product that satisfies release specifications. Procedures should also be in place to deal with receipt of substantially more of the starting material than normally expected. Such procedures should address the disposition of the extra material. Second, manual processing of cells and tissues will exhibit a degree of inherent variability. It is essential to develop processing steps that will successfully and consistently result in appropriate process components and final product, even if the process is confronted with nonstandard or variable tissue materials, such as a T-cell suspension contaminated with red blood cells or low-weight biopsy material. Process validation should take this variability into consideration and ensure that critical manufacturing and testing endpoints consistently meet specifications. The process validation shows that the procedures can produce a product free of microbial contamination. It should also show that there is no cross-contamination among different patient product lots. If possible, the process should be validated for virus clearance as discussed in ICH Q5A: Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin. If this is not possible, cells used for production of the product should be evaluated for their ability to propagate viruses that are known to contaminate these cells or source materials. This should include raw materials used as ancillary products.

As a result of the variability discussed above, the consistency and the robustness of the manufacturing process need to be assessed by testing more than three lots. It is not expected that every manufacturing effort will be successful for patient-specific therapies. However, the success rate should be established and tracked so as to discover any decrease in that rate and to take actions to correct the problem. Well-characterized banked primary cells may be used in the validation of the process if the donors have a range of profiles expected for the patient population to which the therapy will ultimately be directed. Trending of a number of statistically acceptable product administrations can also be appropriate.

### MANUFACTURING OF CELL THERAPY PRODUCTS

#### Introduction

Cell processing for cell therapy applications is a unique form of biologics manufacturing that relies on maintenance of stringent work practices designed to ensure product consistency and prevent contamination by microorganisms or by another patient's cells. Hallmarks of this unique form of manufacturing can include products with limited shelf lives, the need for rigorous control during manual processing steps, a manufacturing environment in which many product lots are simultaneously processed and assembled, raw materials that may or may not be part of the final product, and numerous pieces of processing equipment. By its very nature, cell processing requires a number of operations and manipulations by individuals well trained in aseptic processing techniques. The technical competence of the personnel is particularly crucial to product safety and efficacy with this form of manufacturing. Procedures involving lot segregation, line clearance, and operational discipline must be developed to decrease the chance of mix-up of patient-specific lots.

The degree of control required for cell processing operations is highly dependent upon a number of factors, including the complexity of an aseptic manufacturing process, the primary site of manufacturing, and the mode of administration of the cell product to the patient. Manufacturing processes that involve open manipulation of the cells even in a biological safety cabinet are at greater risk of contamination than the processes done in closed bioreactors or intravenous transfer bag systems that use sterile connection devices and tube-sealing devices. Clean rooms and biological safety cabinets are essential components for processes that involve open manipulations or for patient-specific products. The controlled environment of a carefully designed, constructed, validated, and maintained clean room will minimize the risks of environmental contamination during aseptic processing and decrease the possibility of cross-contamination of patient-specific products. Processes that utilize closed systems do not require clean room environments.

#### Procurement of Source Material

A variety of human- and animal-derived tissues, which can also include whole organs, serve as sources of cells for cell therapy products. Examples include skin, muscle, cartilage, bone, neural tissue, bone marrow, blood vessels, parenchymal cells from organs such as the liver, pancreas, and adrenal glands, and stem cells from adult and fetal tissues. A few general principles in the sourcing of these tissues are as follows: (1) systems must be developed so as to allow the material to be traced back to the donor; (2) steps must be taken to prevent the transmission of an infectious disease from the donor to the recipient; and (3) adherence to aseptic procedures during procurement and initial processing are necessary to ensure the safety of the final product because terminal sterilization of cells is not possible.

#### human tissue

Human-derived tissues may be sourced from normal healthy donors, cadaveric donors, or diseased patients, such as those with cancer. Applicable guidelines and standards for the procurement of human tissue are available from the American Association of Tissue Banks (AATB) and the FDA. Additionally, the federal policy in 45 CFR Part 46 is applicable to all federal or federally supported research. This policy requires that a certified institutional review board review and approve use of any tissue taken from a live human donor. The policy also includes special considerations for research on prisoners, children, and pregnant women or research in other areas involving gestational tissue. In all cases, appropriate written consent must be obtained from the donor or the donor's next of kin, describing which tissue is being procured and for what use it is intended. The donor must meet established guidelines for donor suitability and be tested for the infectious diseases listed in [Table 4](#). The medical history of the donor must be reviewed to ensure the absence of signs and symptoms of these diseases and to rule out issues and behaviors that increase the risk of exposure to such diseases.

Human tissue should be obtained under environmental conditions and controls that provide a high degree of assurance for aseptic recovery. Standard hospital operating room practices are applicable for tissues requiring dissection and surgical procurement. The air quality provided in a typical limited-access operating room is adequate for such procedures. Procurement personnel must be appropriately trained in all aspects of tissue recovery, such as surgical scrubbing, gowning, operating room behavior, anatomy, surgical site preparation, and antisepsis. Special care is required when tissue or organ procurement requires extensive manipulation of the bowel and when sharp dissection may result in the inadvertent puncture of the bowel. Tissue that contains microbial flora (for instance, skin) at the time of procurement can be adequately disinfected by using antimicrobial or bactericidal agents and extensive scrubbing.

Table 4. Infectious Disease Testing for Human Cells and Tissues Used in Cell Therapy Products

Cell Type	Testing:					
	HIV 1, 2	Hepatitis C	Hepatitis B	HTLV	Cytomegalovirus	Treponema pallidum
Autologous stem cells	R	R	R	R		



ther autologous tissue	R	R	R			
Allogeneic stem cells from family-related donors	X	X	X	X	X	X
Other allogeneic tissue	X	X	X	X	X	X

X—required

R—recommended; the labeling stating “tested negative” or “not tested for biohazards” may be required

\* For autologous or allogeneic cord blood donors or fetal tissue, a mother’s sample may be used for testing.

#### human blood and bone marrow

Hematopoietic progenitor cells represent one of the most extensively used cell sources in the field of human transplantation. These cells can be collected from the bone marrow, peripheral blood, placental umbilical cord blood, or fetal liver. The source of cells is somewhat dependent upon the patient, the disease, and the clinical protocol. Regardless of the cell source, methods for processing the cells are similar.

Human-derived blood cells and bone marrow cells may be sourced from normal, healthy donors or patients with hematological disorders. Applicable guidelines and standards for the collection and processing of these materials have been published by the American Association of Blood Banks (AABB), the Foundation for the Accreditation of Hematopoietic Cell Therapy, the National Marrow Donor Registry (NMDR), and the FDA. Similar issues regarding consent, infectious disease testing, and donor medical history apply in the sourcing of blood- or bone marrow-derived cells for allogeneic transplants. In cases where these cells will be subjected to selection, expansion, genetic manipulation, or other complex processing procedures, the testing outlined in [Table 4](#) should be followed.

Bone marrow for clinical use is harvested predominantly by percutaneous needle aspiration of the anterior or posterior iliac crests or the sternum. Standard hospital operating room practices are employed by specially trained personnel. Plastic syringes and commercially available aspiration needles are used to draw 3- to 5-mL volumes of marrow from each site of penetration. The material is transferred to a sterile, balanced salt solution or tissue culture medium containing sufficient anticoagulant, such as heparin, to prevent clotting. Removal of bone spicules may be accomplished by passing the material through stainless steel mesh screens or collection kits consisting of sterile, plastic collection bags with in-line filters having about a 200-µm porosity. The volume of marrow collected is dependent upon the body weights and other characteristics of both the donor and the recipient. The maximum volume to be harvested from a donor is about 10 to 15 mL per kg of body weight.

Circulating hematopoietic, peripheral blood progenitor cells (PBPCs) comprise a small population of peripheral blood mononuclear cells that can be utilized in place of or in addition to bone marrow. PBPCs are collected by apheresis, a procedure by which donor blood is withdrawn from a vein and separated ex vivo into some or all of its component parts. One or more of the components are retained as the harvest and the remaining parts are returned to the donor. Conditioning of the donor may enrich the number of circulating PBPCs in the harvest. Examples of such conditioning include collection during recovery from myelosuppressive chemotherapy and administration of hematopoietic growth factors, such as granulocyte colony-stimulating factor (G-CSF) and granulocyte–macrophage colony-stimulating factor (GM-CSF), or steroids. Collections are also improved by increasing the frequency or volume of apheresis. Apheresis requires one or two large-bore peripheral venous catheters in the upper extremities or a single large-bore, thick-walled, central venous double or triple lumen catheter (Mahurkur type). Two types of apheresis technology are available: the discontinuous-flow cell separators (Haemonetics) and the continuous-flow systems (COBE or Fenwall). Anticoagulation for normal to high flow rates is with a citrate-based material. In a closed system, the risk of contamination is low. The procedure is generally performed by trained, dedicated staff in a blood bank or in a donor center associated with a blood bank.

Placental and umbilical cord blood provides a third source of hematopoietic progenitor cells. Compared to bone marrow and PBPCs, the stem cells of placental and umbilical cord blood have a higher proliferative and self-renewal capacity. Volume of collection and thus cell number are limited and depend upon timing and the presence of a dedicated team of personnel. Collections are made during the third stage of labor. Typically, a closed method of collection is employed and involves cannulation or puncture of the umbilical vein with subsequent collection into plastic syringes or blood collection bags containing citrate-based anticoagulant. The procedure is performed in a controlled-access room away from the site of birth. Cellular content of the collection includes large numbers of erythrocytes, leukocytes, platelets, and target mononuclear cells. An open collection technique, which involves drainage of the blood by gravity from the cut end of the cord into sterile tubes containing anticoagulant, does not afford the same aseptic assurance level as the above-mentioned technique.

A major area of concern with the use of placental and umbilical cord blood relates to potential risks of unknown genetic disorders that may be transmitted to the recipient. Donor suitability is established by the usual infectious disease screening of the mother and the completion of a medical questionnaire. The donation remains anonymous and without any long-term follow-up of the child.

#### animal tissue

The major area of concern with the use of animal tissue relates to the known and unknown risks of potential infectious disease transmission to humans, and as such, the transplantation of animal cells raises unique public health concerns. Introduction of xenogeneic infectious agents into and propagation through the general human population is a risk that must be addressed. Draft Public Health Service (PHS) Guideline on Infectious Disease Issues in Xenotransplantation (August 1996), and any other related regulatory documents that are generated as this field advances, must be consulted when developing xenotransplant cell therapy products. Developers of such products should understand that the product recipients will be subjected to a high level of scrutiny (for instance, clinical and laboratory surveillance or registry in xenotransplantation databases) because of the above-mentioned public health concerns.

The use of animal tissue in the manufacture of cell therapy products requires that the tissue be sourced in a controlled and documented manner and from animals bred and raised in captivity in countries or geographic regions that have appropriate national health status, disease prevention, and control systems. In addition, the care and use of animals should be approved by a certified institutional animal care and use committee. Donor animals must have documented lineage, be obtained from closed herds or colonies, and be under health maintenance and monitoring programs. The facility for housing these animals should be USDA certified (large vertebrate animals) or Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) certified (small vertebrate animals) and should meet the recommendations stated in the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996), which can be obtained from the AAALAC. Such facility should be staffed with veterinarians and other trained personnel who will ensure animal health and disease prevention. The procedures employed in the facility should be documented and records should be kept. Health maintenance and monitoring programs are based on standard veterinary care for the species and include physical examinations, monitoring, laboratory diagnostic tests, and vaccinations. Use of a stepwise batch or all-in-all-out method of movement of source animal through the facility, rather than the continuous replacement movement, is recommended. It allows the decontamination of the facility prior to the introduction of the new set of animals, thereby reducing the chance of disease transmission. Feed components should be documented and should exclude, whenever possible, recycled or rendered materials that may have been associated with the transmission of prior-associated diseases.

To provide a high degree of assurance of product safety, screening of donors and of tissues derived from these donors should be performed at several stages throughout the process to rule out the presence of microbial agents. These control tests should utilize assays that are sufficiently sensitive and specific to detect bacteria, mycoplasma, fungi, or viruses of interest. Donor animals can be screened for certain diseases prior to donation of tissue by applying a variety of serological monitoring tests. Tissues can be subjected to a panel of tests including, but not limited to, the following:

1. test for sterility;
2. test for mycoplasma;
3. test for cultivable viruses in vitro;
4. test for unknown viruses by inoculation of various laboratory animals;
5. tests for xenotropic endogenous retroviruses and other animal retroviruses by in vitro cocultivation techniques, biochemical methods (for instance, to detect viral reverse transcriptase), and molecular biology assays (such as PCR assay for viral genomic sequence detection); and
6. direct detection or observation methods such as electron microscopy, detection of specific viral antigens by fluorescent antibody microscopy, or enzyme immunoassay methods.

Most of these tests are addressed under Analytical Methodologies or under [Biotechnology-Derived Articles](#) [1045](#) and [Viral Safety Evaluation of Biotechnology Products Derived](#)



from [Cell Lines of Human or Animal Origin](#) (1050). Post-tissue retrieval necropsies, sentinel animal programs, and archival storage of donor organs, tissues, blood, and other specimens are additional components of the overall program to ensure the safety of animal tissue for use in cellular therapeutic applications.

Most of the same aseptic procurement issues apply to animal tissue and to human tissue. Again, the tissue should be obtained under environmental conditions and controls that provide a high degree of assurance of aseptic recovery. Specifically designed procurement facilities, usually closely associated with the animal holding facility, are typically employed.

These facilities have specific attributes and design features that may not be available or applicable in the hospital operating-room setting. Such features include the following: (1) staging of various events, such as shaving, sedation, and operating-room preparation, in different rooms that are often separated with air locks for environmental control; (2) high-efficiency particulate air (HEPA) filtration; (3) adjacent but separate facilities for further tissue processing; and (4) dedicated areas for carcass removal. The issues regarding the training of personnel, bowel manipulation and puncture, and disinfection that are applicable to human tissues apply to the surgical procurement of tissue from animals as well (see [Human Tissue](#)).

#### Cell Isolation and Selection

##### general considerations

The general principles for processing human and animal tissues following aseptic procurement are independent of the tissue source. The manufacture of cell products may occur at a clinical site or at a central cell-processing facility. Sites involved in cell processing should ensure reproducibility and safety of the manufactured products through appropriate QC and QA programs.

Regardless of the location, processing should occur in a dedicated area physically separated from the site of procurement. To the greatest extent possible, the facility design and processing procedures should be consistent with those provided by the FDA's Guidelines on Sterile Drug Products Produced by Aseptic Processing (June 1987), or provided under [Pharmaceutical Compounding—Sterile Preparations](#) (797) for processes involving open manipulation. Generally, this requires that properly trained and outfitted processing staff handle blood or tissue samples in a critical zone supplied with class 100 HEPA-filtered air, which is provided by a biological safety cabinet located in a controlled clean room supplied with class 10,000 HEPA-filtered air. The facility and processing areas should be monitored for air quality in a manner that provides a high level of process asepsis. For guidance in this area, see [Microbiological Evaluation of Clean Rooms and Other Controlled Environments](#) (1116). The material should be packaged in sterile, leak-proof containers and transported from the procurement area to the processing area under controlled conditions that maintain cell viability. The fluid medium in which the specimens are bathed during transportation should be optimized to maintain cell and tissue viability. This transport medium can be supplemented with antibiotics. If so, the antibiotic levels in process buffers are decreased and eventually eliminated during subsequent processing steps, so that antibiotics are not present in the final cellular product. In the case of blood products or tissues containing substantial amounts of blood, the transport media or buffered electrolyte solution should contain an anticoagulant such as heparin or a citrate-based material.

##### isolation

Solid organs or tissues are usually dissected to expose a desired region. This material may be used as is for transplantation or it may be processed further. If multicellular organoids (for instance, islets of Langerhans) or single-cell suspensions are desired, the tissue may be subjected to mechanical or enzymatic disaggregation. Physical disaggregation may be accomplished through the use of instruments that impart high shear forces on the material (namely, to homogenize) or break the tissue into smaller pieces. Alternatively, the material can be pressed or passed through screens of defined mesh sizes.

Enzymatic digestion of the extracellular connective tissue, which holds cells together within the tissue, is another common method for dissociating solid tissue. Typically, the tissue is minced into small cubes, usually larger than 1 mm<sup>3</sup>, and incubated in a buffered solution containing a digestive enzyme. Alternatively, the intact organ is infused with a solution to rinse the blood from the tissue followed by the enzymatic solution that aids the digestion. Various enzymes are used to accomplish this. Examples include collagenase, trypsin, elastase, hyaluronidase, papain, and chymotrypsin. Enzymes with nuclease activity, such as deoxyribonuclease, may be added to digest nucleic acids released from damaged cells, preventing excessive cell clumping. At the end of the incubation process, the cell suspension may be subjected to a mild pumping action to further break up multicellular clusters into those of desired size or composition. Enzymatic and physical disaggregation methods are often combined to achieve the desired result.

Because cells isolated from blood and bone marrow products are inherently cell suspensions, mechanical manipulation is limited to plasma removal, which is accomplished by centrifugation and physical removal of clots that occurred during transport via 200-μm filtration.

##### selection

Cell suspensions at this stage may be transferred directly to culture vessels as described for Propagation under Cell Propagation and Differentiation, genetically manipulated as described under Introduction of Genetic Material into Cells, or formulated by various techniques as described under Formulation of Cell Therapy Products. Cell suspensions often consist of a mixture of cell types that may require further processing to isolate a cell population of interest or to decrease the level of an undesirable cell type such as potentially contaminating tumor cells. Various cell isolation and separation techniques exist that provide high yields of pure cell populations.

Each cell type typically possesses specific size and density; therefore, different cell types will sediment at different rates in a centrifugal field or at unit gravity. Cell populations can be selectively sedimented to yield pure fractions by varying the centrifugation forces and the duration of centrifugation. Separation can also be achieved by isopycnic centrifugation, where the cell suspension is centrifuged in a gradient medium that encompasses all of the densities of cells in the sample. In this procedure, the various cell populations sediment to an equilibrium position at the gradient density equal to the density of the cell population. Specifically designed continuous-flow elutriation centrifuges separate cell populations by subjecting a cell suspension to opposite centrifugal and fluid stream forces in a special chamber within the centrifuge rotor mechanism. Cell populations separate within the rotor on the basis of their various sizes and densities, and they are selectively eluted out of the rotor chamber by increasing the fluid stream force. Finally, methods that do not require centrifugation but instead involve the addition of high-density agents, such as hydroxyethyl starch, to the cell suspension will result in cell separation. The mixture is allowed to settle in a tube at unit gravity, resulting in the separation of different cell types based on buoyant density. Concentration and separation procedures such as these frequently result in cell loss due to clumping and aggregation.

Cell separation can also be achieved by applying techniques that take advantage of unique cytological or biochemical characteristics of different cell populations. Soybean agglutinin binds to and agglutinates cells that bear a particular carbohydrate moiety expressed on mature blood cells, but not stem cells, allowing for purification of the stem cells. Lymphocytes possess the CD2 antigen that acts as a receptor for sheep red blood cells. The lymphocytes form rosettes, which then can be separated via differential centrifugation.

Some applications take advantage of the ability of certain cell populations to adhere to the surface of specific solid substrates such as tissue culture plastic, collagen-coated materials, and natural and synthetic polymeric scaffolds. The specifically bound cell type is selectively recovered onto the surface and removed from the initial cell suspension. When placed under the appropriate culture conditions, these cells will multiply and eventually occupy the available surface or void volume of the substrate.

Monoclonal antibodies directed against specific cell surface antigens or receptors can be used for both positive and negative cell selection. For example, a monoclonal antibody-labeled cell population can be removed from the cell suspension immunomagnetically, after exposure to magnetic particles coated with antimonoclonal antibody. The magnetic particles and their bound cells are removed from the cell suspension magnetically. Cells are released from the complex following incubation with reagents, such as specific peptides, that dissociate the monoclonal antibody from the cell. Unlabeled cell suspensions can be poured over or incubated on surfaces such as plastic flasks or microspheres coated with monoclonal antibodies as a means of isolating particular cell populations. In addition, a fluorescence-activated cell sorter (FACS) can be used to separate different cell types by binding antibodies tagged with fluorescent markers to a particular cell type.

Various other techniques purify particular cell populations by destroying unwanted cells present in the mixture. For example, certain cell-bound monoclonal antibodies are able to fix and activate complement, which is added to the cell suspension, resulting in lysis of the cell. Some procedures use cytotoxic agents or mitotic inhibitors to selectively impede or kill unwanted cells in a cell product. These methods typically target an unwanted cell subpopulation with a high growth rate, such as tumor cells. Finally, an antibody can be conjugated to a toxic moiety, such as ricin, allowing delivery of the cytotoxic agent to the targeted cell population. Most of these procedures require several washing steps after the exposure of the cells to the cytotoxic agents to ensure the removal of the dead cells, cell fragments, and cytotoxic agents from the final cell product.

#### Cell Propagation and Differentiation

##### propagation

A key issue for cell therapy products is the ability to manufacture and deliver a therapeutically relevant dose of the required cell population to the patient. Depending on the application, the product may be a pure, homogeneous cell type or it may be a mixture of different functional cell types. Many target cell populations are present at low level or low purity in complex



primary source tissues. In such cases, production of a therapeutic dose may be achieved only by specific enrichment and propagation of the required cells.

Propagation of cells may occur in suspension culture (for example, T cells or hematopoietic stem and progenitor cells), adherent culture (for example, mesenchymal stem cells, embryonic stem cells, neuronal stem cells, or dermal fibroblasts), or a mixture of both (for example, bone marrow stroma expansion). Numerous devices of varying degrees of sophistication and automation exist for cell culture.

In the simplest iteration, cells can be propagated in tissue culture flasks (T flasks), roller bottles, on polymeric scaffolds, or nonrigid, gas-permeable bags inside regular incubator units that are controlled for temperature, humidity, and gas composition. Multilayered plastic cell factories, cell cubes, and multi-bag systems have been developed that enable expansion, harvesting, and formulation to be carried out in a closed system.

Traditional small-scale fermenter units can be used for expansion of cells in suspension culture. It is also possible to expand adherent cells in such units either by providing a surface for attachment (coated beads or disks) or by adapting the cells to propagate in suspension culture. Some culture systems are specifically designed for the propagation of cells for therapeutic applications. These systems tend to be closed systems that use disposable bioreactor cartridges, such as those made of hollow fiber or molded plastic, in automated processing units with direct control of parameters such as temperature, gas composition, and media perfusion rate. These units can provide a completely automated, closed system for expansion and harvesting. In some cases the automated software is set up for patient–donor tracking and will document culture conditions and manipulations for the entire processing run. These features are useful in the design and implementation of QC product-release testing programs and for the QA documentation of processing runs.

In the case of adherent culture, the cells are usually released from the surface upon which they have expanded. Methods of release include physical agitation, enzymatic cleavage with enzymes such as porcine or bovine trypsin, collagenase, or dextranase, chelation of metal ions (for example, with edetate disodium), and competitive inhibition of adhesion or matrix molecules. As described above, consideration must be given to the source, safety, toxicology, and residual testing for any reagent used to release adherent cells during manufacturing.

Some product-specific systems that do not require the release of adherent cells have been developed. In these systems, the cells are expanded upon a synthetic or natural matrix that is then applied topically (for example, in dermal repair products) or the cells are grown inside or outside of fibers for ex vivo perfusion (for example, hepatocytes in hollow-fiber devices to treat liver disease). In these applications, the matrix and device composition must be biocompatible and, in some cases, biodegradable.

In all of the above systems, standard cell culture parameters must be optimized for maximum process efficiency. Such parameters include composition of cellular source material, initial seeding density, media composition, rate of media exchange, temperature, gas composition, and rate of delivery. Depending on the nature of the product, the potential effect of process parameters on the potency and function of the target cells should be defined.

In closed bioreactor systems, it can be difficult to observe or sample cells so as to determine and control the rate of proliferation and thereby the point of harvest. Measurement of traditional fermentation parameters, such as rate of nutrient usage or production of metabolic products, can provide a surrogate method, amenable to validation, with which to evaluate the rate of proliferation and predict when to harvest the cell product. The relationship of such parameters to the viability, potency, and function of the cell product should be well defined. Postexpansion purification and enrichment of target cells by using methods such as those described above may be required.

#### differentiation

Some cell therapies require lineage or functional differentiation of the source cells. For example, hematopoietic stem cell expansion processes normally result in products containing a mixture of multipotent stem cells, lineage-committed progenitor cells, and lineage-differentiated cells. The composition of these products can be manipulated by using different combinations of growth factors and cytokines during the expansion process. The inverse is true for processes in which mature cells are de-differentiated to enable them to then be recommitted to a lineage pathway (for example, chondrocytes in cartilage repair).

Specific examples of ex vivo manipulation are the programming of professional antigen-presenting cells, such as dendritic cells and monocytes or macrophages, and the production of antigen-specific T cells to target various specific disease indications. In these applications, the manipulated cells may be engineered to target and attack a specific tumor or tumor cell type, to induce a specific antibody or other cellular response, or to potentially vaccinate a patient. The processes for production of such products can involve one or more exposures of the relevant cells to disease-specific synthetic immunogens (for example, peptides) or natural immunogens (for example, dead tumor cells, viruses, cell membrane fractions, or purified natural molecules) before, after, or during culture expansion. Alternatively, the target cells may be genetically engineered with a specific gene product, such as an HIV-specific receptor. In some applications, relevant cells are cocultured with tumor cells, other diseased cells, or cells producing a transducible or transfecatable gene construct to generate a specifically targeted product.

Again, prior to delivery, the manipulated target cells may require further purification and enrichment by applying the methods described throughout this section. In the case of certain T-cell products, the desired antigen-specific cells can be cloned and then further expanded to provide the therapeutic dose.

#### Introduction of Genetic Material into Cells

A common extension of cell therapy involves the introduction of genetic material, usually DNA, into cells to alter their pattern of gene expression. For the purpose of this section, it is assumed that the nucleic acid is DNA. Similar scenarios can be applied to RNA or a derivative of DNA, except that the stability and solubility of the particular nucleic acid may dictate modifications of certain steps. This process is often referred to as ex vivo gene therapy, because the cells are removed from the patient or donor and the genetic material is introduced while the cells are outside of the body. Genetically modified cells are then administered to the patient. The genetic material introduced can either cause the expression of new genes and products or cause the inhibition of the expression of already expressed genes and products. The latter represents a type of antisense therapy. The genetic material can be introduced by the same range of reagents that are involved with gene therapy: viral vectors, nucleic acids in a simple formulation (naked DNA), or nucleic acids formulated with agents, such as liposomes, that enhance their ability to penetrate the cell. Most of the steps and considerations discussed above also apply to the ex vivo introduction of genetic material into cells. However, the main goal of ex vivo therapy is to develop robust processes that will work with the majority of patient's or donor's cells. This takes considerably more effort than processes for cell lines.

The method of introduction of new genetic material into cells depends on the biology of the system and the desired stability of gene expression. If a simple retroviral vector such as Molony murine leukemia virus is used for transduction, the cells must be actively dividing because vector DNA is only integrated into the cellular DNA during replication. This usually leads to long-lasting expression of the desired gene product. Adenoviral vectors, naked DNA, or formulated DNA can be introduced into nondividing cells. However, gene expression will be transient, because the introduced DNA will usually be extrachromosomal.

The main challenge is to achieve efficient transduction or transfection, introducing sufficient DNA into the cell before the DNA degrades. In the case of transduction by retroviral vectors, vectors derived from simple retroviruses, cells are stimulated with reagents that cycle them into the S phase (replication) at the time the vector is applied. Most retroviral vectors are stable in cell culture for a period up to a few hours. Because diffusion is minimal, only a small fraction of viral particles will come into contact with cells over this period. The following techniques can be used to increase the number of viral particles that contact the cell in a given time period:

1. maximization of viral particle concentration and minimization of the media volume during the transduction step
2. multiple applications of the virus
3. centrifugation of virus particles onto the cells
4. placing of cells on a filter and slow pulling of viral media through the filter
5. addition of binding-enhancing polymers to the media.

note—Coculturing of the target cells with the viral producer cells is not recommended. This technique increases the chance of a recombinant event occurring and of the production of RCV. Furthermore, any product for which coculturing is used to transduce the human cells would be considered a xenotransplant if the producer cells were not human. The second cell type, whether human or not, may cause inflammation.

Each of the above techniques has its own set of issues that must be addressed in order to develop a robust process. In technique 1, reduction of the volume during transduction results in rapid exhaustion of the medium; therefore, supplemental medium should be added within a few hours. In technique 2, the cells may no longer be in the correct cell cycle phase during later applications or cells may have become refractory because of unproductive transformation during the prior application. Techniques 3 and 4 can work well on a very small scale, but the number of cells that can be transduced may be insufficient to obtain an efficacious dose. In technique 5, polymers may fail to provide a benefit because virus-binding may involve specific receptors whose surface density may prove to be the limiting factor.



similar issues and techniques can apply with other viruses or DNA preparations. The issue of slow diffusion is even more marked for the use of DNA preparations. Factors such as the cell type in which the viral vector was produced, the media used for vector production, and the purity of the vector can have a dramatic effect on the efficiency of transduction.

While certain methods may not require cells to be actively cycling, in practice, most processes will require that cells be capable of replication because of the following considerations:

1. Safety considerations may dictate that only cells that express the new DNA are returned to the patient, which requires that these cells be selected. As described below, the most common selection method utilizes an antibiotic-resistant gene that is co-introduced with the new genetic material.
2. Further propagation may be required to achieve the therapeutic dose of cells.
3. Economic, biological, or technical reasons may dictate that the DNA introduction step be carried out at a low cell number and that the desired cell population then be expanded to the required dose.

Therefore, conditions that enable the cell or maintain the cell's ability to proliferate must be developed in almost all cases. The biology of the cells, the available technology, and the process economics will determine whether cells are propagated before, after, or during the introduction of new genetic material. Most processes do in fact expand the population after the introduction of the new gene.

Whether cells that do not productively express the gene can be administered to patients depends on the biology of the application, the dose required versus the handling capability of the manufacturing system, and most importantly, the toxicity of the nonproductive cell population. Selection of the genetically modified cell population is commonly carried out using an antibiotic-resistance marker gene, such as neomycin, which is co-introduced into the cell with the new genetic material. For neomycin selection, cells in culture are treated with the antibiotic G418 at a concentration and for a period that have been shown to kill cells with nonproductive expression, while allowing the productively expressing cells to proliferate. In this manner it is presumed that cells that are resistant to the antibiotic will also express the DNA of interest. The expression should be tested as a lot-release requirement or verified in a series of mock runs. Because most antibiotics decrease cellular proliferation, optimization of the culture media composition may be necessary for efficient selection and propagation of the gene-modified cells.

Following the antibiotic selection step, a second phase of antibiotic-free cell propagation may be required in order to achieve the desired dose and to rinse residual G418 out of the system. The selected medium and the total time that the cells are in culture can be critical to maintaining the desired expression of the original differentiated functions. An additional issue associated with the use of selection markers is that they generally are nonhuman genes. The expression of these genes usually elicits an immune response.

Process development is often carried out with cells from healthy donors. Consideration should be given to the fact that for very sick patients, it can be difficult to obtain healthy cells that can be stimulated to undergo efficient, sustained replication.

#### Formulation of Cell Therapy Products

##### suspensions

Formulations for cell therapy products depend upon the desired length of storage and whether the cells are administered as a suspension or in combination with a matrix. Regardless of the route of administration, cells that will be administered as a suspension can be frozen or not frozen. The most common formulation for cells that are cryopreserved is a 5% to 10% solution of dimethyl sulfoxide (DMSO), with or without hydroxyethyl starch (generally 6%), and a plasma protein, such as 4% to 10% human serum albumin, in a balanced salt solution.

DMSO prevents dehydration by altering the increased concentration of nonpenetrating extracellular solutions during ice formation at the time of freezing. The high molecular weight polymeric hydroxyethyl solution protects the cells from dehydration as water is incorporated into the extracellular ice crystals. The use of protein often results in maximum recovery and viability of cells after thawing. Serum (5% to 90%) has been used in place of specific proteins. Some cryopreservation formulations are completely free of protein. If the solution contains a buffer, the pH of the buffer should not be affected by changes in temperature. The optimal concentration of cells for cryopreservation depends on the cell type, but it generally ranges from 106 to 107 cells per mL. The purity of the cell population can also affect recovery. For instance, granulocytes can be damaged by the cryopreservative and the cell viability can decrease. These effects are dependent upon the concentration of cryopreservative. Both effects subject the patient to an increased level of infusion-related toxicity, although this is related to the volume administered and the final concentration of the cryopreservative.

Formulations for cell suspensions stored without freezing generally contain cell culture media, often without any protein. Because cells continue to metabolize their media even at the reduced temperatures used for storage, the medium supplies the amino acids and other nutrients that help in maintaining cell viability.

##### products combined with biocompatible matrices

Many cell therapy products are administered in combination with a biocompatible matrix. For instance, wound healing or skin substitute products contain cells seeded on a matrix. The biochemical and physical structure of the matrix and the method for combining cells with the matrix are specific to the application. Some common examples include the following:

1. Cells loaded into a semipermeable membrane device—Usually the pore size of the membrane is large enough to allow the cell-secreted therapeutic factors to pass, but it is small enough to stop immunoglobulins and host cells from making contact with, destroying, or having an immune response to the therapeutic cells. The device can be a single hollow fiber or a semipermeable capsule with cells inside that secrete therapeutic compounds, or it can be part of a larger system of pumps and filters, such as hollow-fiber modules with hepatocytes for the treatment of liver disease.
2. Cells seeded onto a three-dimensional matrix and allowed to propagate and form a tissue-like structure—In the resulting product, the cells are oriented in a unique manner that is important for the intended use of the product (for example, skin substitutes). In some cases, mechanical force has been used for proper cell orientation.
3. Cells encapsulated in a gel or cross-linkable polymer solution—The resulting implantable structure can serve as a culture vessel, as a means to shield the cells from the host's immune system, or as a way to mold cells into a defined shape. Some of the polymers used include alginate, hyaluronic acid, collagen, chitin, or synthetic polymers.
4. Cells adhered to matrices of defined shape that are then implanted—Some examples include osteogenic precursor cells on matrices of demineralized cadaveric human bone, ceramic hydroxyapatite, ceramic hydroxyapatite-tricalcium phosphate, or biodegradable glass, which can be used in the repair of bone defects.

When manufacturing such products, the primary consideration is the sourcing of a quality matrix material. The matrix material should be biocompatible, should not interfere with cell function, and should not trigger an immune response in the patient. If it is intended that the cells proliferate after loading onto or into the matrix, the matrix and the supporting culture system must allow exchange of nutrients and waste products. Cells may form tissue-like structures under favorable conditions and for those applications where this is required. A thick, impermeable matrix will lead to forming regions of necrotic tissue. Many of these devices are designed so that they can be removed from the patient after a certain period of time.

In all cases where cells are combined with biocompatible matrices, the use of closed systems for the manufacture and the delivery of product is preferable. As cell therapy products of this type can be quite intricate, the manufacturing details for such products are outside the scope of this chapter.

#### MANUFACTURING OF GENE THERAPY PRODUCTS

##### Introduction

The principles applicable to the production of pharmaceutical or biological products are also relevant to the production of gene therapy vectors for therapeutic use in humans. The same CGMP requirements can be applied to determine product consistency, process validation, raw material qualification, and compliance of the manufacturing facilities. Manufacturers will face development issues such as scalability, yield, cost efficiency, and product stability.

Most gene therapy vectors have been produced only in relatively small batches necessary to meet the needs of early clinical trials in small numbers of patients. However, areas of rapid progress are large-scale production of vectors, vector purification, and suitable analytical techniques. This section focuses on issues involved with designing vectors for gene therapy and choosing a production technology; it does not focus on specific production technologies.

##### Design Considerations for Gene Vectors

##### types of vectors



A typical gene therapy vector is composed of (1) the vector backbone, viral or plasmid, (2) a promoter, (3) the therapeutic gene of interest, including introns, and (4) a polyadenylation signal. Murine and human retroviruses, adenoviruses, parvoviruses such as adeno-associated virus (AAV), herpes viruses, poxviruses, toga viruses, nonviral plasmid therapy systems, and synthetic antisense-oligonucleotide therapy systems are being developed for gene therapy applications. The properties of these vectors (see Table 5) differ greatly in terms of their capacity to deliver genes to cells. Some viral vectors preferentially target dividing cells while others are capable of transducing both dividing and nondividing cells. There are significant variations in transgene capacity, meaning that there are limitations on the size of the foreign DNA fragment that can be incorporated into the recombinant genome. The ideal gene therapy vector has often been described as one capable of efficient transduction, targeted delivery, and controlled gene expression. The level, timing, and duration of gene expression required will depend on the clinical indication. Low-level, long-term gene expression is thought to be required for some diseases including adenosine deaminase (ADA) deficiency or type A and type B hemophilia. High-level, short-term expression may be more appropriate for cancer when genes that induce apoptosis are used, or for cardiovascular disease where preventing hyperproliferation of smooth-muscle cells may impede restenosis of saphenous vein grafts.

Table 5. Types of Gene Vectors

FAMILY	RETROVIRIDAE	ADENOVIRIDAE	VIRAL PARVOVIRIDAE	HERPES-VIRIDAE	TOGAVIRIDAE	POXVIRIDAE	NONVIRAL
Example Species	Murine Leukemia Virus	HIV	Adenovirus	AAV	Herpes Simplex Virus	Sindbis	Poxvirus (Vaccinia)
Vector Characteristics							
Insert size limit	8 kb	8 kb	4.3 to 34 kb	4 to 5 kb	40 to 150 kb	5 kb	25 to 50 kb
Chromosome integration	Yes	Yes	No; episomal	Can be integrated or episomal	Can be integrated or episomal	No	Yes, but at very low frequency
Therapeutic protein expression	Stable	Stable	Stable or transient	Stable	Stable or transient	Transient	Transient
Vector localization	Nucleus	Nucleus	Nucleus	Nucleus	Nucleus	Cytoplasm	Nucleus
Types of cells transduced	Dividing only	Dividing and quiescent	Dividing and quiescent	Dividing and quiescent	Dividing and quiescent	Dividing and quiescent	Dividing and quiescent
Efficiency of gene transfer	High	High	High	High	High	High	Low
Expression of viral proteins	No	No	Yes, unless viral genes deleted	No	Yes	Yes	No
Other	Tropism can be altered by pseudotyping					Can be used as a plasmid therapy system	

#### vector design criteria

Vectors are designed and selected for disease states on the basis of the following criteria:

1. capacity to accommodate the DNA for the therapeutic gene and its transcription cassette
2. host–vector interactions, both cellular and humoral
3. capacity to target intended cells
4. control of therapeutic gene expression
5. vector replication status
6. capacity for integration into chromosomes of target cells.

Selection of the route of administration and manipulation of the total dose of vector are strategies that can be used to compensate for some features of specific vector systems. The design and selection of a vector system include the evaluation of the disease of interest.

Additionally, there are advantages and disadvantages for the manufacture of each of the different vector systems. Production consistency favors those systems with well-defined fermentation or culture systems, such as plasmid, retroviral, or adenoviral vectors, or chemically defined systems, such as synthetic antisense-oligonucleotide systems. For those viral vector systems that require helper functions (see below), a rationally engineered cell line can overcome the scalability and consistency limitations of cotransfections. Engineered cell lines can also eliminate the possibility of replication-competent recombinant virus appearing in viral culture. Use of a cell line that is adapted to suspension culture can affect scalability and cost efficiency.

#### targeting transduction

To be effective, a vector must first find and transduce its target cell. Viruses have a natural host range that is strongly influenced by the expression levels of specific cell-surface receptors in target tissues, the cell cycle status of the target cells, and the route of administration. Integrins are a class of cell-adhesion receptors known to interact with either the penton base or the fiber protein of adenoviruses. The Coxsackie and adenovirus receptor (CAR) is also known to interact with adenoviruses. However, the expression levels of integrins and of CAR vary according to tissue type, affecting the transduction efficiency of adenoviral vectors. Amphotropic retroviruses infect cells via a sodium-dependent phosphate transporter molecule that is expressed at a detectable level in every human cell type.

The host and tissue range can be modified or targeted by a variety of approaches. Retroviruses, and lentiviruses, in particular, encode an envelope protein that mediates virus binding and entry via a specific host-cell receptor. Envelope proteins from one retrovirus may be interchanged with a protein from another retrovirus or a protein, such as the vesicular stomatitis virus glycoprotein from an entirely different virus. This process is referred to as pseudotyping. Viral protein coats may be modified in several ways. By engineering the fiber and knob of adenovirus, it is possible to change the intrinsic integrin specificity. Similarly, viral coat proteins can be chemically modified for ligand-mediated receptor targeting. It is feasible to create ligand–plasmid fusion molecules for receptor-mediated targeting of nonviral vectors. Some lipid formulations for nonviral vectors incorporate antibody Fab fragments or ligands to target plasmid delivery.

With respect to cell cycling, adenoviruses easily infect both quiescent and rapidly dividing cells, while murine leukemia virus-based retroviral vectors are efficient only when transducing rapidly dividing cells. Lentiviral vectors can infect quiescent cells, including cells of neuronal origin. In general, nonviral vectors have lower transduction efficiencies than viral vectors.

Transduction efficiencies of nonviral vectors are strongly influenced by the formulation used and route of administration.

#### impact of humoral immune system

Regardless of the route of administration, the intended target cell, and the dose, the vector is likely to encounter some component of the immune system as it moves toward the target cell. For viral vectors, the humoral (antibody-based) immune system cannot readily distinguish between wild-type viral infections and recombinant viral vectors because the humoral response is directed against proteins contained in the viral coat or package. Protein-containing formulations of nonviral vectors can also elicit a humoral immune response. Either specific or cross-reacting humoral responses may pre-exist or they may be elicited during dosing, and the antibody response may vary in its capacity to diminish gene transduction in individual patients. It is possible to compensate for the neutralizing activity of the antibodies by increasing the vector dose or by altering the dosing interval to coincide with periods of low antibody titer. Because neutralizing capacity is frequently enhanced upon multiple dosing, effective dosing by repeated administration may be problematic. This issue is generally avoided by the use of nonviral systems. Alternatively, viral vectors can be engineered to evade the immune system. For adenoviral vectors, one approach involves increasing expression of specific viral genes that allow the virus to evade the host's humoral response.



#### impact of cellular immune responses

Once protein expression is under way, cellular immune responses can lead to a rapid removal of both viral and nonviral vector-transduced cells from the body and a decrease in therapeutic effectiveness. Although protein synthesis is not required for cellular immune responses to viral vector envelope proteins, *de novo* synthesis of viral genes can exacerbate host-cellular responses. To reduce potential cellular responses, viral vectors have been designed with specific backbone deletions to eliminate the expression of viral structural genes.

Examples of such vectors include the E1- and E4-deleted adenoviruses, the adenoviruses and herpesviruses in which all viral genes have been deleted (gutless) or are helper dependent, and the recombinant adeno-associated viral vectors. Certain plasmid sequences especially those with the CpG motif, can elicit a strong cellular immune response.

#### antigenicity of gene product

The therapeutic gene product may also be antigenic. When proteins that are retained in the target cell are used, cellular responses may eliminate the target cell. In some cases this is the desired therapeutic effect, particularly in the antigen-based immunotherapy for cancer or a viral disease. However, if sustained protein expression is required, the cellular immune response may decrease the effectiveness of the therapy or eliminate it entirely. The antigenicity of the therapeutic gene may reflect a variety of experimental conditions. If a gene such as the cystic fibrosis transmembrane conductance regulator (CFTR) is truncated to fit within a chosen vector, this modification may result in creation of a distinct antigen. By using the gene that encodes thymidine kinase derived from the herpes simplex virus (HSV), a foreign protein is introduced into a human subject and thus it can function as an antigen. Any patient with a monogenic deficiency disorder is at risk for lack of tolerance to the normal protein that is defective or absent in the disease state (for example, dystrophin in Duchenne muscular dystrophy).

#### complement inactivation

Retroviral vectors are also subject to another host-defense mechanism—the complement component of the immune system. Retroviral vectors are reported to be rapidly inactivated by complement in sera from primates, but not from lower mammals. In considering the replication cycle of retroviruses, it is known that glycosylation epitopes are derived from the host cell during the budding process. Because many retroviral vectors used in gene therapy are murine in origin and have been grown in mouse packaging cell lines, they will have envelopes containing mouse glycoproteins. When retroviral vectors are made in human cells, they are substantially more resistant to human complement. It is reasonable to assume that the mechanism of resistance involves incorporation of natural human cell-membrane complement control proteins that have been incorporated into the vector envelope during the budding stage of particle assembly.

#### vector localization within the target cell

Once the vector reaches the target cell, several factors can affect the level and duration of therapeutic gene expression, and these factors dictate the choice of an appropriate vector system for a specific clinical indication. The localization of the vector genome within the cell, the strength of the gene expression control elements, the stability of the message, and the stability of the translated protein will all affect the therapeutic impact. Alphavirus-based vectors, such as those derived from Sindbis or Semliki Forest virus, reside in the cytoplasm and typically exhibit a very high level of gene expression. Retroviral, adenoviral, and other viral vectors have advantages in gene delivery with their natural mechanisms for nuclear delivery of the therapeutic gene and reasonable levels of gene expression from viral or other promoters. Nonviral plasmid vectors are episomal and are often susceptible to DNA degradation when they are shunted into cell endosomes. However, some nonviral systems incorporate nuclear targeting signals as a means of increasing therapeutic gene-transcription efficiency.

#### tissue-specific promoters

Another means of controlling gene expression is to incorporate tissue-specific promoters to stimulate or to restrict expression of the therapeutic gene. Drug-responsive promoters are being used to control gene expression. Rapamycin, mifepristone, or the tetracycline on systems have been used to repress gene expression. This type of regulation may be required for certain proteins, such as erythropoietin, where constitutive expression may produce toxicity.

#### impact of replication status of vector

Replication status is another important consideration for vector design and selection. Viral vectors are most frequently constructed to be incompetent or replication-defective in order to limit uncontrolled vector spread and pathogenicity. However, when effective therapy requires infection of virtually all the target cells, replication can be engineered to be conditional when specific viral gene interactions are matched with intracellular pathway targets. When these targets are defective or missing, such as in cancer cells, the virus can replicate, but when the target cell is functioning normally, viral replication is repressed. One of the risks inherent in the use of conditionally replicating viral vectors is that the growth of the virus is not absolutely restricted to a single cell type, that is, the system may be leaky. As compensation, the susceptible target cells may be efficiently transduced at a dose that is significantly lower than that necessary for nontarget cells.

Nonviral vectors are normally designed as nonreplicating systems, but some groups are experimenting with replicating nonviral plasmids to increase gene expression levels given the low transduction efficiency of most nonviral systems and to increase the duration of gene expression. Additional preclinical studies are needed to establish the safety of these systems.

Artificial chromosomes have also been designed to take advantage of normal mechanisms for retaining gene expression in rapidly dividing target cells.

#### vector integration

The duration of gene expression is also a function of the stability of the vector genome. Retroviral vectors can stably integrate into the host-cell genome. Adenoviruses do not integrate because their DNA remains episomal. Recombinant adeno-associated virus (AAV) vectors integrate, but because the rep genes responsible for site-specific integration are normally excluded from the construct in order to increase the vector-packaging capacity, integration is not site-specific as it is for wild-type AAV. Nonviral plasmid DNA does not integrate efficiently. However, stable episomes have been observed in certain cell types, such as muscle cells. Site-specific integration can be a desirable feature for vectors intended to correct genetic disorders. Although it is not currently possible, the control of the site of integration is desirable in order to prevent insertional mutagenesis. Insertional mutagenesis has the potential to kill a cell, if a critically functioning gene is inactivated, or to predispose a cell to malignant transformation, if a tumor-suppressor gene is inactivated.

The success of any gene therapy product is dependent on the relationship between the vector-delivery system and the requirements of the disease application in terms of the site, level, and duration of therapeutic gene expression. It is unlikely that there will ever be a universal vector, and the challenge is in fitting the vector to the disease.

#### Manufacturing and Purification Strategies

##### vector construction

Viral and nonviral gene-transfer vectors are constructed by using standard molecular biology protocols. For viral vectors, the vector backbone consists of viral RNA or DNA sequences from which the regions encoding viral structural genes or the regions required for replication have been deleted. The deleted region of the vector is usually modified with specific restriction endonuclease sites used to allow insertion of the gene of interest. For nonviral vectors, the plasmid DNA backbone contains multiple restriction sites for cloning and the bacterial elements necessary for plasmid production. Vector backbones can accommodate single or multiple gene inserts depending on the maximum amount of sequence they can carry. The promoter that facilitates transcription of the gene insert can be a related viral promoter, such as murine leukemia virus long terminal repeat (MuLV LTR), or a heterologous promoter that is either tissue-specific, such as alpha crystalline promoter (of the eye), or constitutive, such as cytomegalovirus (CMV). For example, in a retroviral vector construct containing two gene inserts, transcription of one is regulated from the 5'-LTR-promoter sequence, while a second gene insert can be linked to an internal heterologous promoter from Simian virus 40 (SV40). The complementary DNA (cDNA) containing the therapeutic gene of interest, including its introns, is excised from its source by using restriction enzymes and is inserted at the multiple cloning site of the gene-transfer vector. The polyadenylation signal can be derived from multiple sources such as the SV40 virus or human growth hormone.

Characterization and testing of gene therapy vectors are described under Analytical Methodologies.

##### helper function systems

Recombinant viral vectors are most often modified to be replication-defective, a condition created by deletion or modification of the viral genes needed for replication and production of infectious virus. As a result, viral vectors require help to produce infectious vector particles. Helper functions are often provided by packaging cell lines to deliver the necessary viral element from a source outside of the gene of interest (in trans). Packaging cell lines should be designed to minimize the risk of production of RCV through recombination between the vector and the packaging elements.

Plasmids encoding the necessary elements are introduced into the packaging cell by standard methods such as calcium phosphate-mediated transfection or electroporation. If multiple trans-acting elements are needed, these elements are introduced on separate plasmids in order to increase the number of recombination events needed to form a wild-type viral genome, thus decreasing the frequency of the event. An additional approach to eliminate production of RCV is the elimination of common sequences between the packaging cell



plasmids and the gene therapy vector.

Stable packaging cell lines should be selected and clonal MCBs prepared. In retroviral vector production systems, typically the pro-viral form of the retroviral vector is stably incorporated into the packaging cell, resulting in what is referred to as the producer cell line. A stable, banked packaging cell-producer line will lead to consistency in production and control of adventitious agent contamination. Alternatively, the system can be transient, with the packaging plasmids transfected along with the gene therapy vector for each round of vector production. However, a transient transfection system is less efficient and limited in scalability.

Typical helper function systems are as follows:

1. Retroviral Vector Systems—The murine fibroblast cell line NIH 3T3 has been the basis for several packaging cell lines. The gag, pol, and env functions can be colocated on a single plasmid (PA317) or placed on individual plasmids (psi-CRIP). This increases the number of recombination events required to produce an RCV. The human embryonic kidney cell line 293 has been modified to be a packaging cell line for retroviruses, because use of a human cell line allows production of a retroviral vector that is not affected by the human complement system.
2. Adenoviral Vector Systems—HEK 293 cells are widely used to supply the E1 function necessary for efficient adenoviral replication that is deleted from first-generation adenoviral vectors. Other complementing cell lines, such as E1-modified A549 cells (human lung carcinoma) and the PER.C6 cell line (human embryonic retinoblast), have also been created to supply E1 or other missing functions. The latter contains the E1 region under the control of a phosphoglycerate kinase (PGK) promoter and has no flanking adenoviral sequences in order to eliminate production of replication-competent adenovirus (RCA).
3. AAV Vector Systems—These systems classically use adenovirus-infected human 293 cell lines transiently transfected with AAV helper plasmid containing the rep and cap genes, which are required for AAV replication and capsid formation, respectively, and which are deleted from the AAV vector. The HeLa cell line (from human uterine cervical carcinoma) has also been used as a transient production system. More recently, both of these cell lines have been used to establish stably transfected packaging cell lines that express rep and cap genes and in some cases express the adenoviral functions needed for AAV replication when rep and cap are present (E1a, E1b, E2a, E4, and VA RNA).
4. Gutless Adenoviruses—The manufacturing systems for gutless adenoviruses are similar to classical AAV vector manufacturing systems in that human 293 cells are transiently transfected with helper plasmid containing required adenoviral functions.

#### viral gene therapy vectors

Retrovirus and adenovirus have classically been produced on the laboratory scale by using traditional cultivation methods for anchorage- and serum-dependent cell lines, employing flasks, trays, and roller bottles. Initially, gene therapy vectors were produced by using these exact methods because large volumes of product were not required for early clinical studies. Cell bank systems are used as the source of cells and virus banks as the source of virus for clinical production. In most cases, supernatant is collected, clarified, and stored frozen in bags at  $-70^{\circ}\text{C}$ . In many early clinical trials unpurified supernatant has been used for ex vivo gene transfer.

More recently, larger-scale upstream production methods have been reported including suspension, bioreactor, and fixed-bed or microcarrier culture methods. Some groups have reported adapting their process cells to serum-free culture conditions. Cells are harvested and lysed or supernatant collected. The harvest is clarified and purified to remove host-cell debris, host-cell DNA, and other process-derived contaminants.

Traditionally, viruses are purified by gradient ultracentrifugation, but this is time-consuming and unsuitable for larger-scale production purposes. The selection of downstream process steps and their sequence is determined by the nature of the virus itself and the upstream process used for manufacturing the virus. As processes are being developed for the manufacture of gene therapy vectors, many different purification steps have been reported. These include ion-exchange and sulfonated-cellulose chromatography, zinc ion affinity chromatography, size-exclusion chromatography, and DNase or other nuclease treatments. AAV production and lentiviral production are complicated by a need for transient transfection or cotransfection of plasmid or helper virus. These processes have so far required anchorage-dependent cell lines that are difficult to scale up. The development of stably transfected cell lines would allow large-scale production.

#### plasmid vectors

Plasmids are double-stranded, circular DNA molecules that exist in bacteria as extrachromosomal, self-replicating molecules. They have been modified to serve as cloning systems, to contain multiple restriction endonuclease recognition sites for insertion of the cloned transgene, and to contain selectable genetic markers for identification of cells that carry the recombinant vector. Plasmid-based nonviral vectors are frequently used as gene delivery systems for both *in vivo* and *ex vivo* gene therapies. They are in the form of naked DNA or complexed with lipids or other agents that facilitate transfer across the cell membrane and delivery to the cell nucleus without degradation. An advantage of a plasmid-vector system is the efficient production of large quantities of the vector that is easily characterized and involves no risk of contamination with the RCV.

Nonviral vectors are typically produced by using an *Escherichia coli* bacterial system. Plasmids are transfected into *Escherichia coli*, and an appropriate single bacterial colony is selected and expanded to create an MCB. After reselection of a colony from a bacterial plate inoculated from the MCB, plasmid DNA is isolated from cultures that can range in size from 1 L on a laboratory scale to hundreds of L in bacterial fermenters. Plasmid DNA can be purified by several methods including affinity or ion-exchange chromatography and cesium chloride–ethidium bromide density gradients. Cesium chloride–ethidium bromide density gradients are not recommended for production of clinical-grade material.

#### oligonucleotide vectors

Antisense oligonucleotides are manufactured by synthetic chemistry procedures. Currently, the method of choice is solid-phase phosphoramidite chemistry. Synthesis is linear, rather than convergent, and a high level of efficiency must be maintained at each synthesis step. This is accomplished by using molar excesses of highly pure raw materials to drive the reaction kinetics towards completion. Synthetic oligonucleotide manufacturing may require metric-ton quantities of nucleoside phosphoramidites and other compounds such as activator and sulfur-transfer reagents for commercial-scale manufacturing. An issue for oligonucleotide manufacturing is that during the preparation of raw materials and the synthesis of oligonucleotides, precaution must be taken regarding moisture, because moisture is detrimental to both yield and purity. Purification of the single-strand oligonucleotide product requires removal of residual solvents and synthetic strand by-products. Nevertheless, current oligonucleotide-manufacturing technology is readily scalable and cost-efficient, and it results in products with purity levels similar to those of classical small-molecule pharmaceuticals.

#### formulation of gene therapy products

Final formulations for vector products are still in early development. So far, mannitol, sucrose, lipids, polymers, and serum albumin have been utilized as stabilizers. Aseptic filling of large numbers of vials, using classical manufacturing processes, may be problematic. For example, some viral vectors are thermally sensitive and storage at ultra-low temperatures is often required. Progress is being made in both viral and nonviral vector lyophilization and in the use of stabilizers for liquid formulations.

### ON-SITE PREPARATION AND ADMINISTRATION

#### General Considerations

One or more product modifications or preparative steps may be required prior to administration of the cell or gene therapy product to the patient. These modifications or steps are frequently performed close to the time of administration; and, therefore, they are performed under conditions not under control of the original manufacturing facility. The nature of these modifications is largely dictated by characteristics of the product in relationship to the particular application. These include thawing, washing, or filtration to remove unwanted cells or substances accumulated during storage, transfer to an infusible solution, or compounding with a vehicle or structural material. In addition, patient considerations, such as the need to dose the product according to patient weight or blood volume, may influence these steps.

All product modifications performed between the time of initial product manufacture and final administration to the patient should be viewed as a part of the overall manufacturing process. The practical implications of this concept are that the process controls must be established for all product storage intervals, transport steps, and modifications, starting with a clear definition of critical control points. Operational requirements for performing any of these steps after initial product manufacture include defined physical space with appropriate environmental controls, trained personnel, detailed standard operating procedures, and a comprehensive quality program.

The unique and irreplaceable nature of many cell and gene therapy products, many of which have originated from an autologous or a selected allogeneic tissue source, creates special considerations for product manufacture, release, and administration. It is critical to both anticipate the need for and to establish policies and procedures to guide product modification or administration steps in cases where predefined release criteria cannot be met. These procedures should include a mechanism to obtain medical consultation to assess risk and benefit



considerations for the patient and to provide complete documentation of any decision to modify predefined product specifications.

#### On-Site Preparation product manipulations

Prior to administration, on-site preparation of the cell or gene therapy product may involve one or more manipulations. These manipulations include the following:

1. Change in Final Container—The manufactured product may have been stored or transported in one container but may require transfer to a different container for administration.
2. Change in Physical State or Temperature—A product may require thawing from the frozen state or warming from the refrigerated state.
3. Change in Solution or Suspension—A product may have to be dissolved, diluted, or suspended in a liquid.
4. Addition to Biocompatible Structural Material—A cell or gene therapy product may need to be combined with living, natural, or synthetic structural tissue or matrix. Examples of matrix material include hollow fibers, fibrous sheets, gels, plugs, capsules, sponges, or granules.
5. Admixture or Compounding with Other Nonstructural Materials—A product may require mixing or compounding with drugs, cytokines, biologics, or other nonstructural materials.
6. Filtration or Washing—Unwanted materials in the manufactured product, such as particulates, cellular debris, metabolites, or compounds remaining from previous manipulations may require washing or filtration steps.
7. Sampling—Sampling of the final product immediately prior to administration may be required for certain clinical protocols.

#### facility requirements

Facility requirements for performing on-site preparative steps or administration of cell and gene therapy products depend on the nature of the products, their applications, and the manipulations required. The most important determinant of facility features is the level of risk for microbial contamination associated with each step. Definition of low-risk and high-risk conditions can be made according to a framework similar to that defined for Low-Risk Level CSPs and High-Risk Level CSPs in the CSP Microbial Contamination Risk Levels section under [Pharmaceutical Compounding—Sterile Preparations \(797\)](#).

#### release of final product

Cell and gene therapy products that undergo on-site preparative steps or manipulations must be subjected to appropriate checks or tests to ensure that all quality specifications are met prior to release for patient administration. The nature and extent of manipulations will determine whether release requirements or critical specifications must be added to those required immediately after initial manufacture. Pre-release requirements usually include the following:

1. physical inspection of the product, which typically includes measures to ensure appropriate product appearance with regard to color, turbidity, particulates or foreign matter, container integrity; product temperature; and accuracy and convenience of labeling;
2. review of process records; and
3. for patient-specific products, clerical checking of product labeling or records related to identity of the intended recipient.

In addition, products considered to be high-risk products according to the description under [Pharmaceutical Compounding—Sterile Preparations \(797\)](#) should undergo additional product testing. For all high-risk products, quality assays for the identity, potency, and purity of the active ingredients should be defined and performed. For high-risk products in Category II, sterility and endotoxin testing should be performed.

#### Administration to Patients

##### pre-administration requirements

Depending on the specific cell or gene therapy application, steps may need to be taken by trained patient-care staff to prepare the patient for product administration. These steps are aimed at ensuring that the product will provide the intended therapeutic outcome and at minimizing the risk of adverse effects.

In cases where autologous, selected allogeneic, or xenogeneic tissue is the source of the cell or gene therapy product, determination of patient suitability for the therapy, including the evaluation of histocompatibility between the donor and the recipient, typically occurs prior to the product preparation. However, because of the possibility of changes in clinical status of the patient after the time of tissue collection, such as fever, infection, recurrence or spread of tumors, or organ dysfunction, a thorough re-evaluation of the patient's general condition and suitability for therapy must be performed in close proximity to product administration. This evaluation usually includes a patient history, physical examination, and laboratory studies such as blood counts and chemistries. In addition, baseline physical or functional measurements, laboratory tests, or imaging studies relevant to the specific application may be obtained. Examples include pulmonary function tests for a therapy aimed at improving lung function, measurement of blood levels of an enzyme that is the gene product in a gene therapy application, and nuclear imaging of organs prior to anticancer therapies.

A variety of patient interventions related to route of product administration may be required before product administration. For cellular therapies requiring intravenous administration, patients with poor peripheral venous access may require placement of a central venous catheter. In applications where cells or matrices combined with cells are implanted into the patient, the site of implantation may require preparation in the operating room. This may involve surgically opening the site, removing the degenerated or damaged tissue, trimming of the adjacent tissue to accommodate the implant, and excising the tissue from a second site to be used as an anchor or support for the implant. For instance, in the case of cell products for wound healing, it is critical that the site for grafting be free from infection and that it demonstrates a well-prepared wound bed. In cells intended to repair cartilage defects, the site of damage needs to be prepared so that the cells can be applied to a watertight compartment. For applications involving direct administration of the product into an organ system (for example, bronchioalveolar system) or vascular network (for example, coronary arteries), the patient may require endoscopic or surgical access to these sites.

In all cases, the need for adequate anesthesia and premedication must be carefully evaluated in conjunction with these steps prior to product administration. For example, if it is anticipated that DMSO will remain in a thawed, cryopreserved cellular product, the patient is given an antihistamine prior to administration of the cells to block adverse effects associated with histamine release induced by the DMSO. Pre-administration patient evaluation must also include assessment of concurrent therapies that may interact with the cell or gene therapy product to modify its effects. Some therapies may be considered adjunctive to the cell or gene therapy, such as cytokines that promote proliferation or differentiation of the infused or implanted tissue. Other commonly used drugs such as antibiotics, antineoplastics, anticoagulants, and anti-inflammatory agents must be evaluated for possible effects on the efficacy of the cell or gene therapy product.

#### patient treatment

Some cell or gene therapy products are patient-specific, in that they are manufactured from a selected tissue source, such as autologous, selected allogeneic, or xenogeneic tissue. Certain patient-specific products have a defined potential for benefit or adverse immunoreactivity. Systems must be in place to prevent administration of such a product to the wrong patient. Recommended systems include procedures similar to those used for administration of human blood products, with special attention given to the correct identification of the patient and patient-specific product by at least two people immediately prior to administration.

Cell and gene therapy products can be administered by a variety of routes. These include the intravenous route, the parenteral routes (subcutaneous, intramuscular, and intra-arterial), and the respiratory or gastrointestinal tract route. Other possibilities include direct application of cell or gene therapy products into regional vasculature, organs, tissues, or body cavities by means of needles or catheters or following surgical exposure of the tissue. While parenteral administration can be accomplished in routine outpatient or inpatient facilities, the other means of administration may require specialized facilities, such as an aseptic operating theater or endoscopic suite. In all cases, standard operating procedures and a quality program must be in place to ensure that the product is administered in the intended manner.

#### postadministration monitoring of patient

There should be written policies and procedures for monitoring patient outcomes and managing reports of adverse events. Patient-outcome assessment should include indicators that are likely to detect errors or problems related to the entire manufacturing process, with special attention given to manipulations, storage, or transportation after the initial manufacture of the product. Management of adverse reactions should include procedures for ensuring prompt medical evaluation and treatment of patients with suspected adverse effects and a

stem for reporting and evaluating adverse effects that may point to a potential defect in the administered product. Reporting procedures include providing details required for federal, state, or USP adverse-event reporting programs.

Follow-up and monitoring procedures should be implemented for patients who have received gene therapy vectors or ex vivo gene therapies. To the extent that it is relevant and that it can be assessed, vector or modified cell biodistribution and persistence in vivo should be monitored. With direct administration of vectors, localization to the germ line may be an issue.

Although preclinical studies can be used to address this issue, useful information may be gained through patient monitoring. In the case where a retroviral vector has been administered, patients should be monitored for replication-competent retrovirus (RCR) according to the FDA's Draft Guidance for Industry: Supplemental Guidance on Testing for Replication Competent Retrovirus in Retroviral Vector Based Gene Therapy Products and During Follow-Up of Patients in Clinical Trials Using Retroviral Vectors (October 2000). This involves active monitoring during the first year and archiving of patient samples thereafter if RCR is not detected initially.

Database systems to collate and track patient-monitoring results are essential to management of this information. National registries or publication of data should be considered for establishing the collective safety of gene therapy.

#### ANALYTICAL METHODOLOGIES

##### General Considerations

The complexity and scope of cell and gene therapy products is reflected in the wide range of analytical methods that are used to assess product quality. Approved cell and gene therapy products must comply with applicable sections of 21 CFR 211 and 21 CFR 610 to ensure their identity, dose, potency, purity, and safety. Specific guidance for the identification, development, and validation of analytical methodologies to support cell and virus bank characterization, final-product release, and stability studies is currently provided in the Center for Biologics Evaluation and Research (CBER) Points to Consider for Human and Somatic Cell and Gene Therapy (April 1998); under [Validation of Compendial Procedures \(1225\)](#); and in the ICH guidelines entitled "Q2A Validation of Analytical Procedures", "Q2B Validation of Analytical Procedures: Methodology"; and "Q6B Specification, Tests and Procedures for Biotechnological/Biological Products". Most product-specific analytical methods for cell and gene therapy products have not been standardized. Even well-defined tests such as those described under [Sterility Tests \(71\)](#) may not be directly applicable to certain cell and gene therapy products. For some cell and gene therapy products, large quantities of clinical material may not be available during early clinical development. Some required tests (e.g., sterility) may have to be modified. Consultation with the regulatory authorities is advised.

[Table 6](#) provides an overview of the product-specific testing parameters for the biological component and general methods or approaches being used to satisfy the testing requirements for cell therapy products and for nonviral, viral, and antisense-oligonucleotide gene therapy products. The analysis of cell and gene therapy products relies heavily on biological assays but it also utilizes methodologies developed for biotechnology-derived products (see [Biotechnology-Derived Articles \(1045\)](#)). Antisense-oligonucleotide products are regulated by the FDA Center for Drug Evaluation and Research (CDER) and rely heavily on physicochemical methods. The intent of this section is to outline the types of methods and their specific applications with regard to product characterization, stability, and release testing. Process validation may alleviate the need for certain specific lot-release tests. Development of appropriate reference materials and standards for cell therapy and viral and nonviral gene therapy products should be a part of product development. Reference materials should be fully characterized in order to provide continuity between standards over time. In the case of cellular products the reference material may be a surrogate tissue or simulated product.

Table 6. Analytical Tests for Cell and Gene Therapy Biological Products

		Gene Therapy Products	
Test	Cell Therapy Products	Viral	Nonviral and Antisense-Oligonucleotide
Identity of biological substance	Surface marker determination	Restriction enzyme map	Restriction enzyme map
	Species	PCR	PCR
	Morphology	Immunoassay for expressed gene	Immunoassay for expressed gene
	Bioassay	Sequencing	Sequencing
	Biochemical marker		
Dose	Viable cell number	Particle number	Plasmid-DNA weight
	Enumeration of specific cell population	Transducing units (DNA hybridization assay)	Formulated-complex weight HPLC or capillary electrophoresis assay using authenticated reference standard
	Total DNA	Total protein	
	Total protein	HPLC assay using authenticated reference standard	
Potency	Viable cell number (cells intended for structural repair)	Function of expressed gene (induction of secondary effect and other bioassays)	Function of expressed gene (induction of secondary effect and other bioassays)
	Bioassays: Colony-formation assay Function of expressed gene Induction of secondary effect (e.g., human leukocyte antigen (HLA) induction, secretion of cytokines, and up-regulation of surface marker)		
Purity	Percentage of viable cells	Residual host-cell DNA	Percentage of specific physical form (e.g., percentage supercoiled)
	Percentage of transduced cells	Process contaminants (e.g., serum and cesium chloride)	Residual host-cell DNA
	Percentage of cells with specific surface marker	Residual helper virus	Residual RNA
	Process contaminants (e.g., serum)	Optical density ratio	Residual host-cell proteins
		Residual host-cell proteins	Residual solvents
		Viral protein profile (HPLC assay for defective or immature particles)	Optical density ratio
		Residual RNA	Process contaminants (e.g., cesium chloride and synthetic oligonucleotide by-products)
Safety	Mycoplasma	General safety	
	Sterility	Mycoplasma	Mycoplasma
	Pyrogen and endotoxins	Sterility	Sterility
	Adventitious viruses	Pyrogen and endotoxins	Pyrogen and endotoxins
	Residual virus (for transfected cells)	Adventitious viruses	



Replication-competent vector virus (transfected cells)

RCV

Cell therapy products may require a rapid-release approach if they have a limited shelf life. The rapid-release approach is not usually applied to viral and nonviral gene therapy products because these products are sufficiently stable for completion of testing prior to release. Some formulated nonviral gene therapy products also have limited shelf lives. In such cases, the individual components are tested prior to release and the formulated complex is not tested. The formation and stability of the formulated nonviral gene therapy complex is established through validation studies during product development.

As specified in the CFR, product samples must be retained after product-release testing is completed. Additional samples may need to be retained if rapid-release strategies are employed, so that the product quality can be reassessed by alternative or traditional test methodologies if necessary.

#### New Methodologies and Compendial Perspective

Although the application of compendial methods is encouraged, there are many instances where the analytical method that best addresses the issue is a new method not found among the compendial methods. USP encourages the development of appropriate methods and submission of these methods, once validated, to the USP for addition to the compendia.

One such new methodology is the PCR-based assay. PCR-based assays are utilized in a variety of applications for both cell and gene therapy products. PCR assays can be a viable substitute for long-term bioassays and should be considered when a rapid-release strategy is used. Other applications include the use of PCR-based assays to assess adventitious viral agents in product or in cell and virus banks. PCR might also be used in an identity test of a gene therapy product. In all cases, validation of the new method and assay equipment and qualification of analytical personnel are required.

PCR assays are based on amplification of specific target DNA by using PCR technology. Traditionally, a pair of DNA oligonucleotide primers is used in combination with nucleotides and the Taq polymerase to increase the amount of a specific-size oligomer in a series of alternating cycles of permissive and nonpermissive polymerase temperature conditions. The reaction mix is resolved by gel electrophoresis and visualized by staining with ethidium bromide in order to detect the amplified target (amplicon). RT-PCR involves the use of a reverse transcriptase to create cDNA from RNA prior to performing the PCR reaction, so that the RNA can be detected. PCR and RT-PCR methods can be used qualitatively (positive or negative readout) or quantitatively.

Currently there are two common approaches to quantitation using PCR: (1) competitive PCR that involves use of a spiked mimic and (2) real-time or kinetic PCR that is based on the 5' nuclease activity of the Taq polymerase. In competitive PCR, quantitation is based on the ratio of the amplified mimic to the amplified target. In real-time PCR, the degradation of a dual-labeled, fluorescent, target-specific oligonucleotide probe is monitored in real time, as PCR amplification is occurring. The probe is labeled with a reporter fluorescent dye at the 5' end and a quencher fluorescent dye at the 3' end. When the probe is intact, the fluorescence emission of the reporter is quenched as a result of the physical proximity of the two dyes. The probe sequence is selected so that it hybridizes to the target between the two primer sites. During the extension phase of the PCR cycle, the probe is cleaved by the 5' nuclease activity of the Taq polymerase, while the reporter dye signal is increased by the release of reporter dye from the probe. Additional reporter dye molecules are cleaved during each cycle, resulting in an increase in the fluorescence intensity of the reporter dye proportional to the amount of amplicon produced. The resulting relative increase in reporter fluorescent dye emission is detected in real time during PCR amplification and it allows the threshold cycle number to be related to the target copy number. The threshold cycle number is defined as the PCR cycle number where the increase in reporter fluorescence is detected above the background fluorescence in the assay system. A greater quantity of input DNA or messenger RNA (mRNA) results in a lower threshold cycle, as a result of requiring fewer PCR cycles for reporter fluorescence-emission intensity to reach the threshold. Typically, assays can be designed to detect 1 to 10 copies of the target per reaction.

Like all analytical methods used to release product, PCR assays must be validated. Validation should include the rationale for the selection of primer and probe sequences and a demonstration of the specificity and efficiency of the primers and, for real-time quantitative PCR assays, of the probe. Because primers and probes are the main components of a nucleic acid-based detection system, the performance of the assay is highly dependent on the quality of these reagents. Specificity is generally demonstrated by assessing the resulting PCR product by gel electrophoresis to show that the amplicon is the expected size. For quantitative assays, the design and nature of the quantitation standards must also be addressed.

Assay validation must also address linearity, accuracy, ruggedness, and reproducibility with regard to both the assay itself and the sample preparation, that is, extraction of the sample DNA for PCR or of the sample RNA for RT-PCR. Validation should include a demonstration of the specific limit of detection in the sample type employed, because some sample types contain inhibitors of PCR. Validation should also address the reproducibility of the sampling scheme and the efficiency of nucleic acid extraction and purification procedures to produce the starting material (DNA or RNA). Well-designed spiking studies where samples are spiked both prior to and after extraction can address these issues.

PCR assays are occasionally subject to false-positive results because of the contamination of equipment or samples during handling in preparation for the assay. The most abundant source of contaminating target nucleic acid is the previously generated amplicon. However, the PCR reaction can be modified so that the resultant amplicon is sensitive to uracil-N-glycosylase digestion and can therefore be eliminated. In addition, isolation of sample preparation areas from other phases of the assay and the use of dedicated equipment for each assay phase are generally necessary to prevent amplicon contamination of test samples and, hence, false-positive signals. Assay protocols that include appropriate controls, such as nontarget sequence and nucleic acid-free controls, can aid in determining the source and point of contamination if it occurs. Validation should address the procedures implemented to prevent contamination.

#### Sampling Issues

Sampling for lot-release testing should be based on the potential distribution for the parameter tested. See Stability-Protocol Development under Stability for additional considerations. Samples from each lot need to be retained in case there is a safety or quality issue with the lot. Even if the product has a very short shelf life, these retained samples can be used to detect impurities and other substances. The need for proper design of the sampling scheme is highlighted in safety testing for adventitious agents for cell or viral gene therapy products or in assessment of RCV for viral gene therapy products. In such cases, process validation will assist in determining the appropriate statistically based sampling design.

#### Safety general considerations

Safety testing for cell and gene therapy products focuses on three issues: (1) preventing the unwitting use of contaminated cells, tissues, or gene therapy agents with the potential for transmitting infectious diseases, (2) preventing the use of improperly handled or processed, and consequently contaminated, products, and (3) ensuring safety when cellular and gene therapies are adapted for use other than in their normal functions or setting.

The primary means of assessing safety are the performance of biological assays to measure adventitious agents directly. Molecular biology-based assays that measure adventitious agent DNA or RNA are also used.

#### cell therapy products

Direct transmission of infectious disease is a major concern for cell therapy products. The degree of risk is dependent upon various factors such as whether the cells or tissues are to be used in a person different from the one they were obtained from; whether they are banked, shipped, or processed in a facility that handles cells and tissues from multiple donors; and how extensively they are processed. Improper handling can alter the integrity and function of cell therapy products by introducing microorganisms or by contaminating the therapeutic cell products with other donor or patient cells during collection, processing, or storage.

In addition to transmittable-disease screening and testing of allogeneic donors of all viable and nonviable tissues intended for use as cell therapy products, appropriate labeling and tracking are required. These requirements are not only based on the potential risk of disease transmission from donor to recipient but also on the following: (1) the unusual, but documented, possibility of product-to-product transmission (for example, viral contamination may occur among disrupted bags in the liquid phase of a liquid nitrogen freezer) or (2) the possibility of erroneous administration of a product to the wrong recipient. Specific donor screening and testing requirements for allogeneic cell products are based on those currently required for human blood products. These include (1) specific donor testing for HIV Type 1, HIV Type 2, hepatitis B, hepatitis C, and syphilis and (2) medical history screening for high risk for HIV, hepatitis B, Creutzfeldt-Jakob disease, and tuberculosis. Some of these tests and screening measures are also recommended, but not required, for autologous tissues.

The risk of cross-species infectivity during xenotransplantation is still unknown. Assessing the risk of infection from a new transmissible agent is difficult. In vitro coculture assays involving sensitive human indicator cell lines for the donor species should be developed. In particular, assay of endogenous retrovirus (ERV) present in the xenogeneic cell or tissue is required. In the case of porcine cells and tissues, both PCR and RT-PCR assays for porcine endogenous retrovirus (PERV) have been described and are applied to donor cells and



issues. These tests are also being used for patient monitoring. Assays for PERV antibody have also been developed for patient monitoring. Published studies indicate that the risk of PERV transmission to patients may be low.

Often the shelf life of cell therapy products is shorter than the time required to test for sterility and adventitious agents using traditional cell-based methods. However, as already discussed, development of validated rapid PCR-based methods allows both assessment and timely release. Presence of mycoplasma and a range of specific adventitious viruses and bacteria can be tested by using PCR or DNA- or RNA-hybridization dot blot analysis. Fourteen-day sterility testing is not always a viable alternative for final release of cell therapy products; in such cases, automated methods that rely on colorimetric detection or on continuous monitoring may be acceptable if they are validated. Facility and process validation are necessary adjuncts to ensure safety with regard to sterility and mycoplasma, particularly when rapid-release strategies are employed.

Additional testing for safety may be required when cell therapy products are used in the patient for a purpose other than that which the cells or tissue fulfills in its native state or when placed in a location of the body where such structural function does not normally occur. Testing should be designed to predict product behavior under these settings and should be designed based on the context of use. For example, in the case of a cell therapy product for cancer patients, where the cells are activated by culture on a feeder layer of cells during processing, it may be necessary to test the product cells for the presence of feeder cells. Residual feeder cells in the final product may cause an inflammatory response. In addition, products using non-human feeder cells are considered xenogeneic products. Cell therapies are exempted from general safety testing.

If the cells were modified by a viral gene vector during manufacturing, presence of RCV must be tested. Typically, RCV testing (see *Viral Gene Therapy Products under Safety*) is limited when rapid release is required by shelf life. Again, molecular biology-based methods such as PCR can be used in rapid screening situations. In such cases, during product development, testing that employs cell-based assays (for example, detection of cytopathic effect on indicator cell lines) is performed after release to validate the molecular biology-based test result.

#### viral gene therapy products

One of the primary safety concerns associated with viral vectors used for gene therapy is RCV. Regardless of the virus, these concerns are based on the potential lack of predictability for the pathogenicity of a contaminating virus for a specific route of administration, particularly if it is not the normal route of infection or if humans are not a natural host for the virus.

The pathogenesis of a wild-type adenovirus infection is known but may not be predictive for the routes of administration employed with recombinant adenoviral vectors. For adenoviral vectors, a limit of one RCA per dose is currently considered acceptable; other limits have been established for dose levels greater than  $1 \times 10^9$  particles, specific indications, and routes of administration based on preclinical safety studies and patient-monitoring studies during clinical development. Limits as high as several thousand RCAs per dose have been reported. Typically, RCA levels are determined by using a cell-based assay that allows amplification of the RCA while preventing replication of the product. The cell line recommended for amplification and detection of RCA is the A549 cell line. However, some recombinant adenoviral vectors express therapeutic genes that interfere with analysis on A549 cells. In such cases, a bioassay utilizing two cell lines is used, with the first cell line chosen on the basis of resistance to the effects of expression of the therapeutic gene of interest and with subsequent passage of cell lysate onto A549 cells for amplification and detection of the RCA. RCA is most often detected by visual observation of the cytopathic effect but it may also be detected in the A549 cell culture by using immuno- or PCR-based methods. Quantitation of the RCA level is based on the quantity of sample tested and the detection limit of the assay. Typically, RCA bioassays are validated as being able to detect 1 plaque-forming unit or infectious unit of RCA in the test sample over a wide range of test-sample sizes. Test-sample sizes can range from  $1 \times 10^8$  to  $1 \times 10^{12}$  particles but they are typically based on clinical-dose size. To verify detection limits, spike controls should be included as part of the test, even with validated assays. For recombinant adenoviruses produced using 293 cells, RCA detection by PCR methods can be confounded by detection of residual 293 host-cell DNA (detection of the E1 region). PCR assays, however, can be designed to specifically quantitate host cell DNA contamination and can be made specific to particular forms of slow-growing RCA. Quantitative PCR assays can be used in conjunction with a cell-based method for precise quantitation of RCA levels. When a tested sample is found to be positive, the identity of the RCA is usually confirmed by conducting PCR analysis. This rules out the possibility that contamination of the assay by exogenous wild-type adenovirus or other adventitious agents is responsible for the positive result.

For retroviral vectors, testing for RCR is required for cell banks, viral vector production lots, and any resultant ex vivo product lots (see the FDA's *Draft Guidance for Industry: Supplemental Guidance on Testing for Replication Competent Retrovirus in Retroviral Vector Based Gene Therapy Products and During Follow-Up of Patients in Clinical Trials Using Retroviral Vectors*, October 2000). Standard assays have been designed to detect replication-competent murine leukemia virus (MLV). The pathogenesis and potential long-term toxicity of low-level amphotropic MLV in human beings is not known. Methods commonly used to detect RCR include an amplification of virus titer by application of product to a replication-permissive cell line such as *Mus dunni*. Because infection is limited by the ability of a virus to reach the cells through Brownian motion, procedures (e.g., centrifugation and filtration) that physically bring the virus into contact with the cells may be used to enhance detection. However, high-titer recombinant vector can interfere with the detection of low-level RCR and this interference may be enhanced through such methods. Infected cells are passaged several times to allow viral replication. Culture medium is harvested at the end of the culture period and RCR detected by using an indicator cell line. If the product is an amphotropic MLV, RCR may be detected by using a feline cell-based PG4 S+L- assay, a mink cell-based MiCi S+L- assay, or a marker rescue assay. In S+L- assays, the RCR expresses proteins that lead to transformation and subsequent plaque formation on the monolayer. In a marker rescue assay, RCR infects a cell line that expresses a retroviral vector encoding a marker gene such as  $\beta$ -galactosidase, drug resistance, or a fluorescent protein. The vector is packaged by the proteins supplied to it in trans by the RCR. The potentially vector-laden supernatant is transferred to naive target cells that are then screened for expression of the marker vector.

Testing for RCR is performed by cocultivation of the cell line or amplification of vector supernatant with an RCR replication-permissive cell line, typically *Mus dunni*, for several passages. Culture medium is harvested at the end of this cocultivation process and applied to an appropriate indicator cell line as described above. It is important to note that artifacts may be generated during the cocultivation assay by expression of an endogenous virus in the permissive cell line or through fusion if the vector-producing cell line is cultured directly with a marker rescue cell line. In addition, cocultivation may not be possible for ex vivo cell products that have specific culture requirements or limited culture life spans.

Methodologies for testing the presence of RCR in crude, purified bulk or final vector products are not specified. The FDA has deposited a reference standard of an amphotropic hybrid MLV with the ATCC. This viral stock has been assigned a label titer and should be used in assay validation. Method validation should demonstrate the ability to reproducibly detect a single RCR particle in individual product types because the product and its related impurities can interfere with the detection of RCR. Currently there are no acceptable limits for RCR contamination in products. Any product lot found to contain RCR cannot be used for human use.

Reference standards for assessing RCV in other viral vectors including ecotropic, xenotropic or pseudotyped MLV, adenovirus, and lentivirus have not been developed. Amplification and detection of replication-competent HIV, especially its pseudotyped variants, may warrant special containment and handling procedures.

Additional safety testing usually focuses on methods similar to those described under [Biotechnology-Derived Articles](#) (1045), in Safety Tests—Biologics under [Biological Reactivity Tests](#). [In Vivo](#) (88), and under [Sterility Tests](#) (71). For viral gene therapies produced using a human cell line, performance of the in vitro adventitious agent bioassays using 3 cell lines is recommended on either the bulk or final product. For adenoviral vectors, specific tests for adeno-associated virus are also recommended on either the bulk or final product. For adeno-associated virus, specific tests for adenovirus and herpesvirus are recommended on either the bulk or final product.

#### nonviral gene therapy products

Safety testing usually focuses on methods similar to those described under [Biotechnology-Derived Articles](#) (1045), in Safety Tests—Biologics under [Biological Reactivity Tests](#). [In Vivo](#) (88), and under [Sterility Tests](#) (71). However, the General Safety test is not required for therapeutic DNA plasmid products (even if formulated). Safety testing should be performed on nonviral formulated material. If the shelf life of the formulated nonviral therapy is very short, then the components should be tested individually.

#### Dose-Defining Assays general considerations

An assay that precisely measures the amount of the product is referred to as a dose-defining assay, and it is selected on the basis of its precision and accuracy. An assay that measures therapeutic activity of the product is referred to as a potency assay and it is designed to measure product function. The design of the assay is dependent upon the type of product. In the case of drugs, the assays measuring the amount of active ingredient (dose) are referred to as strength assays.

Product dose can be defined as the concentration or amount of the drug product administered to the patient and it is typically measured as product mass. For cell and gene therapy products, attributes such as viable cell number, milligrams of plasmid or antisense oligonucleotide, or the viral particle number are often used to define the dose of the product.

#### cell therapy products



Cell therapy products may be dosed on the basis of enumeration of one or more cell populations. For ex vivo gene therapy, dose may be based on cell number as well as level of expression of the gene product. For products in the form of a homogeneous, single-cell suspension, viable cell number is the most frequently used assay. Such assays may include enumeration of all cells, total nucleated cells, or another subset of cells. Viability assays are usually based on a cell's ability to exclude a supravital dye, such as trypan blue. Results are expressed as the number of cells that exclude the dye and are therefore considered viable. The compound 7-AAD, a red-fluorescing compound that binds to nuclear proteins and is also excluded by viable cells, may be incorporated into flow-cytometric methods for simultaneous determination of viability and cell-identity markers.

Cell counting may be performed rapidly by manual or automated methods. Manual cell counting by visual enumeration of cells in a hemacytometer chamber is a readily available technique with acceptable accuracy, but a lower degree of precision than most automated methods. Typical instruments for automated cell counting provide reproducible enumeration of nonnucleated cells (e.g., erythrocytes and platelets) and nucleated cells and differential counting of the nucleated cells into mononuclear and polymorphonuclear leukocyte populations. Further discrimination of specific cell populations usually requires cell-surface phenotype analysis by flow-cytometric or other methods (see Cell Therapy Products under Identity). The proportion of a specific subpopulation of cells may be determined by FACS analysis or by flow cytometry.

An example of a cell enumeration assay is the enumeration of CD34-positive (CD34+) hematopoietic progenitor cells, the number being expressed as the number of cells per recipient's body weight. In numerous studies this measurement has been shown to predict hematologic reconstitution following myelosuppressive or ablative therapy in autologous or allogeneic hematopoietic transplantation.

For products that contain cells in a nonhomogeneous suspension, such as cells that form a two- or three-dimensional structure, alternative measures for cell enumeration, such as total area of a cell sheet, wet weight, total protein, and total DNA, have been used. If such measures are used to determine product dose, then supplemental tests must be performed to demonstrate therapeutic activity.

#### viral and nonviral gene therapy products

Particle concentration is a commonly used measure for viral vector product dose. Particle concentration may be measured by physical, biophysical, or in vitro cell-based assays. For example, quantitation of purified adenoviral particles may be determined by using the optical density of a solution of virus in 0.1% (w/v) sodium dodecyl sulfate (SDS) solution, at 260 nm, because a relationship between absorption and particle concentration has been published for adenovirus. The particle number concentration is equivalent to the product of the absorbance at 260 nm in a 1-cm cell, the dilution factor, and  $1.1 \times 10^{12}$  particles.<sup>2</sup> Other methods to determine particle concentration include particle counting by electron microscopy and integration of viral peak area against an authenticated reference standard in an anion-exchange resin-based high-pressure liquid chromatographic (HPLC) assay.

Virus concentration can also be assessed through the measurement of selected structural proteins with known molecular masses and known copy numbers within the virion. For this method, the virus has to be lysed, and the structural proteins have to be separated by using an appropriate, high-recovery chromatographic procedure (e.g., reverse-phase HPLC). The chromatographic separation and the identity and the purity of the selected structural protein has to be verified during assay validation by methods such as SDS polyacrylamide gel electrophoresis (SDS-PAGE), peptide sequencing, and mass spectroscopy. The selected structural proteins have to be quantified, for example, by integrating chromatographic peaks at 214 nm and comparing the area to an authenticated reference standard. The virus concentration can then be calculated based on the molecular mass, the copy number, and the measured mass of the protein. Very importantly, the virus concentration can be estimated simultaneously for multiple structural proteins, allowing the use of this assay in relatively impure virus preparations. This method has been applied to adenovirus and should be applicable to other viral vector types.

Biophysical methods of determining particle number include direct quantitation of vector nucleic acid by radiolabeled-probe hybridization and indirect quantitation by amplification of template nucleic acid (e.g., PCR and RT-PCR) or by signal amplification (e.g., branched-chain DNA using multiple-probe hybridization).

In cases where biophysical methods are not available, bioassays that measure gene-vector titer have been used. These involve infection, transfection, or transduction of a susceptible cell line *in vitro*, followed by some measure of the product uptake. Methods for quantitation or estimation of the number of infection, transfection, or transduction events include plaque-forming unit assays, tissue culture infectious dose, 50% (TCID<sub>50</sub>) assays based on cytopathic effect or immunofluorescent detection of an expressed vector protein, or a quantitative DNA-hybridization assay. Examples are as follows.

For retroviral or lentiviral gene therapy products or AAVs that carry a selectable marker (e.g., that for neomycin resistance) or a reporter gene (e.g.,  $\beta$ -galactosidase) in addition to the therapeutic gene, the infectious titer is commonly determined by measuring the number of transduced or infected cells expressing these nontherapeutic proteins. Vector titer is typically reported as the number of colony-forming units (cfu) per mL for cells transduced with viral vectors containing drug-resistance markers and selected for growth in drug-containing medium. Titer based on  $\beta$ -galactosidase can be expressed in terms of blue (cfu) per mL after staining and counting the cells that convert the  $\beta$ -galactosidase substrate X-Gal into a blue chromophore. For vectors without a marker gene, quantitation of transduction has been measured precisely by using quantitative PCR.

Most nonviral gene therapy products contain plasmid DNA and their usual measure of dose is the DNA mass. The DNA mass may be determined in the formulated state, and, if recombinant protein is included in the formulation, the total combined mass of all formulation components based on a specific ratio can be used. DNA concentrations greater than 500 ng per mL are most simply determined by using optical density measurement at 260 nm. This method is not generally applicable to lipid-formulated DNA. Because RNA and proteins also have significant absorbances at 260 nm, other analyses must be performed to demonstrate that there is minimal contamination with RNA, protein, or residual host-cell chromosomal DNA. Dyes that specifically bind to double-stranded DNA allow the DNA concentrations of less than 500 ng per mL to be measured accurately when calculated against an authenticated DNA standard curve. PicoGreen is one such fluorescent dye and it is minimally affected by single-stranded DNA, RNA, proteins, salts, and detergents. The fluorescent dye Hoechst 33258 also binds to both double-stranded and single-stranded DNA and it can be used to determine DNA concentrations as low as 0.3 ng per mL. The Hoechst 33258 does not bind to protein or RNA and it can accurately determine the DNA concentrations in crude samples.

Methods, such as capillary electrophoresis, employing an authenticated reference material, can also be used to determine the strength of nonviral and antisense-oligonucleotide products.

#### Potency

##### general considerations

Potency is defined as the therapeutic activity of the drug product. Together with dose, potency defines the biological activity of each lot (see General Considerations under Dose-Defining Assays). Potency may be assessed through *in vitro* or *in vivo* bioassays. It is not uncommon for these assays to have coefficients of variation between 30% and 50%. These assays require a well-defined, representative reference material that can be used as a positive control for the assay. The positive control serves to qualify the performance of an individual assay. Potency assay development should focus on characterizing and controlling variability. The high-precision assays are more effective tools in monitoring product quality. Information about potency-assay variability should be incorporated into the stability study design and the proposed statistical approach to assignment of expiration date (see Stability).

#### cell therapy products

Functional assays that can be performed on cellular products are application-related and include viable cell number and a wide range of colony-forming assays, proliferative assays, cell-to-target killing assays, and assays that quantitate gene expression following gene transduction. For hematopoietic progenitor cells prepared from marrow, peripheral blood, or cord blood, traditional colony-forming assay quantitates committed progenitor cells such as colony-forming unit-granulocyte-macrophage (CFU-GM); this assay has been correlated with clinical engraftment outcomes in some studies. More recently, process-monitoring programs incorporate assays such as the long-term culture-initiating cell (LTCIC) assay or the *in vivo* animal models such as competitive repopulation in immunodeficient mice to monitor the activity of the most primitive hematopoietic stem cells. In the case of cells intended for structural repair, proliferation under a set of defined ex vivo conditions may be used as the potency assay. If the cells release an enzyme or active molecule, a potency assay could be based on the units of enzymatic activity or on the total of active molecules released. For instance, the production of insulin in response to changes in glucose levels could be the basis of a potency assay for cells intended to treat diabetes.

Patient-specific products, such as autologous cancer vaccines that elicit an *in vivo* immune response, present a challenge in demonstrating therapeutic activity in an *in vitro* or *in vivo* assay system. Assessment of potency in these circumstances is currently the subject of public-policy debate. Novel approaches to measuring potency, such as the correlation of clinical outcome to other characterization tests such as identity tests, may be appropriate and should be discussed with regulatory authorities early in development. For example, the ability to determine specific cell-surface identity markers by employing flow-cytometric techniques or vital stains may be an acceptable measurement of potency if properly validated and correlated with clinical outcome.



## viral and nonviral gene therapy products

Bioassays employed to measure the potency of viral and nonviral gene therapy products generally involve infection, transfection, or transduction of a susceptible cell line in vitro, followed by some functional measure of the expressed gene of interest. Functional assays for the therapeutic gene (e.g., those measuring enzyme activity and cytokine activity) should generally be used instead of analytical methods such as enzyme-linked immunosorbent assay (ELISA), HPLC, or FACS, which provide information about the level of expression but only infer function. In addition, for viral vectors, infectious titer measurements by themselves are generally not considered an adequate measure of product potency. The design and ultimate suitability of an assay system for determining product potency will depend on the relationship between the intended human target cell in vivo and the following: (1) the transduction or transfection efficiency of the cell line used in vitro (2) the protein expression levels, and (3) the duration of expression required for the therapeutic effect.

In vivo tests may also be used to measure vector-product potency. Readouts may be based on a response per animal (e.g., blood levels of therapeutic protein 24 hours after treatment) or a group response rate (e.g., percentage of animals that elicited an immune response or survived virus challenge). The availability of an appropriate in vivo test system will depend on the vector-host range (for viral vectors), the pharmacokinetics and biodistribution of the vector and its resultant gene product relative to its human counterpart, and the time frame required to observe the therapeutic effect or surrogate. Issues of cost, facilities, validation, and ethics will determine the practicality of an in vivo potency test.

### Purity

#### general considerations

Analytical methods that separate, isolate, and specifically quantify the intended active product components determine product purity. Impurities are either product- or process-related components that can be carried through to the final product. The manufacturing and purification process should be optimized to consistently remove impurities while retaining product activity. The requirement to test for a particular impurity for product lot release will depend on the following: (1) the demonstrated capability of the manufacture and purification process to remove or inactivate the impurity through process validation, and (2) the toxicity potential associated with the impurity.

Examples of process-related impurities associated with cell and gene therapy products include residual production-medium components (e.g., FBS, antibiotics, cytokines, and *Escherichia coli* chromosomal DNA in a plasmid product), ancillary products used in downstream processing (e.g., nucleases such as DNase I), and residual moisture for lyophilized vector products. Impurities may be biactive (e.g., cytokines and hormones) or immunogenic (e.g., product aggregates, degradation products, plasmid-selection markers, and nonhuman-derived proteins) or they may have other deleterious effects (e.g., they may compete with the product) if administered at a dose equal to that of the product. Product-related impurities are specific to each product type. Examples include differentiated cells in a stem-cell therapy product, nicked plasmid forms in nonviral products, and defective or immature virus particles in retroviral or adenoviral vector products. Analytical methodologies to assess purity require quantitation or physical separation of intended product from its impurities. Common sense should drive the need to quantify specific impurities. It may be possible to validate the manufacturing process to the extent that specific lot-release testing for impurities will be very limited. An emphasis may be placed on demonstrating the consistency of the product-impurity profile.

Testing for impurities is often extensive during product characterization and process validation when the consistency of the manufacturing and purification process is being demonstrated. Testing for impurities as part of lot-release testing should reflect the safety risks associated with the impurity and the ability of the process to consistently remove that impurity.

#### cell therapy products

One measure of purity is the percentage of viable cells in the total cell population. Another measure is the percentage of transduced cells or the percentage of cells with a specific marker. If an entire population of cells is the therapeutic agent, methods should determine the relative amounts of each subpopulation. Limits should be defined for each cell subpopulation. These assays may be based on immunological methods utilizing flow cytometry or DNA-hybridization dot blot analysis. Additional tests for process contaminants are performed depending on the specifics of the manufacturing process. For example, a quantitative ELISA for residual serum proteins may be required. If the cells are genetically modified during manufacture, then testing for residual vector may need to be performed.

For gene-modified cell therapies, determining the purity of the cell therapy product depends on the availability of reagents and methods to distinguish therapeutic cells from the other cells present in the product. As in the case of gene therapy products, gene-modified cells can be distinguished from the unmodified cells on the basis of expression of the transgene. FACS using an antibody that detects the therapeutic protein or fluorescent probes that detect expressed RNA allow the separation and quantitation of the transgene expressing and nonexpressing cells. By adding an antibody to a cell-specific phenotypic marker and by using a double-sorting technique, FACS can be used to further identify the subpopulation of cells that are modified.

Endotoxin testing is also required. Biomaterials used with cell therapy products should also be tested for their biocompatibility.

#### viral gene therapy products

Product-related impurities for viral vectors include aggregates and defective and immature particles that may be produced during the manufacture or purification of the recombinant vector. Aggregates of vector may form if the product is highly concentrated, stored under certain conditions (e.g., under certain pH or temperature), or reconstituted after lyophilization. Assays to detect aggregates include particle size analysis by laser light-scattering and the use of nonreducing, nondenaturing PAGE, followed by staining of the gel or transfer and detection of viral proteins by Western blot analysis. Sedimentation rate analysis also allows separation of aggregates from monomers based on size. Optical density analyses of light-scattering are also used to assess vector aggregation.

Defective particles are viral particles that do not contain the appropriate recombinant genome, that is, they contain some other nucleic acid or contain no genome at all, or the vector has some missing, defective, or otherwise altered structural component that impairs its ability to transduce a cell. For viral vector systems that have capsomeric symmetry, which requires the appropriate nucleic acid incorporation for configuration, empty particles may be readily distinguished from those carrying genomes. For enveloped viruses, empty particles may not be as readily separated from those with encapsidated nucleic acid.

For some viral vector products, active viral particles may be separated from defective particles by using analytical HPLC. Anion-exchange resins have been used to separate active adenovirus from defective virus particles. However, this method might not be useful for an adenoviral vector purified by anion-exchange chromatography unless the resin for the assay is different from that used during manufacture. Depending on the nature of the viral vector and its nonactive or defective forms, other methods of separation, such as equilibrium centrifugation in a cesium chloride density gradient, may need to precede the quantitation of the active particle. Ideally, the method of separation will allow quantitation.

Defective particles that carry a noncell-derived oncogenic gene or other undesirable genes may pose a special concern. For example, in murine-based retroviral packaging cell lines, small viral elements called VL30 sequences can be packaged in about one third of all particles. Assays may need to be developed to quantify specific defective particles if they are known to be present in quantities sufficient to pose a safety concern.

Virus quality and the comparability of preparations can also be assessed by measuring selected structural proteins with known molecular masses and known copy numbers within the virion. For this method, the virus is lysed, and the structural proteins are separated by using reverse-phase HPLC or some other high-recovery chromatographic procedure. The chromatographic separation should be validated and the identity of the selected structural proteins verified by methods such as SDS-PAGE, peptide sequencing, or mass spectroscopy. One can fingerprint the batch based on quantification of the selected structural proteins and comparison to a reference standard. When the method incorporates the use of mass spectroscopy, impurities such as structural variants can also be identified. For adenovirus preparations, some precursor and most mature virion proteins can be monitored, thus allowing monitoring of the product and of the immature virion forms.

Host cell-derived proteins may be considered impurities for some viral vector products and may be separated and quantified by PAGE or HPLC or detected by amino acid analysis, Western blot, or immunoassay-based methods. However, for enveloped viruses such as retroviruses, host cell-derived membrane proteins are an integral part of the product. In those vector systems, it may be difficult to determine the presence of contaminating exogenous host-derived proteins.

Presence of specific process-related impurities depends on the manufacture and purification process of each vector or product type. However, most products will need to be tested for residual endotoxin (see [Bacterial Endotoxins Test \(85\)](#)). Acceptable limits of endotoxins have been determined and can be directly applied to viral vector products.

Although genomic DNA derived from continuous cell substrates used to manufacture biological product has been considered historically as potentially tumorigenic, recent studies suggest that the risks are very low. However, every attempt should be made during process development to reduce contaminating DNA levels. The need to test for residual DNA as part of product lot release should be evaluated on a case-by-case basis and may be dependent upon the size distribution of the DNA, its association with the product or its formulation



...ponents, and the route of administration of the product. Quantitative PCR assays have been developed to analyze the amount of residual host-cell DNA by using primers designed to amplify evolutionarily conserved and abundant target sequences, such as 18S for 293 cells.

Quantitation of residual serum components such as bovine serum albumin (BSA) can be achieved by using ELISA and a BSA reference standard. Specific functional or immunological methods may need to be developed for other ancillary products including other culture media or purification process components such as cytokines or enzymes (e.g., nucleases such as DNase I or benzonase).

#### nonviral gene therapy products

Testing is usually performed on the individual components, the plasmid DNA, the lipid or lipoplex components and (recombinant) protein components if any are present in the formulation. Plasmid DNA is characterized for a variety of impurities including residual host-cell DNA, residual RNA, and residual protein. Residual protein testing is frequently included in lot-release testing. Optical density ratios, usually the ratio of the measurement at 260 nm to that at 280 nm, are frequently used in purity specifications for plasmid DNA.

In addition, the plasmid DNA should also be characterized with regard to its form. Plasmid DNA forms include monomeric supercoiled, relaxed monomer, and linear forms. The profile of forms needs to be monitored for product consistency. Additionally, it may be possible to correlate form with *in vivo* transfection behavior. While monomeric supercoiled plasmid has been shown to be more efficient than relaxed monomer, linear, or multimeric forms of the plasmid in transfecting cell lines *in vitro*, *in vitro* transfection has been shown to not always predict *in vivo* behavior. Formulation, delivery method, and route may impact *in vivo* transfection. Agarose gel electrophoresis can resolve these forms of plasmid, which are then detected by UV after ethidium bromide staining. This method provides information about the relative levels of the plasmid forms, but it is not highly quantitative for the individual species. Analytical anion-exchange HPLC can be used as a quantitative assay for monomeric supercoil and percentage of other forms, including concatamers. Other sophisticated methods, such as capillary zone electrophoresis, linear-flow dichroism, and atomic-force microscopy have been proposed as replacements for agarose gel analysis. Until they are validated, these analytical methods may be more appropriate for characterization studies in support of process development and validation rather than for lot-release testing. The appropriate methods for lot release will depend on what effect these alternate plasmid forms have on the product potency. Many of these methods, such as HPLC, are also applicable to the assessment of the purity of antisense-oligonucleotide products and the determination of the level of by-products.

Tests for process-related impurities, such as cesium chloride, must also be conducted. In the case of antisense-oligonucleotide products, residual solvents must be quantified. Lipid and lipoplex formulation components must also be tested for their chemical purity. Testing for specific chemical impurities is commonly performed by using gas chromatography-mass spectroscopy (GC-MS), high-pressure liquid chromatography (HPLC), or thin-layer chromatography (TLC) methods.

If protein is part of the formulated complex, then the protein must also be tested for purity. The methods outlined under [Biotechnology-Derived Articles](#) (1045) or under [Biotechnology-Derived Articles—Capillary Electrophoresis](#) (1053), [Biotechnology-Derived Articles—Isoelectric Focusing](#) (1054), and [Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis](#) (1056) are relevant.

Bacterial protein, DNA, RNA, and endotoxins are the major types of host-derived process contaminants. Standard protein assays (e.g., Lowry, Bradford, or Coomassie), PAGE followed by silver staining or Western blot analysis, or ELISA can be used to detect residual host protein in the nanogram range. Host chromosomal DNA may be detected by slot blot hybridization (detection in picogram range) or by PCR using highly conserved target sequences (e.g., 18S for *Escherichia coli*). However, low background may be unavoidable in PCR-based assays because the recombinant polymerases used for the amplification of target also contain residual bacterial DNA. PAGE or agarose gel electrophoresis followed by fluorescent dye staining may be used to detect residual RNA. Quantitation may not be required given the labile nature of RNA and the low-level toxicity associated with it.

Certain antibiotics, such as kanamycin, that may be used during the fermentation process must be removed during the process, and validation of the process or lot-release testing must be performed to confirm removal during the purification of the plasmid. HPLC is one method that can be used to detect low-level residual antibiotic.

#### lyophilized viral and nonviral vector products

Residual moisture may affect the stability of a lyophilized vector product. The FDA's Guideline for the Determination of Residual Moisture in Dry Biological Products (January 1990) recommends a 1% residual moisture level, although data indicating no adverse effects on product stability at higher levels will be considered acceptable. Residual moisture levels can be determined by using a standard method (see [Water Determination](#) (921)) that is compatible with the formulated product.

#### Identity

##### general considerations

Lot-release testing for cell and gene therapy products must include an identity test. This test serves to specifically identify the product. The complexity of the identity test will depend on the nature of the specific product and the array of products being manufactured. For example, more extensive and rigorous testing may be performed for an autologous gene-modified cell therapy product at a facility where multiple patient products are manufactured than for a viral vector product produced at a site that manufactures a single vector product.

#### cell therapy products

Cell therapy identity tests must be relevant to the cell type and manipulations applied during processing. Differential surface markers (for instance, CD3, CD4, CD34, and CD45) are frequently used to ascertain product identity. Flow-cytometric immunoassay methods are the most common means of detecting and quantifying these markers. In this type of assay, a sample of the cells is stained with fluorescently labeled antibodies directed against specific identity markers and then passed as a single cell suspension in front of a laser source. Identification and quantitation of particular cell subsets is accomplished by multiparameter analysis, usually of size and granularity (measured by forward and side light-scattering) and of one or more identity markers (measured by emitted fluorescence). Simultaneous quantitation of cell viability can be performed by adding 7-amino-actinomycin D (7-AAD) to cell suspensions marked with antibodies conjugated to green (e.g., FITC) or orange (e.g., phycoerythrin) fluorescent compounds.

Analyses, such as isoenzyme analyses, employing biochemical markers are also used. For example, isoenzyme analyses are used to confirm species in the case of xenotransplants. Cell morphology can be used if it can distinguish specific cell types or unique function. Morphology can be combined with doubling-time parameters to better distinguish different cell types.

#### viral gene therapy products

Restriction enzyme mapping and sequencing of the transcription unit DNA are the most commonly used approaches to establishing the identity of viral vectors for characterization purposes. PCR-based methods, restriction enzyme mapping, and transgene expression-based immunoassays are most commonly used to confirm the identity during lot-release testing.

#### nonviral gene therapy products

Restriction enzyme mapping is the most common identity method for plasmid-DNA and antisense-oligonucleotide products. The number of enzymes used to create the vector fingerprint will vary with the complexity of the DNA and the degree of similarity between multiple products. If lipids, lipoplex agents, or proteins are used to formulate the DNA, then their identity must also be tested. Lipids and lipoplex chemicals may be identified by procedures used for traditional pharmaceuticals, such as GC-MS, TLC, and the like. Protein components of the formulation may be identified by peptide mapping or other means outlined under [Biotechnology-Derived Articles—Isoelectric Focusing](#) (1054), [Biotechnology-Derived Articles—Peptide Mapping](#) (1055), and [Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis](#) (1056).

#### STABILITY

##### General Considerations

The shelf lives of cell and gene therapy products will vary widely, depending on the nature of the product, its intended clinical use, its specific attributes, and the recommended storage, packaging, and shipping conditions. Therefore, it is difficult to draft uniform guidelines regarding stability-study duration and testing frequency applicable to all gene and cell therapy products. In all cases, the stability study should be designed on the basis of scientifically sound principles and approaches and a comprehensive understanding of the final therapeutic product and its intended use. Stability of in-process hold steps, cell and virus banks, critical raw materials, and reference standards also needs to be assessed. A well-designed and executed stability program will provide a high degree of assurance that the product is stable within the specified shelf life.



For viral and nonviral vector gene therapy products and cell therapy products that are not patient-specific, the selection of batches to support license application and final-product labeling should be carried out in accordance with the principles of stability testing, such as those described in ICH guideline Q5C, presented under [Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products](#) (1049). Stability data should also be collected for bulk material and other in-process points if material is stored before final processing and filling.

Nonviral DNA plasmid vectors are often formulated with specific mixtures of lipids, proteins, or lipoconjugates to form liposomes or encapsulated complexes. Depending on the formulation used, a shelf life of hours to years can be attained. Where a product has a short shelf life, the final formulation may need to be prepared at the clinic just before administration. Instability is frequently observed as aggregation and precipitation. Formation and stability of the formulated complex must be established through validation studies during product development. Stability data should also be collected for major components of the formulated complex, such as the lipids, the liposomes, and the DNA itself.

For many patient-specific cell products including transduced products, each product is unique and often only one lot is prepared for a single patient. In general, the lots tend to be of small volumes, sometimes less than 10 mL, and they may involve products that cannot be frozen and hence have short shelf lives (between 24 and 72 hours) as the cells continue to metabolize their medium. Protocols to establish stability of patient-specific therapy should use materials from multiple donors and at least three lots. Well-characterized banked primary cells may be used in the validation of storage, shipping, and expiration dating if the donors have a range of profiles expected for the patient population to which the therapy will ultimately be directed. The stability of the product under the holding conditions at the medical center should be validated.

#### Stability-Protocol Development

Formal stability studies to support licensure as well as early phase product stability information gathering should be detailed in a written plan that describes how stability data will be collected and analyzed to support the expiration period of the product. Protocols should follow the format recommended in existing guidelines and include the scope, storage conditions, number of lots to be tested, test schedule, assays to be used, data analysis, and product specifications. Any assay used in a formal stability study for licensure must be validated before the study begins. The specific study design should take into account the reasonably expected possibilities the product may encounter (see Accelerated and Most Appropriate Challenge Conditions) and it should incorporate the latest knowledge in the biological sciences while addressing existing regulatory requirements. For instance, if the final formulation of the product is performed at the clinical site, stability studies on this final formulation should be done to establish the time and conditions under which the product can be held.

Stability studies must verify that the storage conditions maintain the purity and potency of the product, so that the product administered to the patient is still capable of satisfying the stability specifications. These specifications may differ from the release specifications. However, stability specifications must be verified with clinical data. Stability assessment should include assessment of product functionality (potency). The potency assay often has a high degree of inherent variability. Measuring and calculating the decay of product activity by employing the standard statistical methodologies may require multiple, frequent sampling intervals over an extended period of time and may require analysis of more than three production lots to compensate for the variability of the assays. Initial studies to establish a provisional expiration date must be conducted prior to administration to the first patient. Initial studies are also useful for determining which assays are stability-indicating, that is, the best indicators of product degradation. Because existing compendial methods do not address the unique characteristics of cell and gene therapy products, the development of assays that would address these unique characteristics is encouraged.

#### Accelerated and Most Appropriate Challenge Conditions

The stability-indicating profile of a cell or gene therapy product may vary with time under the influence of a wide variety of environmental conditions, including temperature, extremes in physiological storage conditions, and light. Multifactorial degradation pathways must be considered in the development of a program investigating the effects of these parameters on the stability of the products. Studies should include conditions that are outside of the specified storage ranges, that is, challenge conditions such as those encountered during periods of abnormal storage, shipping, or handling. Examples include brief incubator malfunctions, incubator or cold storage failure, periods of extreme temperature fluctuation due to shipping to hot or cold climates, hypobaric conditions experienced in the cargo hold of a commercial airliner, or temperatures likely to be encountered in the surgical suite. A short exposure to an environmental condition well outside of an established limit and a long exposure to an environmental condition just outside of an established acceptable range may be equally detrimental. The slow and constant rate of product degradation at a specified temperature may increase if a different set of storage conditions is applied. The effect of light on the stability-indicating profile should be investigated if it is scientifically warranted. Special attention should be given to products stored in fluids containing light-sensitive or reactive components that may give rise to cytotoxic by-products.

Studies analogous to accelerated aging studies typically used in pharmaceutical stability-monitoring programs are also useful to determine how the product degrades and which assays are stability-indicating. These studies can be the same as some of those mentioned in the preceding paragraph. Other studies include placing a product at 37° or at 18°, while its normal storage temperature is 25 ± 2°, or placing a lyophilized product in a high-humidity environment. Such studies should be performed before formal stability studies begin, so that the formal studies incorporate the validated stability-indicating assays into the protocol.

#### STORAGE AND SHIPPING

##### General Considerations

The storage conditions are chosen to preserve the purity and potency of the product so that the specifications for the product are maintained throughout storage, shipping, and handling at the clinic. Initial studies must be conducted prior to patient administration to determine acceptable storage, shipping, and handling conditions. The initial storage and shipping conditions need not be those envisioned for the commercial product. They should ensure that the product specifications are maintained beyond the initially proposed expiration dating. For products with short shelf lives, storage and shipping conditions, even within a medical center, need to be considered together because shipping constitutes the bulk of storage time after manufacturing. Special consideration should be given to the ability of gas to permeate the shipping container, especially if the cell or gene therapy product is stored or shipped on dry ice. Once stability-indicating methods are developed and the final storage and shipping conditions are chosen, these conditions are validated as discussed under Stability.

Most products with limited shelf lives will be shipped by using reliable overnight courier systems. In some cases, some critical products are hand-carried onto commercial aircraft.

Special permission must be obtained by commercial carriers if scanning by airport X-ray equipment is to be avoided. Cargo shipping studies should be designed during the development of packaging systems to identify stresses to which biological products may be subjected. Bracing and insulating materials should then be chosen and validated to provide a packaging system that will alleviate the extreme conditions of shipping.

#### Cell Therapy Products

**Cryopreservation**— Cryopreservation is the main mode used for the long-term storage of cells, that is, storage of cells for periods longer than 1 year. (See also Suspensions under Formulation of Cell Therapy Products.) The rate of cooling for the cell solutions is important because of the mechanical and dehydration injuries resulting from the formation and growth of ice crystals. The ideal temperatures are dependent upon the type of cells being cryopreserved and the concentration of the cryopreservative. The optimal cooling rate for most cells is between 1° and 3° per minute. Controlled-rate freezers, which can reproducibly produce this optimal cooling rate, are critical when large numbers of vials or large volumes of cells in bags are being frozen. Once cooled to below freezing, cells need to be stored at temperatures below -130°. This can be achieved with electric freezers or with liquid nitrogen. Storage of cells in the vapor phase of a liquid nitrogen freezer reduces the risk of cross-contamination with other material in the freezer. However, a map of freezer temperatures needs to be generated so that cells are not stored so far from the liquid nitrogen that they are subjected to temperatures above -130° as the liquid nitrogen evaporates or during the opening of the freezer. Some cells can be stored at -80° if the cells are to be used within a few weeks.

**Thawing**— Thawing is performed fairly rapidly. If a small amount of cells is to be reinfused or transplanted, DMSO does not need to be removed from the suspension, because most cell preparations can be concentrated adequately to keep the DMSO concentration within tolerable limits. DMSO use has two effects on cells after thawing: cells may clump if damaged, and DMSO reduces cell viability in minutes. If the DMSO needs to be removed or cells need to be concentrated for administration, the defrosted cell suspension is generally serially diluted to avoid osmotic shock and resuspended in a protein-containing medium. Cell viability and potency are generally determined after thawing, but the information may not influence the clinical use of the material.

**Frozen Products**— Frozen cell therapy products are shipped to the medical center on dry ice or in liquid nitrogen dry shippers. Dry shippers may be preferable because temperature is more readily maintained during shipping. Dry ice and liquid nitrogen are both considered hazardous materials during shipping. Storage conditions at the clinic need to be defined. Most



pharmacies do not have access to liquid nitrogen freezers. At best, they have mechanical freezers capable of maintaining the temperature at  $-70^{\circ}\text{C}$ . Clinics that have cell-processing centers or are involved with bone marrow transplantation have liquid nitrogen freezers. If further processing, for example, defrosting and administering, of the cell product is performed at the clinic, the storage conditions and expiration date for the product should be specified. Often the laboratory that is handling the cells and the clinic that will administer the product have to closely collaborate because cells in a concentrated suspension survive for only a few hours.

**Unfrozen Products**— Other cell therapy products are stored unfrozen. Because cells continue to metabolize during storage, their expiration period is typically between 24 and 96 hours.

The expiration date can be extended to several weeks by increasing the volume of storage medium, by reducing the storage temperature, or by attaching a series of bags or compartments that allow the medium to be exchanged without breaching the sterility of the system. These products are shipped in insulating containers with refrigerant packs to maintain a defined temperature range. To stabilize the excursions from these defined and validated temperature ranges, well-designed container configurations with dense foam insulation, which protects the product from shifts in external environmental temperatures, are used. The product purity and potency should not be affected over practical shipment intervals, that is, intervals of 24 to 96 hours, either at higher or lower temperatures. However, if potency may be affected, the shipping box configuration must be re-engineered to maintain optimal potency for the longest possible shipping periods. The product itself should be placed in a lightproof, leakproof container with adequate physical support to ensure stability and prevention of leakage during typical conditions of shipment.

#### Gene Therapy Products

Most gene therapy products can be lyophilized or formulated by means similar to those employed for many recombinant protein products or cell therapy products. These storage formulations typically have expiration periods longer than one year and no unusual shipping requirements. Nonviral gene therapy products, which may be unstable in their final formulation, can have similar expiration periods if they are stored in a multiple-vial kit with the nucleic acid in one vial and a carrier, such as lipids, in the other. The final formulation is performed at the medical center just before administration.

#### LABELING

Product labeling is regulated by the FDA and compliance with existing regulations is required. Labeling of cell and gene therapy products as regulated biologics will be subject to these regulations. For biologics and devices (21 CFR 610 Subpart G and 21 CFR 801, respectively), the labeling requirements are separated into container labeling and package labeling requirements. Both the container label and the package label must include the expiration date. If the container is packaged, then the recommended storage conditions are included on the package label. If the container is not packaged, the recommended storage conditions and all other requirements of a package label must appear on the container label. Labeling must also comply with relevant national and international requirements.

If antibiotics are used in the cell processing and therefore may be present in the final product, the labeling should reflect this. For cell therapy products that must be applied to the patient in a particular physical orientation, labeling that indicates the correct orientation should be apparent even after the package is opened. Similarly, if a device should be only grafted in a defined area, that area should be made readily apparent by labeling that is evident once the package is opened. Unless the product has been screened for pathogenic or microbial contaminants prior to release, appropriate biohazard labeling may be required. For products with very short shelf lives, expiration dating will require adjustment and correction for time zones to provide the user an accurate assessment of shelf life. Clinical procedures will be scheduled around these crucial time frames. For patient-specific products, the patient's full name, initials, or a combination of these will need to appear on the labeling, in addition to lot designation, to ensure that the product will be administered to the appropriate patient.

#### REGULATIONS, STANDARDS, AND NEW METHODOLOGIES

##### Summary of Regulations and Standards

The technologies involved in cell and gene therapy products have been widely documented in the literature and are rapidly evolving. These products can be regulated as drugs, biologics, or devices, or not regulated at all, depending on how they are manufactured and used. The novel approaches permitted by these technologies may make it difficult to determine which FDA centers will be involved in their regulation and the FDA has advised manufacturers to seek clarification in the early stages of development. Regulation is the same as that for biotechnology-derived products. The general requirements are described primarily in the 21 CFR. The federal government has issued many general guidelines as Points to Consider or Guidance documents (see [www.fda.gov](http://www.fda.gov)). ICH guidance documents for many of the quality-related areas are directly relevant to qualifying cell and gene therapy products (see [www.ifpma.org/ich1.html](http://www.ifpma.org/ich1.html)) and some of these documents are reproduced in USP 25 as general informational chapters. National Institutes of Health (NIH) has published Guidelines for Research Involving Recombinant DNA Molecules (see <http://www4.od.nih.gov/oba/rac/guidelines/guidelines.html> for text of the document and its amendments) that require NIH review of research, including clinical research or trials, conducted or sponsored by institutions receiving NIH funding. These guidelines apply to many gene therapy products. AATB has developed guidelines for sourcing allogeneic tissue. Public Health Service (PHS), with input from the NIH, FDA, the Centers for Disease Control and Prevention (CDC), and Health Research Services Administration (HRSA), has developed guidelines for the use of xenogeneic-derived products (Draft Public Health Service (PHS) Guideline on Infectious Disease Issues in Xenotransplantation, August 1996). In addition, ASTM is also developing standards for tissue-engineered medical products.

##### Need for New Methodologies

Cost-effective commercialization of cell and gene therapy products requires the development and validation of new methodologies to assess product quality. USP will adopt such new methodologies when they have been properly validated. Similarly, if reference standards or reference materials are needed and available, they could be included in the USP inventory to allow comparative analysis among various clinical trials or to serve as points of reference by manufacturers of these products for raw materials, process components, and process impurities.

#### DEFINITION OF TERMS

**ADENOVIRUS**— Virus belonging to the family Adenoviridae of DNA viruses having a nonenveloped virion with 252 capsomeres and a diameter between 70 and 90 nm; a single linear molecule of double-stranded DNA (36 to 38 kb); at least 10 structural ether-resistant and acid-stable proteins; virions are released by cell destruction.

**ADENOVIRUS-ASSOCIATED VIRUS (AAV)**— Human parvovirus contains a single-stranded DNA genome and depends on helper viruses (adenovirus, herpesvirus, or vaccinia-virus) for replication. Without coinfection, the wild-type virions integrate at a specific site on chromosome 19 and remain latent.

**ADVENTITIOUS AGENT**— A foreign substitute that is introduced accidentally or inadvertently; not natural or hereditary (as in microbial, chemical, or biochemical contamination of a purified substance).

**ALLOGENEIC**— From an unrelated member of the same species; from the same species, but with a different genotype.

**AMPHOTROPIC VIRUS**— A virus that infects and replicates in cells from multiple species.

**ANCILLARY PRODUCTS**— Components used during manufacturing that should not be present in the final product. Examples: growth factors, cytokines, monoclonal antibodies, cell separation devices, media, and media components.

**ANTISENSE THERAPY**— The use of antisense oligonucleotides (a complementary segment to RNA) to control or inhibit gene expression.

**APHERESIS**— Procedure of withdrawing blood from a donor, removing select components (e.g., platelets or leukocytes), and retransfusing the remainder into the donor.

**AUTologous**— From one's own body.

**BASE PAIR**— Two nucleotide bases on different strands of the nucleic acid molecule that bond together.

**BIOASSAY**— Measurement of the effectiveness of a compound by its effect on animals or cells in comparison with a standard preparation. (See also Potency.)

**BIOLOGICAL PRODUCT**— Any virus, therapeutic serum, toxin, antitoxin, or analogous product applicable to the prevention, treatment, or cure of diseases or injuries in humans. (The term analogous product has been interpreted to include essentially all biotechnology-derived products and procedures including gene therapy, transgenics, and somatic cell therapy.)



TECHNOLOGY— Any technique that uses living organisms (or parts of organisms) to make or modify products, to improve plants or animals, or to develop microorganisms for specific uses. The newer definition refers to the industrial and pharmaceutical use of rDNA, cell fusion, novel bioprocessing techniques, and gene therapy.

B LYMPHOCYTES (B cells)—A class of lymphocytes that produce antibodies and are derived from the bone marrow.

BONE MARROW CELLS—A variety of undifferentiated cells (stem cells) and differentiated cells (lymphocytes, granulocytes, erythrocytes, and platelets) found in the internal cavities of bones or bone marrow.

BONE MARROW TRANSPLANTATION—Transplantation of bone marrow cells that are capable of maintaining the hematological functions indefinitely. Technique used in the treatment of immunological disorders (severe combined immune deficiencies such as ADA deficiency), hematological disorders (anemia), metabolic disorders (Gaucher's disease), and malignant diseases (leukemia, lymphoma, or solid tumor).

CD34—Cluster of Differentiation cell-surface marker 34. CD34 is a protein that distinguishes stem and progenitor cells from more mature blood cells.

CELL LINES—Cells that are derived from primary culture embryos, tissue, or organs. Such cell lines may have a finite life span or be immortalized (made to replicate indefinitely).

CELL THERAPY—Therapy that uses whole cells to treat a disease, condition, or injury.

CGMP—Current good manufacturing practice. The FDA outlines CGMP in the 21 CFR and in the Federal Register and its Points to Consider.

CHONDROCYTES—Cells that produce the components of cartilage.

CLONAL—Genes, cells, or entire organisms derived from and genetically identical to a single common ancestor gene, cell, or organism.

CLONOGENIC ASSAY—Procedure based on the ability to give rise to a clone of cells.

COMPLEMENTARY DNA (cDNA)—DNA synthesized from an mRNA rather than a DNA template. It is used for cloning or as a DNA probe for locating specific genes.

CYTOKINE—Any factor that acts on cells; usually a protein that promotes growth.

CYTOPLASM—Cellular material that is within the cell membrane and surrounds the nucleus.

CYTOTOXIC—Able to cause cell death.

DENDRITIC CELL—Cells that sensitize T cells to antigens.

DIFFERENTIATION—A process of biochemical and structural changes by which cells become specialized in form and function.

DIPLOID CELL—A cell with two complete sets of chromosomes (see Haploid Cell).

ECOTROPIC VIRUS—A virus that infects and replicates in cells from only the original host species.

ELECTROPORATION—Physical means of gene transfer (using a brief electrical field), involving creation of temporary pores in cell membrane to introduce DNA.

ELISA—Enzyme-linked immunosorbent assay. An immunoassay that utilizes an enzyme-labeled antigen or antibody to detect the binding of a molecule to a solid matrix.

ENDOTHELIAL CELLS—Epithelial cells of mesodermal origin that line the internal cavities of the body, such as heart and blood and lymph vessels.

ENGRAFTMENT—Process whereby cells, tissues, or organs are implanted or transplanted into another organism. Refers both to the mechanical and the biological processes necessary to have a fully functional graft.

ENVELOPED VIRUSES—Viruses containing a lipoprotein bilayer surrounding the capsid and acquired by budding through the cell membrane of the host cells.

EPIDERMAL—Pertaining to the outermost and nonvascular layer of the skin derived from embryonic ectoderm.

EPISOMAL—Pertaining to any accessory extrachromosomal genetic material.

EPITHELIAL CELLS—Cells from the linings of various organs. Examples: respiratory, intestinal, or vascular epithelial cells.

EXTRACORPOREAL—Situated or performed outside of the body.

EX VIVO—Procedure performed outside of the living organism.

FIBROBLASTS—Connective tissue cells that have the capacity to produce collagen.

FLUORESCENCE-ACTIVATED CELL SORTER (FACS)—A machine that sorts cells based on fluorescent markers attached to them.

FORMULATED—Prepared in accordance with a prescribed method or conditions.

FUSION—Joining of the membrane of two cells, creating a daughter that contains some of the same properties from each parent cell. It is used in making hybridoma cells in which antibody-producing cells are fused to mouse myeloma cells.

G-418—The antibiotic used to select and isolate cells that contain neomycin-resistance gene.

GENE CONSTRUCT—Expression vector that contains the coding sequence of the protein and the necessary elements for its expression.

GENE THERAPY—Therapy that uses DNA to treat a disease or condition. FDA defines gene therapy products as products containing genetic material administered to modify or manipulate the expression of genetic material to alter the biological properties of living cells.

GENOME—Total hereditary material of a cell.

GERM CELL—Reproductive cell (sperm or egg), gamete, or sex cell.

GRAFT VERSUS HOST DISEASE (GVHD)—Rejection of the transplanted tissue by the host. It is the leading cause of patient death when mismatched allogeneic tissue is used.

GRAFT VERSUS LEUKEMIA (GVL)—Rejection of host leukemia cells by donor T cells.

GRANULOCYTE—One of three types of white blood cells. These cells digest bacteria and other parasites.

GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR (GM-CSF)—A natural hormone that stimulates white blood cell production, particularly that of granulocytes and monocytes.

GROWTH FACTORS—Factors responsible for regulatory cell proliferation, function, and differentiation.

HAPLOID—A cell with half the usual number of chromosomes or only one chromosome set. Germ cells are haploid.

HELPER VIRUS—Aids the development of a defective virus by supplying or restoring the activity of a viral gene or by enabling the defective virus to form a functional envelope.

HEMACYTOMETER—A device used to manually count cells.

HEMATOPOIETIC—Pertaining to or affecting the formation of blood cells.

HEPATOCYTES—The predominant cell type in the liver that has an important role in metabolism and is a source of serum proteins. These cells are generally not dividing, but when injured they can divide and regenerate until the injured cells are replaced.

HERPES SIMPLEX VIRUS (HSV)—A DNA virus that is a member of the family Herpesviridae. It can infect both warm- and cold-blooded vertebrates by contact between moist mucosal

surfaces.

HUMAN LEUKOCYTE ANTIGEN (HLA)—Proteins controlled by the major histocompatibility complex. These proteins play a key role in determining transplant compatibility.

HUMORAL—Pertaining to elements found in body fluids (for example, humoral immunity and neutralizing antibodies).

HYBRIDIZATION DOT BLOT (DNA or RNA)—A technique for detecting, analyzing, and identifying protein; similar to the Western blot but without electrophoretic separation of proteins.

IMMUNOASSAY—Technique for identifying substances based on the use of antibodies.

IMMUNOFLUORESCENCE—Technique for identifying a fluorescent label.

IMMUNOGEN—Substance capable of inducing an immune response; a form of antigen that induces immune response, as opposed to a tolerogen that induces tolerance.

IMPLANTATION VS TRANSPLANTATION—Implantation is the insertion or grafting of a biological, living, inert, or radioactive material into the body. Transplantation is the grafting of tissues from the patient's own body or from another person's body.

INSERTIONAL MUTAGENESIS—A type of mutation that is caused by the insertion of a foreign gene into a host-cell chromosome. There are multiple negative consequences of such an event, including death of a cell if an essential gene is inactivated or predisposition to cancer if a tumor suppressor gene is inactivated.

INTEGRATION—Assimilation of genetic material (DNA) into the chromosome of a recipient cell.

INTERLEUKIN (IL)—Lymphokine that regulates the growth and development of white blood cells. More than 12 have been identified.

INTRABODIES—Intracellular antibodies that are not secreted and that are designed to bind and inactivate target molecules inside cells.

IN VIVO—Procedure performed in the living organism.

IN VITRO—Procedure performed outside of the living organism. It may involve cells or tissues derived from the organisms.

ISLET CELLS— $\beta$ -islet cells of the pancreas that secrete insulin.

ISOGENIC—Of the same genotype.

KERATINOCYTES—Differentiated epidermal cells that constitute the top layer of cells in the skin.

LEUKEMIA—Malignant neoplasm of the blood-forming tissues.

LINEAGE (COMMITTED PROGENITOR CELLS, DIFFERENTIATED CELLS)—Specific path of cell differentiation that can be traced to a single cell of origin.

LIPOPLEX—A formulation of lipids and polymers and/or proteins.

LIPOSOME—A spherical lipid bilayer enclosing an aqueous compartment.

LYMPHOKINE—Class of soluble proteins produced by white blood cells that play a role in the immune response.

LYMPHOMA—Form of cancer that affects the lymphatic tissue.

MICROINJECTION—Physical means of gene transfer involving a direct injection of the cell with a syringe and a needle.

MACROPHAGE—Any of many forms of mononuclear phagocytes that are found in tissues and arise from hematopoietic stem cells in the bone marrow.

MOCK RUN—A test run that deliberately omits some critical reagents.

MONOCLONAL ANTIBODIES—Antibodies that are derived from a single cell clone.

MONOCYTES—One of the three types of white blood cells. They are precursors to macrophages.

MYELOSUPPRESSION—Inhibition of bone marrow activity resulting in depletion of red cells, white cells, and platelets.

MYOCYTES—Fundamental cell units in the muscle. Target cells for insertion of genes that encode secretory proteins.

NEOMYCIN—Antibiotic derived from Streptomyces fradiae.

NAKED DNA—Isolated, purified, and uncomplexed DNA (no protein or lipid).

OLIGONUCLEOTIDE—A polymer consisting of a small number of nucleotides, usually 5 to 30.

ONCOGENES—Genes associated with neoplastic proliferation (cancer) following a mutation or perturbation in their expressions.

ONCOGENIC—Cancer-causing.

OSTEOGENIC CELLS—Derived from or involved in the growth or repair of bone.

PACKAGING CELL LINE—Cell line that produces all of the proteins required for packaging and production of viral vectors in an active form, but does not produce replication-competent virus.

p53GENE—Gene whose mutation is the most common alteration observed in human cancers. It is not required for normal development, but the lack of this gene highly increases the potential risk of cancer.

PARVOVIRUS—DNA viruses of the family Parvoviridae. Host range includes many vertebrate species.

PERCUTANEOUS—Performed through the skin. An example of a percutaneous procedure is the injection of an agent or removal of a tissue (sample for biopsy) with a needle.

PERITONEAL MESOTHELIUM—Lining of the peritoneal cavity consisting of a single sheet of cells covering a broad surface. It has abundant lymphatic drainage and permits diffusion of macromolecules.

PLASMID—A small circular form of DNA that carries certain genes and is capable of replicating independently in a host cell.

POLYCLONAL—Derived from a population of cells consisting of many clonal types.

PROCESS VALIDATION—Means for providing documentation that the manufacturing process is controlled, reproducible, and capable of consistently producing a product that meets predetermined specifications.

PRODUCER CELL LINE—An established cell line used to produce virus vectors, often at a large scale.

POLYMERASE CHAIN REACTION (PCR)—Technique to amplify a target DNA or RNA sequence of nucleotides by several hundred thousand-fold.

POTENCY—A quantitative measure of biological activity based on the attribute of the product linked to the relevant biological properties.

PROGENITOR CELL—Parent or ancestral cell, usually one that is already committed to differentiate into a specific type or lineage of cells.

PROMOTER—DNA sequence that is located at the front of a gene and controls gene expression. It is required for binding of RNA polymerase to initiate transcription.

RADIOIMMUNOASSAY (RIA)—Technique for quantifying a substance by measuring the reactivity of radioactively labeled forms of the substance.



RECOMBINANT-DNA— DNA produced by joining fragments of DNA from different sources by in vitro manipulations.

REPLICATION-COMPETENT VIRUS— A virus that can complete an entire replication cycle without a need for a helper virus; an autonomously replicating virus.

RESTRICTION ENDONUCLEASE— An endonuclease that recognizes a specific sequence of bases within double-stranded DNA.

RETROVIRUS— A virus that contains the reverse transcriptase, which converts viral RNA into DNA that then integrates into the host cell in a form called a provirus.

SERUM-FREE— Refers to cell growth medium that lacks a serum component.

SOMATIC CELLS— Cells other than the germ cells.

S PHASE— Part of the cell cycle during which DNA replication occurs.

STEM CELL— Immortal cell that is capable of proliferating and differentiating into different types of specialized cells. Each major tissue system is thought to have its own putative stem cell.

STROMAL— Refers to cellular support elements that contain essential nutrients or growth factors.

SUPRAVITAL DYE— A dye that stains only living cells.

SUSPENSION CULTURE— Cells capable of growth in suspension, not requiring substrate (attachment) on which to grow.

T CELLS— Lymphocytes that acquire functional repertoires and the concept of self in the thymus and are responsible for cell-mediated immunity. There are several subsets of T cells (helper T cells, suppressor T cells, and cytotoxic T cells).

TCID<sub>50</sub> ASSAY— Tissue Culture Infectious Dose, 50% Assay. An assay measuring the amount of product at which 50% of culture cells in the assay are killed (cytopathic effect) or are expressing a vector protein.

TRANSDUCTION— Transfer and expression of genetic material into a cell by means of a virus or phage vector.

TRANSFECTION— Transfer of DNA into cells by physical means such as by calcium phosphate coprecipitation.

TRANSGENE— Refers to the foreign or therapeutic DNA that is part of a vector construct.

TUMORGENICITY— Having the properties of inducing a malignant neoplasm.

VECTOR— The agent (plasmid, virus, or liposome—protein or DNA—protein complex) used to introduce DNA into a cell.

VIABILITY— State of being alive and functional.

VIRION— An elementary viral particle consisting of genetic material (nucleocapsid) and a protein covering.

VIRUS— Submicroscopic organism that contains genetic information necessary for reproduction. It is an obligate intracellular parasite.

WESTERN BLOT— An electroblotting method in which proteins are transferred from a gel to a thin, rigid support (e.g., nitrocellulose membrane) and detected by binding radioactively labeled antibody or antibody coupled to an enzyme, allowing use of a precipitating chromogenic or chemiluminescent substrate.

XENOGENEIC— From a different species.

XENOTRANSPLANTATION— Transplantation of organs from one species to another (e.g., from pigs to humans).

ZOONOSIS— The disease of animals transmitted to humans via routine exposure to or consumption of the source material.

#### ABBREVIATIONS

AABB	American Association of Blood Banks
7-AAD	7-amino-actinomycin D
AATB	American Association of Tissue Banks
AAV	adenov-associated virus
ADA	amino deaminase
BSE	bovine spongiform encephalopathy (mad cow disease)
CBER	FDA Center for Biologics Evaluation and Research
CDC	The Centers for Disease Control and Prevention
CDER	FDA Center for Drug Evaluation and Research
CDRH	Center for Devices and Radiological Health
cfu	colony-forming unit
CGMP	current good manufacturing practice
CSF	colony-stimulating factor
cDNA	complementary DNA
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FACS	fluorescence-activated cell sorter
GVHD	graft versus host disease
GM-CSF	granulocyte-macrophage colony-stimulating factor
HSV	herpes simplex virus
HRSA	Health Research Services Administration
HLA	human leukocyte antigen
kb	kilobase
NIH	National Institutes of Health
NMDR	National Marrow Donor Registry
PBPC	peripheral blood progenitor cell
PCR	polymerase chain reaction

QC-QA	quality control-quality assurance
RCA	replication-competent adenovirus
RCR	replication-competent retrovirus
RCV	replication-competent virus
rDNA	recombinant DNA
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCID <sub>50</sub>	tissue culture infectious dose, 50%

1 The term tissue engineering is not used in this chapter. Definitions and information on tissue engineering are being developed by the ASTM.

2 From CBER's Points to Consider for Human and Somatic Cell and Gene Therapy, April 1998

## 1048 QUALITY OF BIOTECHNOLOGICAL PRODUCTS: ANALYSIS OF THE EXPRESSION CONSTRUCT IN CELLS USED FOR PRODUCTION OF r-DNA DERIVED PROTEIN PRODUCTS<sup>1</sup>

### I. INTRODUCTION

This document presents guidance regarding the characterization of the expression construct for the production of recombinant DNA (r-DNA) protein products in eukaryotic and prokaryotic cells. The document is intended to describe the types of information that are considered valuable in assessing the structure of the expression construct used to produce r-DNA derived proteins. The document is not intended to cover the entire quality aspect of r-DNA derived medicinal products.

The expression construct is defined as the expression vector containing the coding sequence of the recombinant protein. Segments of the expression construct should be analyzed using nucleic acid techniques in conjunction with other tests performed on the purified recombinant protein for assuring the quality and consistency of the final product. Analysis of the expression construct at the nucleic acid level should be considered as part of the overall evaluation of quality, taking into account that this testing only evaluates the coding sequence of a recombinant gene and not the translational fidelity nor other characteristics of the recombinant protein, such as secondary structure, tertiary structure, and posttranslational modifications.

### II. RATIONALE FOR THE ANALYSIS OF THE EXPRESSION CONSTRUCT

The purpose of analyzing the expression construct is to establish that the correct coding sequence of the product has been incorporated into the host cell and is maintained during culture to the end of production. The genetic sequence of recombinant proteins produced in living cells can undergo mutations that could alter the properties of the protein with potential adverse consequences to patients. No single experimental approach can be expected to detect all possible modifications to a protein. Protein analytical techniques can be used to assess the amino acid sequence of the protein and structural features of the expressed protein due to posttranslational modifications such as proteolytic processing, glycosylation, phosphorylation, and acetylation. Data from nucleic acid analysis may be useful because protein analytical methods may not detect all changes in protein structure resulting from mutations in the sequence coding for the recombinant protein. The relative importance of nucleic acid analysis and protein analysis will vary from product to product.

Nucleic acid analysis can be used to verify the coding sequence and the physical state of the expression construct. The nucleic acid analysis is performed to ensure that the expressed protein will have the correct amino acid sequence, but is not intended to detect low levels of variant sequences. Where the production cells have multiple integrated copies of the expression construct, not all of which may be transcriptionally active, examination of the transcription product itself by analysis of m-RNA or c-DNA may be more appropriate than analysis of genomic DNA. Analytical approaches that examine a bulk population of nucleic acids, such as those performed on pooled clones or material amplified by the polymerase chain reaction, may be considered as an alternative to approaches that depend on selection of individual DNA clones. Other techniques could be considered that allow for rapid and sensitive confirmation of the sequence coding for the recombinant protein in the expression construct.

The following sections describe information that should be supplied regarding the characterization of the expression construct during the development and validation of the production system. Analytical methodologies should be validated for the intended purpose of confirmation of sequence. The validation documentation should, at a minimum, include estimates of the limits of detection for variant sequences. This should be performed for either nucleic acid or protein sequencing methods. The philosophy and recommendations for analysis expressed in this document should be reviewed periodically to take advantage of new advances in technology and scientific information.

### III. CHARACTERIZATION OF THE EXPRESSION SYSTEM

#### A. Expression Construct and Cell Clone Used to Develop the Master Cell Bank (MCB)

The manufacturer should describe the origin of the nucleotide sequence coding for the protein. This should include identification and source of the cell from which the nucleotide sequence was originally obtained. Methods used to prepare the DNA coding for the protein should be described.

The steps in the assembly of the expression construct should be described in detail. This description should include the source and function of the component parts of the expression construct, e.g., origins of replication, antibiotic resistance genes, promoters, enhancers, and whether or not the protein is being synthesized as a fusion protein. A detailed component map and a complete annotated sequence of the plasmid should be given, indicating those regions that have been sequenced during the construction and those taken from the literature. Other expressed proteins encoded by the plasmid should be indicated. The nucleotide sequence of the coding region of the gene of interest and associated flanking regions that are inserted into the vector, up to and including the junctions of insertion, should be determined by DNA sequencing of the construct.

A description of the method of transfer of the expression construct into the host cell should be provided. In addition, methods used to amplify the expression construct and criteria used to select the cell clone for production should be described in detail.

#### B. Cell Bank System

Production of the recombinant protein should be based on well-defined MCB and Working Cell Banks (WCB). A cell bank is a collection of ampules of uniform composition stored under defined conditions, each containing an aliquot of a single pool of cells. The MCB is generally derived from the selected cell clone containing the expression construct. The WCB is derived by expansion of one or more ampules of the MCB. The cell line history and production of the cell banks should be described in detail, including methods and reagents used during culture, in vitro cell age, and storage conditions. All cell banks should be characterized for relevant phenotypic and genotypic markers, which could include the expression of the recombinant protein or presence of the expression construct.

The expression construct in the MCB should be analyzed as described below. If the testing cannot be carried out on the MCB, it should be carried out on each WCB.

Restriction endonuclease mapping or other suitable techniques should be used to analyze the expression construct for copy number, for insertions or deletions, and for the number of integration sites. For extrachromosomal expression systems, the percent of host cells retaining the expression construct should be determined.

The protein coding sequence for the recombinant protein product of the expression construct should be verified. For extrachromosomal expression systems, the expression construct should be isolated and the nucleotide sequence encoding the product should be verified without further cloning. For cells with chromosomal copies of the expression construct, the nucleotide sequence encoding the product could be verified by recloning and sequencing of chromosomal copies. Alternatively, the nucleic acid sequence encoding the product could be verified by techniques such as sequencing of pooled c-DNA clones or material amplified by the polymerase chain reaction. The nucleic acid sequence should be identical, within the limits of detection of the methodology, to that determined for the expression construct as described in section III.A., and should correspond to that expected for the protein sequence.

#### C. Limit for In Vitro Cell Age for Production



The limit for in vitro cell age for production should be based on data derived from production cells expanded under pilot plant-scale or full-scale conditions to the proposed in vitro cell age or beyond. Generally, the production cells are obtained by expansion of the WCB; the MCB could be used to prepare the production cells with appropriate justification.

The expression construct of the production cells should be analyzed once for the MCB as described in section III.B., except that the protein coding sequence of the expression construct in the production cells could be verified by either nucleic acid testing or analysis of the final protein product. Increases in the defined limit for in vitro cell age for production should be supported by data from cells that have been expanded to an in vitro cell age that is equal to or greater than the new limit for in vitro cell age.

#### IV. CONCLUSION

The characterization of the expression construct and the final purified protein are both important to ensure the consistent production of a r-DNA derived product. As described above, analytical data derived from both nucleic acid analysis and evaluation of the final purified protein should be evaluated to ensure the quality of a recombinant protein product.

#### GLOSSARY OF TERMS

##### Expression Construct

The expression vector that contains the coding sequence of the recombinant protein and the elements necessary for its expression.

##### Flanking Control Regions

Noncoding nucleotide sequences that are adjacent to the 5' and 3' end of the coding sequence of the product that contain important elements that affect the transcription, translation, or stability of the coding sequence. These regions include, e.g., promoter, enhancer, and splicing sequences, and do not include origins of replication and antibiotic resistance genes.

##### Integration Site

The site where one or more copies of the expression construct is integrated into the host cell genome.

##### In Vitro Cell Age

Measure of time between thaw of the MCB vial(s) to harvest of the production vessel measured by elapsed chronological time in culture, by population doubling level of the cells, or by passage level of the cells when subcultivated by a defined procedure for dilution of the culture.

##### Master Cell Bank (MCB)

An aliquot of a single pool of cells that generally has been prepared from the selected cell clone under defined conditions, dispensed into multiple containers, and stored under defined conditions. The MCB is used to derive all working cell banks. The testing performed on a new MCB (from a previous initial cell clone, MCB, or WCB) should be the same as for the MCB unless justified.

##### Pilot Plant Scale

The production of a recombinant protein by a procedure fully representative of and simulating that to be applied on a full commercial manufacturing scale. The methods of cell expansion, harvest, and product purification should be identical except for the scale of production.

##### Relevant Genotypic and Phenotypic Markers

Those markers permitting the identification of the strain of the cell line that should include the expression of the recombinant protein or presence of the expression construct.

##### Working Cell Bank (WCB)

The WCB is prepared from aliquots of a homogeneous suspension of cells obtained from culturing the MCB under defined culture conditions.

1 This guideline was developed within the Expert Working Group (Quality) of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and has been subject to consultation by the regulatory parties, in accordance with the ICH process. This document has been endorsed by the ICH Steering Committee at Step 4 of the ICH process, November 29, 1995. At Step 4 of the process, the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and the USA. This guideline was published in the Federal Register on February 23, 1996 (61 FR 7006) and is applicable to drug and biological products. Although this guideline does not create or confer any rights for or on any person and does not operate to bind FDA or the industry, it does represent the agency's current thinking on the production of r-DNA derived protein products. For additional copies of this guideline, contact the Drug Information Branch, HFD-210, CDER, FDA, 5600 Fishers Lane, Rockville, MD 20857 (Phone: 301-827-4573) or the Manufacturers Assistance and Communication Staff (HFM-42), CBER, FDA, 1401 Rockville Pike, Rockville, MD 20852-1448. Send one self-addressed adhesive label to assist the offices in processing your request. An electronic version of this guidance is also available via Internet using the World Wide Web (WWW) (connect to the CDER Home Page at <http://www.fda.gov/cder> and go to the "Regulatory Guidance" section).

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1049 QUALITY OF BIOTECHNOLOGICAL PRODUCTS: STABILITY TESTING OF BIOTECHNOLOGICAL/ BIOLOGICAL PRODUCTS<sup>1</sup>

#### I. INTRODUCTION (1)

The guidance stated in the ICH harmonized tripartite guideline entitled "Stability Testing of New Drug Substances and Products" (issued by ICH on October 27, 1993) applies in general to biotechnological/biological products. However, biotechnological/biological products have distinguishing characteristics to which consideration should be given in any well-defined testing program designed to confirm their stability during the intended storage period. For such products in which the active components are typically proteins and/or polypeptides, maintenance of molecular conformation and, hence, of biological activity, is dependent on noncovalent as well as covalent forces. The products are particularly sensitive to environmental factors such as temperature changes, oxidation, light, ionic content, and shear. To ensure maintenance of biological activity and to avoid degradation, stringent conditions for their storage are usually necessary.

The evaluation of stability may necessitate complex analytical methodologies. Assays for biological activity, where applicable, should be part of the pivotal stability studies. Appropriate physicochemical, biochemical, and immunochemical methods for the analysis of the molecular entity and the quantitative detection of degradation products should also be part of the stability program whenever purity and molecular characteristics of the product permit use of these methodologies.

With these concerns in mind, the applicant should develop the proper supporting stability data for a biotechnological/biological product and consider many external conditions that can affect the product's potency, purity, and quality. Primary data to support a requested storage period for either drug substance or drug product should be based on long-term, real-time, real-condition stability studies. Thus, the development of a proper long-term stability program becomes critical to the successful development of a commercial product. The purpose of this document is to give guidance to applicants regarding the type of stability studies that should be provided in support of marketing applications. It is understood that during the review and evaluation process, continuing updates of initial stability data may occur.



## II. SCOPE OF THE ANNEX (2)

The guidance stated in this annex to "Stability Testing of New Drug Substances and Products" applies to well-characterized proteins and polypeptides, their derivatives and products of which they are components, and which are isolated from tissues, body fluids, cell cultures, or produced using recombinant deoxyribonucleic acid (r-DNA) technology. Thus, the document covers the generation and submission of stability data for products such as cytokines (interferons, interleukins, colony-stimulating factors, tumor necrosis factors), erythropoietins, plasminogen activators, blood plasma factors, growth hormones and growth factors, insulins, monoclonal antibodies, and vaccines consisting of well-characterized proteins or polypeptides. In addition, the guidance outlined in the following sections may apply to other types of products, such as conventional vaccines, after consultation with the appropriate regulatory authorities. The document does not cover antibiotics, allergenic extracts, heparins, vitamins, whole blood, or cellular blood components.

## III. TERMINOLOGY (3)

For the basic terms used in this annex, the reader is referred to the "Glossary" in "Stability Testing of New Drug Substances and Products." However, because manufacturers of biotechnological/biological products sometimes use traditional terminology, traditional terms are specified in parentheses to assist the reader. A supplemental glossary is also included that explains certain terms used in the production of biotechnological/biological products.

## IV. SELECTION OF BATCHES (4)

### A. Drug Substance (Bulk Material) (4.1)

Where bulk material is to be stored after manufacture, but before formulation and final manufacturing, stability data should be provided on at least three batches for which manufacture and storage are representative of the manufacturing scale of production. A minimum of 6 months stability data at the time of submission should be submitted in cases where storage periods greater than 6 months are requested. For drug substances with storage periods of less than 6 months, the minimum amount of stability data in the initial submission should be determined on a case-by-case basis. Data from pilot-plant scale batches of drug substance produced at a reduced scale of fermentation and purification may be provided at the time the dossier is submitted to the regulatory agencies with a commitment to place the first three manufacturing scale batches into the long-term stability program after approval.

The quality of the batches of drug substance placed into the stability program should be representative of the quality of the material used in preclinical and clinical studies and of the quality of the material to be made at manufacturing scale. In addition, the drug substance (bulk material) made at pilot-plant scale should be produced by a process and stored under conditions representative of that used for the manufacturing scale. The drug substance entered into the stability program should be stored in containers that properly represent the actual holding containers used during manufacture. Containers of reduced size may be acceptable for drug substance stability testing provided that they are constructed of the same material and use the same type of container/closure system that is intended to be used during manufacture.

### B. Intermediates (4.2)

During manufacture of biotechnological/biological products, the quality and control of certain intermediates may be critical to the production of the final product. In general, the manufacturer should identify intermediates and generate in-house data and process limits that assure their stability within the bounds of the developed process. Although the use of pilot-plant scale data is permissible, the manufacturer should establish the suitability of such data using the manufacturing scale process.

### C. Drug Product (Final Container Product) (4.3)

Stability information should be provided on at least three batches of final container product representative of that which will be used at manufacturing scale. Where possible, batches of final container product included in stability testing should be derived from different batches of bulk material. A minimum of 6 months data at the time of submission should be submitted in cases where storage periods greater than 6 months are requested. For drug products with storage periods of less than 6 months, the minimum amount of stability data in the initial submission should be determined on a case-by-case basis. Product expiration dating should be based upon the actual data submitted in support of the application. Because dating is based upon the real-time/real-temperature data submitted for review, continuing updates of initial stability data should occur during the review and evaluation process. The quality of the final container product placed on stability studies should be representative of the quality of the material used in the preclinical and clinical studies. Data from pilot-plant scale batches of drug product may be provided at the time the dossier is submitted to the regulatory agencies with a commitment to place the first three manufacturing scale batches into the long-term stability program after approval. Where pilot-plant scale batches were submitted to establish the dating for a product and, in the event that the product produced at manufacturing scale does not meet those long-term stability specifications throughout the dating period or is not representative of the material used in preclinical and clinical studies, the applicant should notify the appropriate regulatory authorities to determine a suitable course of action.

### D. Sample Selection (4.4)

Where one product is distributed in batches differing in fill volume (e.g., 1 milliliter (mL), 2 mL, or 10 mL), unitage (e.g., 10 units, 20 units, or 50 units), or mass (e.g., 1 milligram (mg), 2 mg, or 5 mg), samples to be entered into the stability program may be selected on the basis of a matrix system and/or by bracketing.

Matrixing, i.e., the statistical design of a stability study in which different fractions of samples are tested at different sampling points, should only be applied when appropriate documentation is provided that confirms that the stability of the samples tested represents the stability of all samples. The differences in the samples for the same drug product should be identified as, for example, covering different batches, different strengths, different sizes of the same closure, and, possibly, in some cases, different container/closure systems. Matrixing should not be applied to samples with differences that may affect stability, such as different strengths and different containers/closures, where it cannot be confirmed that the products respond similarly under storage conditions.

Where the same strength and exact container/closure system is used for three or more fill contents, the manufacturer may elect to place only the smallest and largest container size into the stability program, i.e., bracketing. The design of a protocol that incorporates bracketing assumes that the stability of the intermediate condition samples are represented by those at the extremes. In certain cases, data may be needed to demonstrate that all samples are properly represented by data collected for the extremes.

## V. STABILITY-INDICATING PROFILE (5)

On the whole, there is no single stability-indicating assay or parameter that profiles the stability characteristics of a biotechnological/biological product. Consequently, the manufacturer should propose a stability-indicating profile that provides assurance that changes in the identity, purity, and potency of the product will be detected.

At the time of submission, applicants should have validated the methods that comprise the stability-indicating profile, and the data should be available for review. The determination of which tests should be included will be product-specific. The items emphasized in the following subsections are not intended to be all-inclusive, but represent product characteristics that should typically be documented to demonstrate product stability adequately.

### A. Protocol (5.1)

The dossier accompanying the application for marketing authorization should include a detailed protocol for the assessment of the stability of both drug substance and drug product in support of the proposed storage conditions and expiration dating periods. The protocol should include all necessary information that demonstrates the stability of the biotechnological/biological product throughout the proposed expiration dating period including, for example, well-defined specifications and test intervals. The statistical methods that should be used are described in the tripartite guideline on stability.

### B. Potency (5.2)

When the intended use of a product is linked to a definable and measurable biological activity, testing for potency should be part of the stability studies. For the purpose of stability testing of the products described in this guideline, potency is the specific ability or capacity of a product to achieve its intended effect. It is based on the measurement of some attribute of the product and is determined by a suitable *in vivo* or *in vitro* quantitative method. In general, potencies of biotechnological/biological products tested by different laboratories can be compared in a meaningful way only if expressed in relation to that of an appropriate reference material. For that purpose, a reference material calibrated directly or indirectly against the corresponding national or international reference material should be included in the assay.



Potency studies should be performed at appropriate intervals as defined in the stability protocol and the results should be reported in units of biological activity calibrated, whenever possible, against nationally or internationally recognized standards. Where no national or international reference standards exist, the assay results may be reported in in-house derived units using a characterized reference material.

In some biotechnological/biological products, potency is dependent upon the conjugation of the active ingredient(s) to a second moiety or binding to an adjuvant. Dissociation of the active ingredient(s) from the carrier used in conjugates or adjuvants should be examined in real-time/real-temperature studies (including conditions encountered during shipment). The assessment of the stability of such products may be difficult because, in some cases, in vitro tests for biological activity and physicochemical characterization are impractical or provide inaccurate results. Appropriate strategies (e.g., testing the product before conjugation/binding, assessing the release of the active compound from the second moiety, in vivo assays) or the use of an appropriate surrogate test should be considered to overcome the inadequacies of in vitro testing.

#### C. Purity and Molecular Characterization (5.3)

For the purpose of stability testing of the products described in this guideline, purity is a relative term. Because of the effect of glycosylation, deamidation, or other heterogeneities, the absolute purity of a biotechnological/biological product is extremely difficult to determine. Thus, the purity of a biotechnological/biological product should be typically assessed by more than one method and the purity value derived is method-dependent. For the purpose of stability testing, tests for purity should focus on methods for determination of degradation products.

The degree of purity, as well as the individual and total amounts of degradation products of the biotechnological/biological product entered into the stability studies, should be reported and documented whenever possible. Limits of acceptable degradation should be derived from the analytical profiles of batches of the drug substance and drug product used in the preclinical and clinical studies.

The use of relevant physicochemical, biochemical, and immunochemical analytical methodologies should permit a comprehensive characterization of the drug substance and/or drug product (e.g., molecular size, charge, hydrophobicity) and the accurate detection of degradation changes that may result from deamidation, oxidation, sulfoxidation, aggregation, or fragmentation during storage. As examples, methods that may contribute to this include electrophoresis (SDS09Page, immunoelectrophoresis, Western blot, isoelectrofocusing), high-resolution chromatography (e.g., reversed-phase chromatography, gel filtration, ion exchange, affinity chromatography), and peptide mapping.

Wherever significant qualitative or quantitative changes indicative of degradation product formation are detected during long-term, accelerated, and/or stress stability studies, consideration should be given to potential hazards and to the need for characterization and quantification of degradation products within the long-term stability program. Acceptable limits should be proposed and justified, taking into account the levels observed in material used in preclinical and clinical studies.

For substances that cannot be properly characterized or products for which an exact analysis of the purity cannot be determined through routine analytical methods, the applicant should propose and justify alternative testing procedures.

#### D. Other Product Characteristics (5.4)

The following product characteristics, though not specifically relating to biotechnological/biological products, should be monitored and reported for the drug product in its final container:

Visual appearance of the product (color and opacity for solutions/suspensions; color, texture, and dissolution time for powders), visible particulates in solutions or after the reconstitution of powders or lyophilized cakes, pH, and moisture level of powders and lyophilized products.

Sterility testing or alternatives (e.g., container/closure integrity testing) should be performed at a minimum initially and at the end of the proposed shelf life.

Additives (e.g., stabilizers, preservatives) or excipients may degrade during the dating period of the drug product. If there is any indication during preliminary stability studies that reaction or degradation of such materials adversely affect the quality of the drug product, these items may need to be monitored during the stability program.

The container/closure has the potential to affect the product adversely and should be carefully evaluated (see below).

### VI. STORAGE CONDITIONS (6)

#### A. Temperature (6.1)

Because most finished biotechnological/biological products need precisely defined storage temperatures, the storage conditions for the real-time/real-temperature stability studies may be confined to the proposed storage temperature.

#### B. Humidity (6.2)

Biotechnological/biological products are generally distributed in containers protecting them against humidity. Therefore, where it can be demonstrated that the proposed containers (and conditions of storage) afford sufficient protection against high and low humidity, stability tests at different relative humidities can usually be omitted. Where humidity-protecting containers are not used, appropriate stability data should be provided.

#### C. Accelerated and Stress Conditions (6.3)

As previously noted, the expiration dating should be based on real-time/real-temperature data. However, it is strongly suggested that studies be conducted on the drug substance and drug product under accelerated and stress conditions. Studies under accelerated conditions may provide useful support data for establishing the expiration date, provide product stability information or future product development (e.g., preliminary assessment of proposed manufacturing changes such as change in formulation, scale-up), assist in validation of analytical methods for the stability program, or generate information that may help elucidate the degradation profile of the drug substance or drug product. Studies under stress conditions may be useful in determining whether accidental exposures to conditions other than those proposed (e.g., during transportation) are deleterious to the product and also for evaluating which specific test parameters may be the best indicators of product stability. Studies of the exposure of the drug substance or drug product to extreme conditions may help to reveal patterns of degradation; if so, such changes should be monitored under proposed storage conditions. Although the tripartite guideline on stability describes the conditions of the accelerated and stress study, the applicant should note that those conditions may not be appropriate for biotechnological/biological products. Conditions should be carefully selected on a case-by-case basis.

#### D. Light (6.4)

Applicants should consult the appropriate regulatory authorities on a case-by-case basis to determine guidance for testing.

#### E. Container/Closure (6.5)

Changes in the quality of the product may occur due to the interactions between the formulated biotechnological/biological product and container/closure. Where the lack of interactions cannot be excluded in liquid products (other than sealed ampules), stability studies should include samples maintained in the inverted or horizontal position (i.e., in contact with the closure), as well as in the upright position, to determine the effects of the closure on product quality. Data should be supplied for all different container/closure combinations that will be marketed.

In addition to the standard data necessary for a conventional single-use vial, the applicant should demonstrate that the closure used with a multiple-dose vial is capable of withstanding the conditions of repeated insertions and withdrawals so that the product retains its full potency, purity, and quality for the maximum period specified in the instructions-for-use on containers, packages, and/or package inserts. Such labeling should be in accordance with relevant national/regional requirements.

#### F. Stability after Reconstitution of Freeze-Dried Product (6.6)

The stability of freeze-dried products after their reconstitution should be demonstrated for the conditions and the maximum storage period specified on containers, packages, and/or package inserts. Such labeling should be in accordance with relevant national/regional requirements.

### VII. TESTING FREQUENCY (7)



The shelf lives of biotechnological/biological products may vary from days to several years. Thus, it is difficult to draft uniform guidelines regarding the stability study duration and testing frequency that would be applicable to all types of biotechnological/biological products. With only a few exceptions, however, the shelf lives for existing products and potential future products will be within the range of 0.5 to 5 years. Therefore, the guidance is based upon expected shelf lives in that range. This takes into account the fact that degradation of biotechnological/biological products may not be governed by the same factors during different intervals of a long storage period.

When shelf lives of 1 year or less are proposed, the real-time stability studies should be conducted monthly for the first 3 months and at 3 month intervals thereafter. For products with proposed shelf lives of greater than 1 year, the studies should be conducted every 3 months during the first year of storage, every 6 months during the second year, and annually thereafter.

While the testing intervals listed above may be appropriate in the preapproval or prelicense stage, reduced testing may be appropriate after approval or licensure where data are available that demonstrate adequate stability. Where data exist that indicate the stability of a product is not compromised, the applicant is encouraged to submit a protocol that supports elimination of specific test intervals (e.g., 9-month testing) for postapproval/postlicensure, long-term studies.

#### VIII. SPECIFICATIONS (8)

Although biotechnological/biological products may be subject to significant losses of activity, physicochemical changes, or degradation during storage, international and national regulations have provided little guidance with respect to distinct release and end of shelf life specifications. Recommendations for maximum acceptable losses of activity, limits for physicochemical changes, or degradation during the proposed shelf life have not been developed for individual types or groups of biotechnological/biological products but are considered on a case-by-case basis. Each product should retain its specifications within established limits for safety, purity, and potency throughout its proposed shelf life. These specifications and limits should be derived from all available information using the appropriate statistical methods. The use of different specifications for release and expiration should be supported by sufficient data to demonstrate that the clinical performance is not affected, as discussed in the tripartite guideline on stability.

#### IX. LABELING (9)

For most biotechnological/biological drug substances and drug products, precisely defined storage temperatures are recommended. Specific recommendations should be stated, particularly for drug substances and drug products that cannot tolerate freezing. These conditions, and where appropriate, recommendations for protection against light and/or humidity, should appear on containers, packages, and/or package inserts. Such labeling should be in accordance with relevant national and regional requirements.

#### X. GLOSSARY (10)

- **Conjugated Product**—A conjugated product is made up of an active ingredient (e.g., peptide, carbohydrate) bound covalently or noncovalently to a carrier (e.g., protein, peptide, inorganic mineral) with the objective of improving the efficacy or stability of the product.
- **Degradation Product**—A molecule resulting from a change in the drug substance (bulk material) brought about over time. For the purpose of stability testing of the products described in this guideline, such changes could occur as a result of processing or storage (e.g., by deamidation, oxidation, aggregation, proteolysis). For biotechnological/biological products, some degradation products may be active.
- **Impurity**—Any component of the drug substance (bulk material) or drug product (final container product) that is not the chemical entity defined as the drug substance, an excipient, or other additives to the drug product.
- **Intermediate**—For biotechnological/biological products, a material produced during a manufacturing process that is not the drug substance or the drug product but for which manufacture is critical to the successful production of the drug substance or the drug product. Generally, an intermediate will be quantifiable and specifications will be established to determine the successful completion of the manufacturing step before continuation of the manufacturing process. This includes material that may undergo further molecular modification or be held for an extended period before further processing.
- **Manufacturing Scale Production**—Manufacture at the scale typically encountered in a facility intended for product production for marketing.
- **Pilot-Plant Scale**—The production of the drug substance or drug product by a procedure fully representative of and simulating that to be applied at manufacturing scale. The methods of cell expansion, harvest, and product purification should be identical except for the scale of production.

1 This guideline was developed within the Expert Working Group (Quality) of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and has been subject to consultation by the regulatory parties, in accordance with the ICH process. This document has been endorsed by the ICH Steering Committee at Step 4 of the ICH process, November 20, 1995. At Step 4 of the process, the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and the USA. This guideline was published in the Federal Register on July 10, 1996 (61 FR 36466) and is applicable to drug and biological products. Although this guideline does not create or confer any rights for or on any person and does not operate to bind FDA or the industry, it does represent the agency's current thinking on stability testing of biotechnological/biological products. For additional copies of this guideline, contact the Drug Information Branch, HFD-210, CDER, FDA, 5600 Fishers Lane, Rockville, MD 20857 (Phone: 301-827-4573) or the Manufacturers Assistance and Communication Staff (HFM-42), CBER, FDA, 1401 Rockville Pike, Rockville, MD 20852-1448. Send one self-addressed adhesive label to assist the offices in processing your request. An electronic version of this guidance is also available via Internet using the World Wide Web (WWW) (connect to the CDER Home Page at <http://www.fda.gov/cder> and go to the "Regulatory Guidance" section).

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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1050 VIRAL SAFETY EVALUATION OF BIOTECHNOLOGY PRODUCTS DERIVED FROM CELL LINES OF HUMAN OR ANIMAL ORIGIN

#### I. INTRODUCTION

This document is concerned with testing and evaluation of the viral safety of biotechnology products derived from characterized cell lines of human or animal origin (i.e., mammalian, avian, insect), and outlines data that should be submitted in the marketing application/registration package. For the purposes of this document, the term virus excludes nonconventional transmissible agents like those associated with Bovine Spongiform Encephalopathy (BSE) and scrapie. Applicants are encouraged to discuss issues associated with BSE with the regulatory authorities.

The scope of the document covers products derived from cell cultures initiated from characterized cell banks. It covers products derived from *in vitro* cell culture, such as interferons, monoclonal antibodies, and recombinant deoxyribonucleic acid (DNA)-derived products including recombinant subunit vaccines, and also includes products derived from hybridoma cells grown *in vivo* as ascites. In this latter case, special considerations apply and additional information on testing cells propagated *in vivo* is contained in [Appendix 1](#). Inactivated vaccines, all live vaccines containing self-replicating agents, and genetically engineered live vectors are excluded from the scope of this document.

The risk of viral contamination is a feature common to all biotechnology products derived from cell lines. Such contamination could have serious clinical consequences and can arise from the contamination of the source cell lines themselves (cell substrates) or from adventitious introduction of virus during production. To date, however, biotechnology products derived from cell lines have not been implicated in the transmission of viruses. Nevertheless, it is expected that the safety of these products with regard to viral contamination can be reasonably assured only by the application of a virus testing program and assessment of virus removal and inactivation achieved by the manufacturing process, as outlined below.

Three principal, complementary approaches have evolved to control the potential viral contamination of biotechnology products:



Selecting and testing cell lines and other raw materials, including media components, for the absence of undesirable viruses which may be infectious and/or pathogenic for humans.

(2) Assessing the capacity of the production processes to clear infectious viruses;

(3) Testing the product at appropriate steps of production for absence of contaminating infectious viruses.

All testing suffers from the inherent limitation of quantitative virus assays, i.e., that the ability to detect low viral concentrations depends for statistical reasons on the size of the sample. Therefore, no single approach will necessarily establish the safety of a product. Confidence that infectious virus is absent from the final product will in many instances not be derived solely from direct testing for their presence, but also from a demonstration that the purification regimen is capable of removing and/or inactivating the viruses.

The type and extent of viral tests and viral clearance studies needed at different steps of production will depend on various factors and should be considered on a case-by-case and step-by-step basis. The factors that should be taken into account include the extent of cell bank characterization and qualification, the nature of any viruses detected, culture medium constituents, culture methods, facility and equipment design, the results of viral tests after cell culture, the ability of the process to clear viruses, and the type of product and its intended clinical use.

The purpose of this document is to describe a general framework for virus testing, experiments for the assessment of viral clearance, and a recommended approach for the design of viral tests and viral clearance studies. Related information is described in the appendices and selected definitions are provided in the glossary.

Manufacturers should adjust the recommendations presented here to their specific product and its production process. The approach used by manufacturers in their overall strategy for ensuring viral safety should be explained and justified. In addition to the detailed data that is provided, an overall summary of the viral safety assessment would be useful in facilitating the review by regulatory authorities. This summary should contain a brief description of all aspects of the viral safety studies and strategies used to prevent virus contamination as they pertain to this document.

## II. POTENTIAL SOURCES OF VIRUS CONTAMINATION

Viral contamination of biotechnology products may arise from the original source of the cell lines or from adventitious introduction of virus during production processes.

### A. Viruses That Could Occur in the Master Cell Bank (MCB)

Cells may have latent or persistent virus infection (e.g., herpesvirus) or endogenous retrovirus which may be transmitted vertically from one cell generation to the next, since the viral genome persists within the cell. Such viruses may be constitutively expressed or may unexpectedly become expressed as an infectious virus. Viruses can be introduced into the MCB by several routes such as: (1) Derivation of cell lines from infected animals; (2) use of virus to establish the cell line; (3) use of contaminated biological reagents such as animal serum components; (4) contamination during cell handling.

### B. Adventitious Viruses That Could Be Introduced During Production

Adventitious viruses can be introduced into the final product by several routes including, but not limited to, the following: (1) Use of contaminated biological reagents such as animal serum components; (2) use of a virus for the induction of expression of specific genes encoding a desired protein; (3) use of a contaminated reagent, such as a monoclonal antibody affinity column; (4) use of a contaminated excipient during formulation; and (5) contamination during cell and medium handling. Monitoring of cell culture parameters can be helpful in the early detection of potential adventitious viral contamination.

## III. CELL LINE QUALIFICATION: TESTING FOR VIRUSES

An important part of qualifying a cell line for use in the production of a biotechnology product is the appropriate testing for the presence of virus.

### A. Suggested Virus Tests for MCB, Working Cell Bank (WCB) and Cells at the Limit of In Vitro Cell Age Used for Production

[Table 1](#) shows examples of virus tests to be performed once only at various cell levels, including MCB, WCB, and cells at the limit of in vitro cell age used for production.

Table 1. Examples of Virus Tests to be Performed Once At Various Cell Levels

	MCB	WCB <sup>1</sup>	Cells at the Limit <sup>2</sup>
Tests for Retroviruses and Other Endogenous Viruses			
Infectivity	+	-	+
Electron microscopy <sup>3</sup>	+ <sup>3</sup>	-	+ <sup>3</sup>
Reverse transcriptase <sup>4</sup>	+ <sup>4</sup>	-	+ <sup>4</sup>
Other virus-specific tests <sup>5</sup>	as appropriate <sup>5</sup>	-	as appropriate <sup>5</sup>
Tests for Nonendogenous or Adventitious Viruses			
In vitro Asays	+	- <sup>6</sup>	+
In vivo Assays	+	- <sup>6</sup>	+
Antibody production tests <sup>7</sup>	+ <sup>7</sup>	-	-
Other virus-specific tests <sup>8</sup>	+ <sup>8</sup>	-	-

1. See text—section III.A.2.

2. Cells at the limit: Cells at the limit of in vitro cell age used for production (See text—section III.A.3.).

3. May also detect other agents.

4. Not necessary if positive by retrovirus infectivity test.

5. As appropriate for cell lines which are known to have been infected by such agents.

6. For the first WCB, this test should be performed on cells at the limit of in vitro cell age, generated from that WCB; for WCB's subsequent to the first WCB, a single in vitro and in vivo test can be done either directly on the WCB or on cells at the limit of in vitro cell age.

7. e.g., MAP, RAP, HAP—usually applicable for rodent cell lines.

8. e.g., tests for cell lines derived from human, nonhuman primate, or other cell lines as appropriate.

### 1. Master Cell Bank

Extensive screening for both endogenous and nonendogenous viral contamination should be performed on the MCB. For heterohybrid cell lines in which one or more partners are human or nonhuman primate in origin, tests should be performed in order to detect viruses of human or nonhuman primate origin because viral contamination arising from these cells may pose a particular hazard.

Testing for nonendogenous viruses should include in vitro and in vivo inoculation tests and any other specific tests, including species-specific tests such as the mouse antibody production (MAP) test, that are appropriate, based on the passage history of the cell line, to detect possible contaminating viruses.

### 2. Working Cell Bank



Each WCB as a starting cell substrate for drug production should be tested for adventitious virus either by direct testing or by analysis of cells at the limit of in vitro cell age, initiated from the WCB. When appropriate nonendogenous virus tests have been performed on the MCB and cells cultured up to or beyond the limit of in vitro cell age have been derived from the WCB and used for testing for the presence of adventitious viruses, similar tests need not be performed on the initial WCB. Antibody production tests are usually not necessary for the WCB. An alternative approach in which full tests are carried out on the WCB rather than on the MCB would also be considered acceptable.

### 3. Cells at the Limit of In Vitro Cell Age Used for Production

The limit of in vitro cell age used for production should be based on data derived from production cells expanded under pilot-plant scale or commercial-scale conditions to the proposed in vitro cell age or beyond. Generally, the production cells are obtained by expansion of the WCB; the MCB could also be used to prepare the production cells. Cells at the limit of in vitro cell age should be evaluated once for those endogenous viruses that may have been undetected in the MCB and WCB. The performance of suitable tests (e.g., in vitro and in vivo) at least once on cells at the limit of in vitro cell age used for production would provide further assurance that the production process is not prone to contamination by adventitious virus. If any adventitious viruses are detected at this level, the process should be carefully checked in order to determine the cause of the contamination, and should be completely redesigned if necessary.

### B. Recommended Viral Detection and Identification Assays

Numerous assays can be used for the detection of endogenous and adventitious viruses. [Table 2](#) outlines examples for these assays. They should be regarded as assay protocols recommended for the present, but the list is not all-inclusive or definitive. Since the most appropriate techniques may change with scientific progress, proposals for alternative techniques, when accompanied by adequate supporting data, may be acceptable. Manufacturers are encouraged to discuss these alternatives with the regulatory authorities. Other tests may be necessary depending on the individual case. Assays should include appropriate controls to ensure adequate sensitivity and specificity. Wherever a relatively high possibility of the presence of a specific virus can be predicted from the species of origin of the cell substrate, specific tests and/or approaches may be necessary. If the cell line used for production is of human or nonhuman primate origin, additional tests for human viruses, such as those causing immunodeficiency diseases and hepatitis, should be performed unless otherwise justified. The polymerase chain reaction (PCR) may be appropriate for detection of sequences of other human viruses as well as for other specific viruses. The following is a brief description of a general framework and philosophical background within which the manufacturer should justify what was done.

Table 2. Examples of the Use and Limitations of Assays Which May be Used to Test for Virus

Test	Test Article	Detection Capability	Detection Limitation
Antibody production	Lysate of cells and their culture medium	Specific viral antigens	Antigens not infectious for animal test system
in vivo virus screen	Lysate of cells and their culture medium	Broad range of viruses pathogenic for humans	Agents failing to replicate or produce diseases in the test system
in vitro virus screen for:		Broad range of viruses pathogenic for humans	Agents failing to replicate or produce diseases in the test system
1. Cell bank characterization	1. Lysate of cells and their culture medium (for co-cultivation, intact cells should be in the test article)		
2. Production screen	2. Unprocessed bulk harvest or lysate of cells and their cell culture medium from the production reactor		
TEM on:		Virus and virus-like particles	Qualitative assay with assessment of identity
1. Cell substrate	1. Viable cells		
2. Cell culture supernatant	2. Cell-free culture supernatant		
Reverse transcriptase (RT)	Cell-free culture supernatant	Retroviruses and expressed retroviral RT	Only detects enzymes with optimal activity under preferred conditions. Interpretation may be difficult due to presence of cellular enzymes; background with some concentrated samples
Retrovirus (RV) infectivity	Cell-free culture supernatant	Infectious retroviruses	RV failing to replicate or form discrete foci or plaques in the chosen test system
Cocultivation	Viable cells	Infectious retroviruses	RV failing to replicate
1. Infectivity endpoint			1. See above under RV infectivity
2. TEM endpoint			2. See above under TEM <sup>1</sup>
3. RT endpoint			3. See above under RT
PCR (Polymerase chain reaction)	Cells, culture fluid and other materials	Specific virus sequences	Primer sequences must be present. Does not indicate whether virus is infectious.

1 In addition, difficult to distinguish test article from indicator cells.

#### 1. Tests for Retroviruses

For the MCB and for cells cultured up to or beyond the limit of in vitro cell age used for production, tests for retroviruses, including infectivity assays in sensitive cell cultures and electron microscopy (EM) studies, should be carried out. If infectivity is not detected and no retrovirus or retrovirus-like particles have been observed by EM, reverse transcriptase (RT) or other appropriate assays should be performed to detect retroviruses that may be noninfectious. Induction studies have not been found to be useful.

#### 2. In Vitro Assays

In vitro tests are carried out by the inoculation of a test article (see [Table 2](#)) into various susceptible indicator cell cultures capable of detecting a wide range of human and relevant animal viruses. The choice of cells used in the test is governed by the species of origin of the cell bank to be tested, but should include a human and/or a nonhuman primate cell line susceptible to human viruses. The nature of the assay and the sample to be tested are governed by the type of virus which may possibly be present based on the origin or handling of the cells. Both cytopathic and hemadsorbing viruses should be sought.

#### 3. In Vivo Assays

A test article (see [Table 2](#)) should be inoculated into animals, including suckling and adult mice, and in embryonated eggs to reveal viruses that cannot grow in cell cultures. Additional animal species may be used, depending on the nature and source of the cell lines being tested. The health of the animals should be monitored and any abnormality should be investigated to establish the cause of the illness.

#### 4. Antibody Production Tests

Species-specific viruses present in rodent cell lines may be detected by inoculating test article (see [Table 2](#)) into virus-free animals and examining the serum antibody level or enzyme activity after a specified period. Examples of such tests are the mouse antibody production (MAP) test, rat antibody production (RAP) test, and hamster antibody production (HAP) test.

The viruses currently screened for in the antibody production assays are discussed in [Table 3](#).

Table 3. Virus Detected in Antibody Production Tests



AP	HAP	RAP
Ectromelia Virus <sup>2,3</sup>	Lymphocytic Choriomeningitis Virus (LCM) <sup>1,3</sup>	Hantaan Virus <sup>1,3</sup>
Hantaan Virus <sup>1,3</sup>	Pneumonia Virus of Mice (PVM) <sup>2,3</sup>	Kilham Rat Virus (KRV) <sup>2,3</sup>
K Virus <sup>2</sup>	Reovirus Type 3 (Reo3) <sup>1,3</sup>	Mouse Encephalomyelitis Virus (Theilers, GDVII) <sup>2</sup>
Lactic Dehydrogenase Virus (LDM) <sup>1,3</sup>	Sendai Virus <sup>1,3</sup>	Pneumonia Virus of Mice (PVM) <sup>2,3</sup>
Lymphocytic Choriomeningitis Virus (LCM) <sup>1,3</sup>	SV5	Rat Coronavirus (RCV) <sup>2</sup>
Minute Virus of Mice <sup>2,3</sup>		Reovirus Type 3 (Reo3) <sup>1,3</sup>
Mouse Adenovirus (MAV) <sup>2,3</sup>		Sendai Virus <sup>1,3</sup>
Mouse Cytomegalovirus (MCMV) <sup>2,3</sup>		Slalocryoadenitis Virus (SDAV) <sup>2</sup>
Mouse Encephalomyelitis Virus (Theilers, GDVII) <sup>2</sup>		Toolan Virus (HI) <sup>2,3</sup>
Mouse Hepatitis Virus (MHV) <sup>2</sup>		
Mouse Rotavirus (EDIM) <sup>2,3</sup>		
Pneumonia Virus of Mice (PVM) <sup>2,3</sup>		
Polyoma Virus <sup>2</sup>		
Reovirus Type 3 (Reo3) <sup>1,3</sup>		
Sendai Virus <sup>1,3</sup>		
Thymic Virus <sup>2</sup>		

1. Viruses for which there is evidence of capacity for infecting humans or primates.

2. Viruses for which there is no evidence of capacity for infecting humans.

3. Virus capable of replicating in vitro in cells of human or primate origin.

#### C. Acceptability of Cell Lines

It is recognized that some cell lines used for the manufacture of product will contain endogenous retroviruses, other viruses, or viral sequences. In such circumstances, the action plan recommended for manufacture is described in section V. of this document. The acceptability of cell lines containing viruses other than endogenous retroviruses will be considered on an individual basis by the regulatory authorities, by taking into account a risk/benefit analysis based on the benefit of the product and its intended clinical use, the nature of the contaminating viruses, their potential for infecting humans or for causing disease in humans, the purification process for the product (e.g., viral clearance evaluation data), and the extent of the virus tests conducted on the purified bulk.

#### IV. TESTING FOR VIRUSES IN UNPROCESSED BULK

The unprocessed bulk constitutes one or multiple pooled harvests of cells and culture media. When cells are not readily accessible (e.g., hollow fiber or similar systems), the unprocessed bulk would constitute fluids harvested from the fermenter. A representative sample of the unprocessed bulk, removed from the production reactor prior to further processing, represents one of the most suitable levels at which the possibility of adventitious virus contamination can be determined with a high probability of detection. Appropriate testing for viruses should be performed at the unprocessed bulk level unless virus testing is made more sensitive by initial partial processing (e.g., unprocessed bulk may be toxic in test cell cultures, whereas partially processed bulk may not be toxic).

In certain instances, it may be more appropriate to test a mixture consisting of both intact and disrupted cells and their cell culture supernatants removed from the production reactor prior to further processing. Data from at least three lots of unprocessed bulk at pilot-plant scale or commercial scale should be submitted as part of the marketing application/registration package.

It is recommended that manufacturers develop programs for the ongoing assessment of adventitious viruses in production batches. The scope, extent, and frequency of virus testing on the unprocessed bulk should be determined by taking several points into consideration, including the nature of the cell lines used to produce the desired products, the results and extent of virus tests performed during the qualification of the cell lines, the cultivation method, raw material sources, and results of viral clearance studies. In vitro screening tests, using one or several cell lines, are generally employed to test unprocessed bulk. If appropriate, a PCR test or other suitable methods may be used.

Generally, harvest material in which adventitious virus has been detected should not be used to manufacture the product. If any adventitious viruses are detected at this level, the process should be carefully checked to determine the cause of the contamination, and appropriate actions taken.

#### V. RATIONALE AND ACTION PLAN FOR VIRAL CLEARANCE STUDIES AND VIRUS TESTS ON PURIFIED BULK

It is important to design the most relevant and rational protocol for virus tests from the MCB level, through the various steps of drug production, to the final product including evaluation and characterization of viral clearance from unprocessed bulk. The evaluation and characterization of viral clearance plays a critical role in this scheme. The goal should be to obtain the best reasonable assurance that the product is free of virus contamination.

In selecting viruses to use for a clearance study, it is useful to distinguish between the need to evaluate processes for their ability to clear viruses that are known to be present and the desire to estimate the robustness of the process by characterizing the clearance of nonspecific "model" viruses (described later). Definitions of "relevant," specific, and nonspecific "model" viruses are given in the glossary. Process evaluation requires knowledge of how much virus may be present in the process, such as the unprocessed bulk, and how much can be cleared in order to assess product safety. Knowledge of the time dependence for inactivation procedures is helpful in assuring the effectiveness of the inactivation process. When evaluating clearance of known contaminants, in-depth, time-dependent inactivation studies, demonstration of reproducibility of inactivation/removal, and evaluation of process parameters should be provided. When a manufacturing process is characterized for robustness of clearance using nonspecific "model" viruses, particular attention should be paid to nonenveloped viruses in the study design. The extent of viral clearance characterization studies may be influenced by the results of tests on cell lines and unprocessed bulk. These studies should be performed as described in section VI. below.

**Table 4** presents an example of an action plan in terms of process evaluation and characterization of viral clearance as well as virus tests on purified bulk, in response to the results of virus tests on cells and/or the unprocessed bulk. Various cases are considered. In all cases, characterization of clearance using nonspecific "model" viruses should be performed. The most common situations are Cases A and B. Production systems contaminated with a virus other than a rodent retrovirus are normally not used. Where there are convincing and well justified reasons for drug production using a cell line from Cases C, D, or E, these should be discussed with the regulatory authorities. With Cases C, D, and E, it is important to have validated effective steps to inactivate/remove the virus in question from the manufacturing process.

Table 4. Action Plan for Process Assessment of Viral Clearance and Virus Tests on Purified Bulk

	Case A	Case B	Case C <sup>2</sup>	Case D <sup>2</sup>	Case E <sup>2</sup>
Status					
Presence of virus <sup>1</sup>	—	—	+	+	(+) <sup>3</sup>
Virus-like particles <sup>1</sup>	—	—	—	—	(+) <sup>3</sup>
Retrovirus-like particles <sup>1</sup>	—	+	—	—	(+) <sup>3</sup>
Virus identified	not applicable	+	+	+	—



Action	not applicable	- <sup>4</sup>	- <sup>4</sup>	+	unknown
Process characterization of viral clearance using nonspecific "model" viruses	yes <sup>5</sup>	yes <sup>5</sup>	yes <sup>5</sup>	yes <sup>5</sup>	yes <sup>7</sup>
Process evaluation of viral clearance using "relevant" or specific "model" viruses	no	yes <sup>6</sup>	yes <sup>6</sup>	yes <sup>6</sup>	yes <sup>7</sup>
Test for virus in purified bulk	not applicable	yes <sup>8</sup>	yes <sup>8</sup>	yes <sup>8</sup>	yes <sup>8</sup>

1 Results of virus tests for the cell substrate and/or at the unprocessed bulk level. Cell cultures used for production which are contaminated with viruses will generally not be acceptable. Endogenous viruses (such as retroviruses) or viruses that are an integral part of the MCB may be acceptable if appropriate viral clearance evaluation procedures are followed.

2 The use of source material which is contaminated with viruses, whether or not they are known to be infectious and/or pathogenic in humans, will only be acceptable under very exceptional circumstances.

3 Virus has been observed by either direct or indirect methods.

4 Believed to be nonpathogenic.

5 Characterization of clearance using nonspecific "model" viruses should be performed.

6 Process evaluation for "relevant" viruses or specific "model" viruses should be performed.

7 See text under Case E.

8 The absence of detectable virus should be confirmed for purified bulk by means of suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing authorization, data from at least 3 lots of purified bulk manufactured at pilot-plant or commercial scale should be provided. However for cell lines such as CHO cells for which the endogenous particles have been extensively characterized and adequate clearance has been demonstrated, it is not usually necessary to assay for the presence of the noninfectious particles in purified bulk.

Case A: Where no virus, virus-like particle, or retrovirus-like particle has been demonstrated in the cells or in the unprocessed bulk, virus removal and inactivation studies should be performed with nonspecific "model" viruses as previously stated.

Case B: Where only a rodent retrovirus (or a retrovirus-like particle that is believed to be nonpathogenic, such as rodent A- and R-type particles) is present, process evaluation using a specific "model" virus, such as a murine leukemia virus, should be performed. Purified bulk should be tested using suitable methods having high specificity and sensitivity for the detection of the virus in question. For marketing authorization, data from at least three lots of purified bulk at pilot-plant scale or commercial scale should be provided. Cell lines such as Chinese hamster ovary (CHO), C127, baby hamster kidney (BHK), and murine hybridoma cell lines have frequently been used as substrates for drug production with no reported safety problems related to viral contamination of the products. For these cell lines in which the endogenous particles have been extensively characterized and clearance has been demonstrated, it is not usually necessary to assay for the presence of the noninfectious particles in purified bulk. Studies with nonspecific "model" viruses, as in Case A, are appropriate.

Case C: When the cells or unprocessed bulk are known to contain a virus, other than a rodent retrovirus, for which there is no evidence of capacity for infecting humans (such as those identified by footnote 2 in [Table 3](#), except rodent retroviruses (Case B)), virus removal and inactivation evaluation studies should use the identified virus. If it is not possible to use the identified virus, "relevant" or specific "model" viruses should be used to demonstrate acceptable clearance. Time-dependent inactivation for identified (or "relevant" or specific "model") viruses at the critical inactivation step(s) should be obtained as part of process evaluation for these viruses. Purified bulk should be tested using suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing authorization, data from at least three lots of purified bulk manufactured at pilot-plant scale or commercial scale should be provided.

Case D: Where a known human pathogen, such as those indicated by footnote 1 in [Table 3](#), is identified, the product may be acceptable only under exceptional circumstances. In this instance, it is recommended that the identified virus be used for virus removal and inactivation evaluation studies and specific methods with high specificity and sensitivity for the detection of the virus in question be employed. If it is not possible to use the identified virus, "relevant" and/or specific "model" viruses (described later) should be used. The process should be shown to achieve the removal and inactivation of the selected viruses during the purification and inactivation processes. Time-dependent inactivation data for the critical inactivation step(s) should be obtained as part of process evaluation. Purified bulk should be tested using suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing authorization, data from at least three lots of purified bulk manufactured at pilot-plant scale or commercial scale should be provided.

Case E: When a virus that cannot be classified by currently available methodologies is detected in the cells or unprocessed bulk, the product is usually considered unacceptable since the virus may prove to be pathogenic. In the very rare case where there are convincing and well justified reasons for drug production using such a cell line, this should be discussed with the regulatory authorities before proceeding further.

## VI. EVALUATION AND CHARACTERIZATION OF VIRAL CLEARANCE PROCEDURES

Evaluation and characterization of due virus removal and/or inactivation procedures play an important role in establishing the safety of biotechnology products. Many instances of contamination in the past have occurred with agents whose presence was not known or even suspected, and though this happened to biological products derived from various source materials other than fully characterized cell lines, assessment of viral clearance will provide a measure of confidence that any unknown, unsuspected, and harmful viruses may be removed. Studies should be carried out in a manner that is well documented and controlled.

The objective of viral clearance studies is to assess process step(s) that can be considered to be effective in inactivating/removing viruses and to estimate quantitatively the overall level of virus reduction obtained by the process. This should be achieved by the deliberate addition ("spiking") of significant amounts of a virus to the crude material and/or to different fractions obtained during the various process steps and demonstrating its removal or inactivation during the subsequent steps. It is not considered necessary to evaluate or characterize every step of a manufacturing process if adequate clearance is demonstrated by the use of fewer steps. It should be borne in mind that other steps in the process may have an indirect effect on the viral inactivation/removal achieved. Manufacturers should explain and justify the approach used in studies for evaluating virus clearance.

The reduction of virus infectivity may be achieved by removal of virus particles or by inactivation of viral infectivity. For each production step assessed, the possible mechanism of loss of viral infectivity should be described with regard to whether it is due to inactivation or removal. For inactivation steps, the study should be planned in such a way that samples are taken at different times and an inactivation curve constructed (see section VI.B.5.).

Viral clearance evaluation studies are performed to demonstrate the clearance of a virus known to be present in the MCB and/or to provide some level of assurance that adventitious viruses which could not be detected, or might gain access to the production process, would be cleared. Reduction factors are normally expressed on a logarithmic scale, which implies that, while residual virus infectivity will never be reduced to zero, it may be greatly reduced mathematically.

In addition to clearance studies for viruses known to be present, studies to characterize the ability to remove and/or inactivate other viruses should be conducted. The purpose of studies with viruses exhibiting a range of biochemical and biophysical properties that are not known or expected to be present is to characterize the robustness of the procedure rather than to achieve a specific inactivation or removal goal. A demonstration of the capacity of the production process to inactivate or remove viruses is desirable (see section VI.C.). Such studies are not performed to evaluate a specific safety risk. Therefore, a specific clearance value need not be achieved.

### A. The Choice of Viruses for the Evaluation and Characterization of Viral Clearance

Viruses for clearance evaluation and process characterization studies should be chosen to resemble viruses which may contaminate the product and to represent a wide range of physico-chemical properties in order to test the ability of the system to eliminate viruses in general. The manufacturer should justify the choice of viruses in accordance with the aims of the evaluation and characterization study and the guidance provided in this document.

#### 1. "Relevant" Viruses and "Model" Viruses



A major issue in performing a viral clearance study is to determine which viruses should be used. Such viruses fall into three categories: "Relevant" viruses, specific "model" viruses, and nonspecific "model" viruses.

"Relevant" viruses are viruses used in process evaluation of viral clearance studies which are either the identified viruses, or of the same species as the viruses that are known, or likely to contaminate the cell substrate or any other reagents or materials used in the production process. The purification and/or inactivation process should demonstrate the capability to remove and/or inactivate such viruses. When a "relevant" virus is not available or when it is not well adapted to process evaluation of viral clearance studies (e.g., it cannot be grown *in vitro* to sufficiently high titers), a specific "model" virus should be used as a substitute. An appropriate specific "model" virus may be a virus which is closely related to the known or suspected virus (same genus or family), having similar physical and chemical properties to the observed or suspected virus.

Cell lines derived from rodents usually contain endogenous retrovirus particles or retrovirus-like particles, which may be infectious (C-type particles) or noninfectious (cytoplasmic A- and R-type particles). The capacity of the manufacturing process to remove and/or inactivate rodent retroviruses from products obtained from such cells should be determined. This may be accomplished by using a murine leukemia virus, a specific "model" virus in the case of cells of murine origin. When human cell lines secreting monoclonal antibodies have been obtained by the immortalization of B lymphocytes by Epstein-Barr Virus (EBV), the ability of the manufacturing process to remove and/or inactivate a herpes virus should be determined. Pseudorabies virus may also be used as a specific "model" virus.

When the purpose is to characterize the capacity of the manufacturing process to remove and/or inactivate viruses in general, i.e., to characterize the robustness of the clearance process, viral clearance characterization studies should be performed with nonspecific "model" viruses with differing properties. Data obtained from studies with "relevant" and/or specific "model" viruses may also contribute to this assessment. It is not necessary to test all types of viruses. Preference should be given to viruses that display a significant resistance to physical and/or chemical treatments. The results obtained for such viruses provide useful information about the ability of the production process to remove and/or inactivate viruses in general. The choice and number of viruses used will be influenced by the quality and characterization of the cell lines and the production process.

Examples of useful "model" viruses representing a range of physico-chemical structures and examples of viruses which have been used in viral clearance studies are given in Appendix 2 and [Table A-1](#).

Table A-1. Examples of Viruses Which Have Been Used in Viral Clearance Studies

Virus	Family	Genus	Natural Host	Genome	Env	Size (nm)	Shape	Resistance <sup>1</sup>
Vesicular Stomatitis Virus	Rhabdo	Vesiculo-virus	Equine Bovine	RNA	yes	70 × 150	Bullet	Low
Parainfluenza Virus	Paramyxo	Paramyxo-virus	Various	RNA	yes	100–200	Pleo/Spher	Low
MuLV	Retro	Type C oncovirus	Mouse	RNA	yes	80–110	Spherical	Low
Sindbis Virus	Toga	Alphavirus	Human	RNA	yes	60–70	Spherical	Low
BVDV	Flavi	Pestivirus	Bovine	RNA	yes	50–70	Pleo/Spher	Low
Pseudo-rabies Virus	Herpes		Swine	DNA	yes	120–200	Spherical	Med
Poliovirus Sabin Type 1	Picorna	Enterovirus	Human	RNA	no	25–30	Icosahedral	Med
Encephalomyo-carditis Virus (EMC)	Picorna	Cardio-virus	Mouse	RNA	no	25–30	Icosahedral	Med
Reovirus 3	Reo	Orthoreo-virus	Various	DNA	no	60–80	Spherical	Med
SV 40	Papova	Polyoma-virus	Monkey	DNA	no	40–50	Icosahedral	Very high
Parvoviruses (canine, porcine)	Parvo	Parvovirus	Canine Porcine	DNA	no	18–24	Icosahedral	Very high

1 Resistance to physico-chemical treatments based on studies of production processes. Resistance is relative to the specific treatment and it is used in the context of the understanding of the biology of the virus and the nature of the manufacturing process. Actual results will vary according to the treatment. These viruses are examples only and their use is not considered mandatory.

## 2. Other Considerations

Additional points to be considered are as follows:

(a) Viruses which can be grown to high titer are desirable, although this may not always be possible.

(b) There should be an efficient and reliable assay for the detection of each virus used, for every stage of manufacturing that is tested.

(c) Consideration should be given to the health hazard which certain viruses may pose to the personnel performing the clearance studies.

## B. Design and Implications of Viral Clearance Evaluation and Characterization Studies

### 1. Facility and Staff

It is inappropriate to introduce any virus into a production facility because of good manufacturing practice (GMP) constraints. Therefore, viral clearance studies should be conducted in a separate laboratory equipped for virological work and performed by staff with virological expertise in conjunction with production personnel involved in designing and preparing a scaled-down version of the purification process.

### 2. Scaled-down Production System

The validity of the scaling down should be demonstrated. The level of purification of the scaled-down version should represent as closely as possible the production procedure. For chromatographic equipment, column bed-height, linear flow-rate, flow-rate-to-bed-volume ratio (i.e., contact time), buffer and gel types, pH, temperature, and concentration of protein, salt, and product should all be shown to be representative of commercial-scale manufacturing. A similar elution profile should result. For other procedures, similar considerations apply.

Deviations that cannot be avoided should be discussed with regard to their influence on the results.

### 3. Analysis of Step-wise Elimination of Virus

When viral clearance studies are being performed, it is desirable to assess the contribution of more than one production step to virus elimination. Steps which are likely to clear virus should be individually assessed for their ability to remove and inactivate virus and careful consideration should be given to the exact definition of an individual step. Sufficient virus should be present in the material of each step to be tested so that an adequate assessment of the effectiveness of each step is obtained. Generally, virus should be added to in-process material of each step to be tested. In some cases, simply adding high titer virus to unpurified bulk and testing its concentration between steps will be sufficient. Where virus removal results from separation procedures, it is recommended that, if appropriate and if possible, the distribution of the virus load in the different fractions be investigated. When virucidal buffers are used in multiple steps within the manufacturing process, alternative strategies such as parallel spiking in less virucidal buffers may be carried out as part of the overall process assessment. The virus titer before and after each step being tested should be determined. Quantitative infectivity assays should have adequate sensitivity and reproducibility and should be performed with sufficient replicates to ensure adequate statistical validity of the result. Quantitative assays not associated with infectivity may be used if justified. Appropriate virus controls should be included in all infectivity assays to ensure the sensitivity of the method. Also, the statistics of sampling virus when at low concentrations should be considered ([Appendix 3](#)).

### 4. Determining Physical Removal Versus Inactivation

Reduction in virus infectivity may be achieved by the removal or inactivation of virus. For each production step assessed, the possible mechanism of loss of viral infectivity should be described with regard to whether it is due to inactivation or removal. If little clearance of infectivity is achieved by the production process, and the clearance of virus is considered to be a major factor in the safety of the product, specific or additional inactivation/removal steps should be introduced. It may be necessary to distinguish between removal and inactivation



... a particular step, for example, when there is a possibility that a buffer used in more than one clearance step may contribute to inactivation during each step, i.e., the contribution of inactivation by a buffer shared by several chromatographic steps and the removal achieved by each of these chromatographic steps should be distinguished.

#### 5. Inactivation Assessment

For assessment of viral inactivation, unprocessed crude material or intermediate material should be spiked with infectious virus and the reduction factor calculated. It should be recognized that virus inactivation is not a simple, first order reaction and is usually more complex, with a fast "phase 1" and a slow "phase 2." The study should, therefore, be planned in such a way that samples are taken at different times and an inactivation curve constructed. It is recommended that studies for inactivation include at least one time point less than the minimum exposure time and greater than zero, in addition to the minimum exposure time. Additional data are particularly important where the virus is a "relevant" virus known to be a human pathogen and an effective inactivation process is being designed. However, for inactivation studies in which nonspecific "model" viruses are used or when specific "model"

viruses are used as surrogates for virus particles, such as the CHO intracytoplasmic retrovirus-like particles, reproducible clearance should be demonstrated in at least two independent studies. Whenever possible, the initial virus load should be determined from the virus that can be detected in the spiked starting material. If this is not possible, the initial virus load may be calculated from the titer of the spiking virus preparation. Where inactivation is too rapid to plot an inactivation curve using process conditions, appropriate controls should be performed to demonstrate that infectivity is indeed lost by inactivation.

#### 6. Function and Regeneration of Columns

Over time and after repeated use, the ability of chromatography columns and other devices used in the purification scheme to clear virus may vary. Some estimate of the stability of the viral clearance after several uses may provide support for repeated use of such columns. Assurance should be provided that any virus potentially retained by the production system would be adequately destroyed or removed prior to reuse of the system. For example, such evidence may be provided by demonstrating that the cleaning and regeneration procedures do not inactivate or remove virus.

#### 7. Specific Precautions

(a) Care should be taken in preparing the high-titer virus to avoid aggregation which may enhance physical removal and decrease inactivation, thus distorting the correlation with actual production.

(b) Consideration should be given to the minimum quantity of virus which can be reliably assayed.

(c) The study should include parallel control assays to assess the loss of infectivity of the virus due to such reasons as the dilution, concentration, filtration or storage of samples before titration.

(d) The virus "spike" should be added to the product in a small volume so as not to dilute or change the characteristics of the product. Diluted, test-protein sample is no longer identical to the product obtained at commercial scale.

(e) Small differences in, for example, buffers, media, or reagents can substantially affect viral clearance.

(f) Virus inactivation is time-dependent; therefore, the amount of time a spiked product remains in a particular buffer solution or on a particular chromatography column should reflect the conditions of the commercial-scale process.

(g) Buffers and product should be evaluated independently for toxicity or interference in assays used to determine the virus titer, as these components may adversely affect the indicator cells. If the solutions are toxic to the indicator cells, dilution, adjustment of the pH, or dialysis of the buffer containing spiked virus might be necessary. If the product itself has anti-viral activity, the clearance study may need to be performed without the product in a "mock" run, although omitting the product or substituting a similar protein that does not have anti-viral activity could affect the behavior of the virus in some production steps. Sufficient controls to demonstrate the effect of procedures used solely to prepare the sample for assay (e.g., dialysis, storage) on the removal/inactivation of the spiking virus should be included.

(h) Many purification schemes use the same or similar buffers or columns repetitively. The effects of this approach should be taken into account when analyzing the data. The effectiveness of virus elimination by a particular process may vary with the manufacturing stage at which it is used.

(i) Overall reduction factors may be underestimated where production conditions or buffers are too cytotoxic or virucidal and should be discussed on a case-by-case basis. Overall reduction factors may also be overestimated due to inherent limitations or inadequate design of viral clearance studies.

#### C. Interpretation of Viral Clearance Studies; Acceptability

The object of assessing virus inactivation/removal is to evaluate and characterize process steps that can be considered to be effective in inactivating/removing viruses and to estimate quantitatively the overall level of virus reduction obtained by the manufacturing process. For virus contaminants, as in Cases B through E, it is important to show that not only is the virus eliminated or inactivated, but that there is excess capacity for viral clearance built into the purification process to assure an appropriate level of safety for the final product. The amount of virus eliminated or inactivated by the production process should be compared to the amount of virus which may be present in unprocessed bulk.

To carry out this comparison, it is important to estimate the amount of virus in the unprocessed bulk. This estimate should be obtained using assays for infectivity or other methods such as transmission electron microscopy (TEM). The entire purification process should be able to eliminate substantially more virus than is estimated to be present in a single-dose-equivalent of unprocessed bulk. See [Appendix 4](#) for calculation of virus reduction factors and [Appendix 5](#) for calculation of estimated particles per dose.

Manufacturers should recognize that clearance mechanisms may differ between virus classes. A combination of factors should be considered when judging the data supporting the effectiveness of virus inactivation/removal procedures. These include:

- (i) The appropriateness of the test viruses used;
- (ii) The design of the clearance studies;
- (iii) The log reduction achieved;
- (iv) The time dependence of inactivation;
- (v) The potential effects of variation in process parameters on virus inactivation/removal;
- (vi) The limits of assay sensitivities;
- (vii) The possible selectivity of inactivation/removal procedure(s) for certain classes of viruses.

Effective clearance may be achieved by any of the following: Multiple inactivation steps, multiple complementary separation steps, or combinations of inactivation and separation steps.

Since separation methods may be dependent on the extremely specific physico-chemical properties of a virus which influence its interaction with gel matrices and precipitation properties, "model" viruses may be separated in a different manner than a target virus. Manufacturing parameters influencing separation should be properly defined and controlled. Differences may originate from changes in surface properties such as glycosylation. However, despite these potential variables, effective removal can be obtained by a combination of complementary separation steps or combinations of inactivation and separation steps. Therefore, well-designed separation steps, such as chromatographic procedures, filtration steps, and extractions, can be effective virus removal steps provided that they are performed under appropriately controlled conditions. An effective virus removal step should give a reproducible reduction of virus load shown by at least two independent studies.

An overall reduction factor is generally expressed as the sum of the individual factors. However, reduction in virus titer of the order of 1 log<sub>10</sub> or less would be considered negligible and would be ignored unless justified.

If little reduction of infectivity is achieved by the production process, and the removal of virus is considered to be a major factor in the safety of the product, a specific, additional inactivation/removal step or steps should be introduced. For all viruses, manufacturers should justify the acceptability of the reduction factors obtained. Results would be evaluated on the basis of the factors listed above.

#### D. Limitations of Viral Clearance Studies



Viral clearance studies are useful for contributing to the assurance that an acceptable level of safety in the final product is achieved but do not by themselves establish safety. However, a number of factors in the design and execution of viral clearance studies may lead to an incorrect estimate of the ability of the process to remove virus infectivity. These factors include the following:

1. Virus preparations used in clearance studies for a production process are likely to be produced in tissue culture. The behavior of a tissue culture virus in a production step may be different from that of the native virus, for example, if native and cultured viruses differ in purity or degree of aggregation.
2. Inactivation of virus infectivity frequently follows a biphasic curve in which a rapid initial phase is followed by a slower phase. It is possible that virus escaping a first inactivation step may be more resistant to subsequent steps. For example, if the resistant fraction takes the form of virus aggregates, infectivity may be resistant to a range of different chemical treatments and to heating.
3. The ability of the overall process to remove infectivity is expressed as the sum of the logarithm of the reductions at each step. The summation of the reduction factors of multiple steps, particularly of steps with little reduction (e.g., below 1 log<sub>10</sub>), may overestimate the true potential for virus elimination. Furthermore, reduction values achieved by repetition of identical or near identical procedures should not be included unless justified.
4. The expression of reduction factors as logarithmic reductions in titer implies that, while residual virus infectivity may be greatly reduced, it will never be reduced to zero. For example, a reduction in the infectivity of a preparation containing 8 log<sub>10</sub> infectious units per milliliter (mL) by a factor of 8 log<sub>10</sub> leaves zero log<sub>10</sub> per mL or one infectious unit per mL, taking into consideration the limit of detection of the assay.
5. Pilot-plant scale processing may differ from commercial-scale processing despite care taken to design the scaled-down process.
6. Addition of individual virus reduction factors resulting from similar inactivation mechanisms along the manufacturing process may overestimate overall viral clearance.

#### E. Statistics

The viral clearance studies should include the use of statistical analysis of the data to evaluate the results. The study results should be statistically valid to support the conclusions reached (see Appendix 3).

#### F. Reevaluation of Viral Clearance

Whenever significant changes in the production or purification process are made, the effect of that change, both direct and indirect, on viral clearance should be considered and the system re-evaluated as needed. For example, changes in production processes may cause significant changes in the amount of virus produced by the cell line; changes in process steps may change the extent of viral clearance.

## VII. SUMMARY

This document suggests approaches for the evaluation of the risk of viral contamination and for the removal of virus from product, thus contributing to the production of safe biotechnology products derived from animal or human cell lines, and emphasizes the value of many strategies, including:

- A. Thorough characterization/screening of cell substrate starting material in order to identify which, if any, viral contaminants are present;
- B. Assessment of risk by determination of the human tropism of the contaminants;
- C. Establishment of an appropriate program of testing for adventitious viruses in unprocessed bulk;
- D. Careful design of viral clearance studies using different methods of virus inactivation or removal in the same production process in order to achieve maximum viral clearance; and
- E. Performance of studies which assess virus inactivation and removal.

## GLOSSARY

Adventitious Virus. See Virus.

Cell Substrate. Cells used to manufacture product.

Endogenous Virus. See Virus.

Inactivation. Reduction of virus infectivity caused by chemical or physical modification.

In Vitro Cell Age. A measure of the period between thawing of the MCB vial(s) and harvest of the production vessel measured by elapsed chronological time in culture, population doubling level of the cells, or passage level of the cells when subcultivated by a defined procedure for dilution of the culture.

Master Cell Bank (MCB). An aliquot of a single pool of cells which generally has been prepared from the selected cell clone under defined conditions, dispensed into multiple containers, and stored under defined conditions. The MCB is used to derive all working cell banks. The testing performed on a new MCB (from a previous initial cell clone, MCB, or WCB) should be the same as for the original MCB, unless justified.

Minimum Exposure Time. The shortest period for which a treatment step will be maintained.

Nonendogenous Virus. See Virus.

Process Characterization of Viral Clearance. Viral clearance studies in which nonspecific "model" viruses are used to assess the robustness of the manufacturing process to remove and/or inactivate viruses.

Process Evaluation Studies of Viral Clearance. Viral clearance studies in which "relevant" and/or specific "model" viruses are used to determine the ability of the manufacturing process to remove and/or inactivate these viruses.

Production Cells. Cell substrate used to manufacture product.

Unprocessed Bulk. One or multiple pooled harvests of cells and culture media. When cells are not readily accessible, the unprocessed bulk would constitute fluid harvested from the fermenter.

Virus. Intracellularly replicating infectious agents that are potentially pathogenic, possess only a single type of nucleic acid (either ribonucleic acid (RNA) or DNA), are unable to grow and undergo binary fission, and multiply in the form of their genetic material.

Adventitious Virus. Unintentionally introduced contaminant virus.

Endogenous Virus. Viral entity whose genome is part of the germ line of the species of origin of the cell line and is covalently integrated into the genome of animal from which the parental cell line was derived. For the purposes of this document, intentionally introduced, nonintegrated viruses such as EBV used to immortalize cell substrates or Bovine Papilloma Virus fit in this category.

Nonendogenous Virus. Virus from external sources present in the MCB.

Nonspecific Model Virus. A virus used for characterization of viral clearance of the process when the purpose is to characterize the capacity of the manufacturing process to remove and/or inactivate viruses in general, i.e., to characterize the robustness of the purification process.

Relevant Virus. Virus used in process evaluation studies which is either the identified virus, or of the same species as the virus that is known, or likely to contaminate the cell substrate or any other reagents or materials used in the production process.



specific Model Virus. Virus which is closely related to the known or suspected virus (same genus or family), having similar physical and chemical properties to those of the observed or suspected virus.

Viral Clearance. Elimination of target virus by removal of viral particles or inactivation of viral infectivity.

Virus-like Particles. Structures visible by electron microscopy which morphologically appear to be related to known viruses.

Virus Removal. Physical separation of virus particles from the intended product.

Working Cell Bank (WCB). The WCB is prepared from aliquots of a homogeneous suspension of cells obtained from culturing the MCB under defined culture conditions.

#### APPENDIX 1

##### Products Derived from Characterized Cell Banks Which Were Subsequently Grown In Vivo

For products manufactured from fluids harvested from animals inoculated with cells from characterized banks, additional information regarding the animals should be provided.

Whenever possible, animals used in the manufacture of biotechnological/biological products should be obtained from well defined, specific pathogen-free colonies. Adequate testing for appropriate viruses, such as those listed in [Table 3](#), should be performed. Quarantine procedures for newly arrived as well as diseased animals should be described, and assurance provided that all containment, cleaning, and decontamination methodologies employed within the facility are adequate to contain the spread of adventitious agents. This may be accomplished through the use of a sentinel program. A listing of agents for which testing is performed should also be included. Veterinary support services should be available on-site or within easy access. The degree to which the vivarium is segregated from other areas of the manufacturing facility should be described. Personnel practices should be adequate to ensure safety.

Procedures for the maintenance of the animals should be fully described. These would include diet, cleaning and feeding schedules, provisions for periodic veterinary care if applicable, and details of special handling that the animals may require once inoculated. A description of the priming regimen(s) for the animals, the preparation of the inoculum, and the site and route of inoculation should also be included.

The primary harvest material from animals may be considered an equivalent stage of manufacture to unprocessed bulk harvest from a bioreactor. Therefore, all testing considerations previously outlined in section IV. of this document should apply. In addition, the manufacturer should assess the bioburden of the unprocessed bulk, determine whether the material is free of mycoplasma, and perform species-specific assay(s) as well as in vivo testing in adult and suckling mice.

#### APPENDIX 2

##### The Choice of Viruses for Viral Clearance Studies

###### A. Examples of Useful "Model" Viruses:

###### 1. Nonspecific "model" viruses representing a range of physico-chemical structures:

- SV40 (Polyomavirus maccacae 1), human polio virus 1 (Sabin), animal parvovirus or some other small, nonenveloped viruses;
- a parainfluenza virus or influenza virus, Sindbis virus or some other medium-to-large, enveloped, RNA viruses;
- a herpes virus (e.g., HSV-1 or a pseudorabies virus), or some other medium-to-large, DNA viruses.

These viruses are examples only and their use is not mandatory.

###### 2. For rodent cell substrates murine retroviruses are commonly used as specific "model" viruses.

###### B. Examples of Viruses That Have Been Used in Viral Clearance Studies

Several viruses that have been used in viral clearance studies are listed in [Table A-1](#). However, since these are merely examples, the use of any of the viruses in the table is not considered mandatory and manufacturers are invited to consider other viruses, especially those that may be more appropriate for their individual production processes. Generally, the process should be assessed for its ability to clear at least three different viruses with differing characteristics.

#### APPENDIX 3

##### A. Statistical Considerations for Assessing Virus Assays

Virus titrations suffer the problems of variation common to all biological assay systems. Assessment of the accuracy of the virus titrations and reduction factors derived from them and the validity of the assays should be performed to define the reliability of a study. The objective of statistical evaluation is to establish that the study has been carried out to an acceptable level of virological competence.

1. Assay methods may be either quantal or quantitative. Quantal methods include infectivity assays in animals or in tissue-culture-infectious-dose (TCID) assays, in which the animal or cell culture is scored as either infected or not. Infectivity titers are then measured by the proportion of animals or culture infected. In quantitative methods, the infectivity measured varies continuously with the virus input. Quantitative methods include plaque assays where each plaque counted corresponds to a single infectious unit. Both quantal and quantitative assays are amenable to statistical evaluation.

2. Variation can arise within an assay as a result of dilution errors, statistical effects, and differences within the assay system which are either unknown or difficult to control. These effects are likely to be greater when different assay runs are compared (between-assay variation) than when results within a single assay run are compared (within-assay variation).

3. The 95 percent confidence limits for results of within-assay variation normally should be on the order of  $\pm 0.5 \log_{10}$  of the mean. Within-assay variation can be assessed by standard textbook methods. Between-assay variation can be monitored by the inclusion of a reference preparation, the estimate of whose potency should be within approximately  $0.5 \log_{10}$  of the mean estimate established in the laboratory for the assay to be acceptable. Assays with lower precision may be acceptable with appropriate justification.

4. The 95 percent confidence limits for the reduction factor observed should be calculated wherever possible in studies of clearance of "relevant" and specific "model" viruses. If the 95 percent confidence limits for the viral assays of the starting material are  $+s$ , and for the viral assays of the material after the step are  $+a$ , the 95 percent confidence limits for the reduction factor are

$$\pm \sqrt{s^2 + a^2}$$

###### B. Probability of Detection of Viruses at Low Concentrations

At low virus concentrations (e.g., in the range of 10 to 1,000 infectious particles per L) it is evident that a sample of a few milliliters may or may not contain infectious particles. The probability,  $p$ , that this sample does not contain infectious viruses is:

$$p = ((V-v) / V)n$$

where  $V$  (L) is the overall volume of the material to be tested;  $v$  (L) is the volume of the sample; and  $n$  is the absolute number of infectious particles statistically distributed in  $V$ .



If  $V \gg v$ , this equation can be approximated by the Poisson distribution:

$$p = e^{-cv}$$

where  $c$  is the concentration of infectious particles per L.

$$\text{or, } c = \ln p / -v$$

As an example, if a sample volume of 1 mL is tested, the probabilities  $p$  at virus concentrations ranging from 10 to 1,000 infectious particles per L are:

c	10	1,000
p	0.99	0.90

This indicates that for a concentration of 1,000 viruses per L, in 37 percent of sampling, 1 mL will not contain a virus particle.

If only a portion of a sample is tested for virus and the test is negative, the amount of virus which would have to be present in the total sample in order to achieve a positive result should be calculated and this value taken into account when calculating a reduction factor. Confidence limits at 95 percent are desirable. However, in some instances, this may not be practical due to material limitations.

#### APPENDIX 4

##### Calculation of Reduction Factors in Studies to Determine Viral Clearance

The virus reduction factor of an individual purification or inactivation step is defined as the  $\log_{10}$  of the ratio of the virus load in the pre-purification material and the virus load in the post-purification material which is ready for use in the next step of the process. If the following abbreviations are used:

Starting material: vol  $v'$ ; titer  $10a'$ ;

virus load:  $(v')(10a')$ ,

Final material: vol  $v''$ ; titer  $10a''$ ;

virus load:  $(v'')(10a'')$ ,

the individual reduction factors  $R_i$  are calculated according to

$$10R_i = (v')(10a') / (v'')(10a'')$$

This formula takes into account both the titers and volumes of the materials before and after the purification step.

Because of the inherent imprecision of some virus titrations, an individual reduction factor used for the calculation of an overall reduction factor should be greater than 1.

The overall reduction factor for a complete production process is the sum logarithm of the reduction factors of the individual steps. It represents the logarithm of the ratio of the virus load at the beginning of the first process clearance step and at the end of the last process clearance step. Reduction factors are normally expressed on a logarithmic scale which implies that, while residual virus infectivity will never be reduced to zero, it may be greatly reduced mathematically.

#### APPENDIX 5

##### Calculation of Estimated Particles per Dose

This is applicable to those viruses for which an estimate of starting numbers can be made, such as endogenous retroviruses.

Example:

###### I. Assumptions

Measured or estimated concentration of virus in cell culture harvest =  $10^6$ /mL

Calculated viral clearance factor =  $> 10^{15}$

Volume of culture harvest needed to make a dose of product = 1 L (103 mL)

###### II. Calculation of Estimated Particles/Dose

$$\begin{aligned} & \frac{(10^6 \text{ virus units/mL}) \times (10^3 \text{ mL/dose})}{\text{Clearance factor} > 10^{15}} \\ &= \frac{10^9 \text{ particles/dose}}{\text{Clearance factor} > 10^{15}} \\ &= < 10^{-6} \text{ particles/dose} \end{aligned}$$

Therefore, less than one particle per million doses would be expected.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Tina S. Morris, Ph.D.</a> Director, Biologics and Biotechnology 1-301-816-8397	(BBV05) Biologics and Biotechnology - Vaccines and Virology

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#### 1051 CLEANING GLASS APPARATUS

Success in conducting many Pharmacopeial assays and tests depends upon the utmost cleanliness of the glassware apparatus used. For example, the accuracy of the assays of heparin sodium and vitamin B12 activity, as well as the pyrogen and total organic carbon tests, are particularly dependent upon scrupulously clean glassware.

One effective method used in the past for cleaning glassware is the application of hot nitric acid. A second traditional method for removing organic matter that does not require heat is the use of a chromic acid-sulfuric acid mixture. However, the chromic acid wash is not recommended because of the hazardous and toxic nature of the material.

Several safer alternatives, including the use of cleansing agents, such as trisodium phosphate and synthetic detergents, have proven highly useful, but require prolonged rinsing. It may be useful to rinse with diluted nitric or sulfuric acid prior to rinsing with water. This operation will facilitate removal of residual alkaline material.



For optical measurements, special care is required for cleaning containers, but the use of both chromic acid and highly alkaline solutions should be avoided.

Effective removal of organic matter is very important for testing pharmaceutical waters in accordance with the general test chapter [Total Organic Carbon \(643\)](#). It has been demonstrated that an alkaline detergent with potassium hydroxide as the primary ingredient\* leaves the least amount of organic matter residuals. Heating in a muffle furnace produces comparable results and is the least labor-intensive procedure; however, it requires specialized equipment.

In all cases, it is important to verify that the cleaning procedure is appropriate for the particular test or assay being undertaken. This can be accomplished via blank runs, scientific judgments, residuals data from cleansing agent and detergent manufacturers, or other controls. Specifically, special care is required for cleaning containers for optical measurement applications; the use of highly alkaline and the no longer recommended chromic acid solutions should be avoided. Finally, a statement should be included in the cleaning protocol describing how the success of the cleaning procedure will be assessed.

\* CIP 100; available from Steris Corporation, Mentor, Ohio, 44060-1824.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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### 1052 BIOTECHNOLOGY-DERIVED ARTICLES—AMINO ACID ANALYSIS

This chapter provides guidance and procedures used for characterization of biotechnology-derived articles by amino acid analysis. This chapter is harmonized with the corresponding chapter in JP and EP. Other characterization tests, also harmonized, are shown in [Biotechnology-Derived Articles—Capillary Electrophoresis \(1053\)](#), [Biotechnology-Derived Articles—Isoelectric Focusing \(1054\)](#), [Biotechnology-Derived Articles—Peptide Mapping \(1055\)](#), [Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis \(1056\)](#), and [Biotechnology-Derived Articles—Total Protein Assay \(1057\)](#).

#### INTRODUCTION

Amino acid analysis refers to the methodology used to determine the amino acid composition or content of proteins, peptides, and other pharmaceutical preparations. Proteins and peptides are macromolecules consisting of covalently bonded amino acid residues organized as a linear polymer. The sequence of the amino acids in a protein or peptide determines the properties of the molecule. Proteins are considered large molecules that commonly exist as folded structures with a specific conformation, while peptides are smaller and may consist of only a few amino acids. Amino acid analysis can be used to quantify protein and peptides, to determine the identity of proteins or peptides based on their amino acid composition, to support protein and peptide structure analysis, to evaluate fragmentation strategies for peptide mapping, and to detect atypical amino acids that might be present in a protein or peptide. It is necessary to hydrolyze a protein/peptide to its individual amino acid constituents before amino acid analysis. Following protein/peptide hydrolysis, the amino acid analysis procedure can be the same as that practiced for free amino acids in other pharmaceutical preparations. The amino acid constituents of the test sample are typically derivatized for analysis.

#### APPARATUS

Methods used for amino acid analysis are usually based on a chromatographic separation of the amino acids present in the test sample. Current techniques take advantage of the automated chromatographic instrumentation designed for analytical methodologies. An amino acid analysis instrument will typically be a low-pressure or high-pressure liquid chromatograph capable of generating mobile phase gradients that separate the amino acid analytes on a chromatographic column. The instrument must have postcolumn derivatization capability, unless the sample is analyzed using precolumn derivatization. The detector is usually a UV-visible or fluorescence detector depending on the derivatization method used. A recording device (e.g., integrator) is used for transforming the analog signal from the detector and for quantitation. It is preferred that instrumentation be dedicated particularly for amino acid analysis.

#### GENERAL PRECAUTIONS

Background contamination is always a concern for the analyst in performing amino acid analysis. High-purity reagents are necessary (e.g., low-purity hydrochloric acid can contribute to glycine contamination). Analytical reagents are changed routinely every few weeks using only high-pressure liquid chromatography (HPLC) grade solvents. Potential microbial contamination and foreign material that might be present in the solvents are reduced by filtering solvents before use, keeping solvent reservoirs covered, and not placing amino acid analysis instrumentation in direct sunlight.

Laboratory practices can determine the quality of the amino acid analysis. Place the instrumentation in a low traffic area of the laboratory. Keep the laboratory clean. Clean and calibrate pipets according to a maintenance schedule. Keep pipet tips in a covered box; the analysts may not handle pipet tips with their hands. The analysts may wear powder-free latex or equivalent gloves. Limit the number of times a test sample vial is opened and closed because dust can contribute to elevated levels of glycine, serine, and alanine.

A well-maintained instrument is necessary for acceptable amino acid analysis results. If the instrument is used on a routine basis, it is to be checked daily for leaks, detector and lamp stability, and the ability of the column to maintain resolution of the individual amino acids. Clean or replace all instrument filters and other maintenance items on a routine schedule.

#### REFERENCE STANDARD MATERIAL

Acceptable amino acid standards are commercially available\* for amino acid analysis and typically consist of an aqueous mixture of amino acids. When determining amino acid composition, protein or peptide standards are analyzed with the test material as a control to demonstrate the integrity of the entire procedure. Highly purified bovine serum albumin has been used as a protein standard for this purpose.

#### CALIBRATION OF INSTRUMENTATION

Calibration of amino acid analysis instrumentation typically involves analyzing the amino acid standard, which consists of a mixture of amino acids at a number of concentrations, to determine the response factor and range of analysis for each amino acid. The concentration of each amino acid in the standard is known. In the calibration procedure, the analyst dilutes the amino acid standard to several different analyte levels within the expected linear range of the amino acid analysis technique. Then, replicates at each of the different analyte levels can be analyzed. Peak areas obtained for each amino acid are plotted versus the known concentration for each of the amino acids in the standard dilution. These results will allow the analyst to determine the range of amino acid concentrations where the peak area of a given amino acid is an approximately linear function of the amino acid concentration. It is important that the analyst prepare the samples for amino acid analysis so that they are within the analytical limits (e.g., linear working range) of the technique employed in order to obtain accurate and repeatable results.

Four to six amino acid standard levels are analyzed to determine a response factor for each amino acid. The response factor is calculated as the average peak area or peak height per nmol of amino acid present in the standard. A calibration file consisting of the response factor for each amino acid is prepared and is used to calculate the concentration of each amino acid present in the test sample. This calculation involves dividing the peak area corresponding to a given amino acid by the response factor for that amino acid to give the nmol of the amino acid. For routine analysis, a single-point calibration may be sufficient; however, the calibration file is updated frequently and tested by the analysis of analytical controls to ensure



its integrity.

#### REPEATABILITY

Consistent high quality amino acid analysis results from an analytical laboratory require attention to the repeatability of the assay. During analysis of the chromatographic separation of the amino acids or their derivatives, numerous peaks can be observed on the chromatogram that corresponds to the amino acids. The large number of peaks makes it necessary to have an amino acid analysis system that can repeatedly identify the peaks based on retention time and integrate the peak areas for quantitation. A typical repeatability evaluation involves preparing a standard amino acid solution and analyzing many replicates (i.e., six analyses or more) of the same standard solution. The relative standard deviation (RSD) is determined for the retention time and integrated peak area of each amino acid. An evaluation of the repeatability is expanded to include multiple assays conducted over several days by different analysts. Multiple assays include the preparation of standard dilutions from starting materials to determine the variation due to sample handling. Often, the amino acid composition of a standard protein (e.g., bovine serum albumin) is analyzed as part of the repeatability evaluation. By evaluating the replicate variation (i.e., RSD), the laboratory can establish analytical limits to ensure that the analyses from the laboratory are under control. It is desirable to establish the lowest practical variation limits to ensure the best results.

Areas to focus on to lower the variability of the amino acid analysis include sample preparation, high background spectral interference due to the quality of reagents and/or to laboratory practices, instrument performance and maintenance, data analysis and interpretation, and analyst performance and habits. All parameters involved are fully investigated in the scope of the validation work.

#### SAMPLE PREPARATION

Accurate results from amino acid analysis require purified protein and peptide samples. Buffer components (e.g., salts, urea, detergents) can interfere with the amino acid analysis and are removed from the sample before analysis. Methods that utilize postcolumn derivatization of the amino acids are generally not affected by buffer components to the extent seen with precolumn derivatization methods. It is desirable to limit the number of sample manipulations to reduce potential background contamination, to improve analyte recovery, and to reduce labor. Common techniques used to remove buffer components from protein samples include the following methods: (1) injecting the protein sample onto a reverse-phase HPLC system, removing the protein with a volatile solvent containing a sufficient organic component, and drying the sample in a vacuum centrifuge; (2) dialysis against a volatile buffer or water; (3) centrifugal ultrafiltration for buffer replacement with a volatile buffer or water; (4) precipitating the protein from the buffer using an organic solvent (e.g., acetone); and (5) gel filtration.

#### INTERNAL STANDARDS

It is recommended that an internal standard be used to monitor physical and chemical losses and variations during amino acid analysis. An accurately known amount of internal standard can be added to a protein solution prior to hydrolysis. The recovery of the internal standard gives the general recovery of the amino acids from the protein solution. Free amino acids, however, do not behave in the same way as protein-bound amino acids during hydrolysis because their rates of release or destruction are variable. Therefore, the use of an internal standard to correct for losses during hydrolysis may give unreliable results. It will be necessary to take this particular point into consideration when interpreting the results.

Internal standards can also be added to the mixture of amino acids after hydrolysis to correct for differences in sample application and changes in reagent stability and flow rates. Ideally, an internal standard is an unnaturally occurring primary amino acid that is commercially available and inexpensive. It should also be stable during hydrolysis, its response factor should be linear with concentration, and it needs to elute with a unique retention time without overlapping other amino acids. Commonly used amino acid standards include norleucine, nitrotyrosine, and  $\alpha$ -aminobutyric acid.

#### PROTEIN HYDROLYSIS

Hydrolysis of protein and peptide samples is necessary for amino acid analysis of these molecules. The glassware used for hydrolysis must be very clean to avoid erroneous results.

Glove powders and fingerprints on hydrolysis tubes may cause contamination. To clean glass hydrolysis tubes, boil tubes for 1 hour in 1 N hydrochloric acid or soak tubes in concentrated nitric acid or in a mixture of concentrated hydrochloric acid and concentrated nitric acid (1:1). Clean hydrolysis tubes are rinsed with high-purity water followed by a rinse with HPLC grade methanol, dried overnight in an oven, and stored covered until use. Alternatively, pyrolysis of clean glassware at 500 $^{\circ}$  for 4 hours may be used to eliminate contamination from hydrolysis tubes. Adequate disposable laboratory material can also be used.

Acid hydrolysis is the most common method for hydrolyzing a protein sample before amino acid analysis. The acid hydrolysis technique can contribute to the variation of the analysis due to complete or partial destruction of several amino acids. Tryptophan is destroyed; serine and threonine are partially destroyed; methionine might undergo oxidation; and cysteine is typically recovered as cystine (but cystine recovery is usually poor because of partial destruction or reduction to cysteine). Application of adequate vacuum (less than 200  $\mu$ m of mercury or 26.7 Pa) or introduction of an inert gas (argon) in the headspace of the reaction vessel can reduce the level of oxidative destruction. In peptide bonds involving isoleucine and valine, the amido bonds of Ile-Ile, Val-Val, Ile-Val, and Val-Ile are partially cleaved; and asparagine and glutamine are deamidated, resulting in aspartic acid and glutamic acid, respectively. The loss of tryptophan, asparagine, and glutamine during an acid hydrolysis limits quantitation to 17 amino acids. Some of the hydrolysis techniques described are used to address these concerns. Some of the hydrolysis techniques described (i.e., Methods 4–11) may cause modifications to other amino acids. Therefore, the benefits of using a given hydrolysis technique are weighed against the concerns with the technique and are tested adequately before employing a method other than acid hydrolysis.

A time-course study (i.e., amino acid analysis at acid hydrolysis times of 24, 48, and 72 hours) is often employed to analyze the starting concentration of amino acids that are partially destroyed or slow to cleave. By plotting the observed concentration of labile amino acids (i.e., serine and threonine) versus hydrolysis time, the line can be extrapolated to the origin to determine the starting concentration of these amino acids. Time-course hydrolysis studies are also used with amino acids that are slow to cleave (e.g., isoleucine and valine). During the hydrolysis time course, the analyst will observe a plateau in these residues. The level of this plateau is taken as the residue concentration. If the hydrolysis time is too long, the residue concentration of the sample will begin to decrease, indicating destruction by the hydrolysis conditions.

An acceptable alternative to the time-course study is to subject an amino acid calibration standard to the same hydrolysis conditions as the test sample. The amino acid in free form may not completely represent the rate of destruction of labile amino acids within a peptide or protein during the hydrolysis. This is especially true for peptide bonds that are slow to cleave (e.g., Ile-Val bonds). However, this technique will allow the analyst to account for some residue destruction. Microwave acid hydrolysis has been used and is rapid but it requires special equipment as well as special precautions. The optimal conditions for microwave hydrolysis must be investigated for each individual protein/peptide sample. The microwave hydrolysis technique typically requires only a few minutes, but even a deviation of 1 minute may give inadequate results (e.g., incomplete hydrolysis or destruction of labile amino acids). Complete proteolysis, using a mixture of proteases, has been used but can be complicated, requires the proper controls, and is typically more applicable to peptides than proteins. [note—During initial analyses of an unknown protein, experiments with various hydrolysis time and temperature conditions are conducted to determine the optimal conditions.]

#### Method 1

Acid hydrolysis using hydrochloric acid containing phenol is the most common procedure used for protein/peptide hydrolysis preceding amino acid analysis. The addition of phenol to the reaction prevents the halogenation of tyrosine.

Hydrolysis Solution: 6 N hydrochloric acid containing 0.1% to 1.0% of phenol.

#### Procedure—

Liquid Phase Hydrolysis—Place the protein or peptide sample in a hydrolysis tube, and dry. [note—The sample is dried so that water in the sample will not dilute the acid used for the hydrolysis.] Add 200  $\mu$ L of Hydrolysis Solution per 500  $\mu$ g of lyophilized protein. Freeze the sample tube in a dry ice–acetone bath, and flame seal in vacuum. Samples are typically hydrolyzed at 110 $^{\circ}$  for 24 hours in vacuum or inert atmosphere to prevent oxidation. Longer hydrolysis times (e.g., 48 and 72 hours) are investigated if there is a concern that the protein is not completely hydrolyzed.

Vapor Phase Hydrolysis—This is one of the most common acid hydrolysis procedures, and it is preferred for microanalysis when only small amounts of the sample are available. Contamination of the sample from the acid reagent is also minimized by using vapor phase hydrolysis. Place vials containing the dried samples in a vessel that contains an appropriate amount of Hydrolysis Solution. The Hydrolysis Solution does not come in contact with the test sample. Apply an inert atmosphere or vacuum (less than 200  $\mu$ m of mercury or 26.7 Pa)



to the headspace of the vessel, and heat to about  $110^{\circ}\text{C}$  for a 24-hour hydrolysis time. Acid vapor hydrolyzes the dried sample. Any condensation of the acid in the sample vials is minimized. After hydrolysis, dry the test sample in vacuum to remove any residual acid.

#### Method 2

Tryptophan oxidation during hydrolysis is decreased by using mercaptoethanesulfonic acid (MESA) as the reducing acid.

Hydrolysis Solution: 2.5 M MESA solution.

Vapor Phase Hydrolysis— About 1 to 100  $\mu\text{g}$  of the protein/peptide under test is dried in a hydrolysis tube. The hydrolysis tube is placed in a larger tube with about 200  $\mu\text{L}$  of the Hydrolysis Solution. The larger tube is sealed in vacuum (about 50  $\mu\text{m}$  of mercury or 6.7 Pa) to vaporize the Hydrolysis Solution. The hydrolysis tube is heated to between  $170^{\circ}\text{C}$  to  $185^{\circ}\text{C}$  for about 12.5 minutes. After hydrolysis, the hydrolysis tube is dried in vacuum for 15 minutes to remove the residual acid.

#### Method 3

Tryptophan oxidation during hydrolysis is prevented by using thioglycolic acid (TGA) as the reducing acid.

Hydrolysis Solution: a solution containing 7 M hydrochloric acid, 10% of trifluoroacetic acid, 20% of thioglycolic acid, and 1% of phenol.

Vapor Phase Hydrolysis— About 10 to 50  $\mu\text{g}$  of the protein/peptide under test is dried in a sample tube. The sample tube is placed in a larger tube with about 200  $\mu\text{L}$  of the Hydrolysis Solution. The larger tube is sealed in vacuum (about 50  $\mu\text{m}$  of mercury or 6.7 Pa) to vaporize the TGA. The sample tube is heated to  $166^{\circ}\text{C}$  for about 15 to 30 minutes. After hydrolysis, the sample tube is dried in vacuum for 5 minutes to remove the residual acid. Recovery of tryptophan by this method may be dependent on the amount of sample present.

#### Method 4

Cysteine-cystine and methionine oxidation is performed with formic acid before the protein hydrolysis.

Oxidation Solution— The formic acid is prepared fresh by mixing formic acid and 30 percent hydrogen peroxide (9:1), and incubating at room temperature for 1 hour.

Procedure— The protein/peptide sample is dissolved in 20  $\mu\text{L}$  of formic acid, and heated at  $50^{\circ}\text{C}$  for 5 minutes; then 100  $\mu\text{L}$  of the Oxidation Solution is added. In this reaction, cysteine is converted to cysteic acid and methionine is converted to methionine sulfone. The oxidation is allowed to proceed for 10 to 30 minutes. The excess reagent is removed from the sample in a vacuum centrifuge. This technique may cause modifications to tyrosine residues in the presence of halides. The oxidized protein can then be acid hydrolyzed using Method 1 or Method 2.

#### Method 5

Cysteine-cystine oxidation is accomplished during the liquid phase hydrolysis with sodium azide.

Hydrolysis Solution: 6 N hydrochloric acid containing 0.2% of phenol, to which sodium azide is added to obtain a final concentration of 0.2% (w/v). The added phenol prevents halogenation of tyrosine.

Liquid Phase Hydrolysis— The protein/peptide hydrolysis is conducted at about  $110^{\circ}\text{C}$  for 24 hours. During the hydrolysis, the cysteine-cystine present in the sample is converted to cysteic acid by the sodium azide present in the Hydrolysis Solution. This technique allows better tyrosine recovery than Method 4, but it is not quantitative for methionine. Methionine is converted to a mixture of the parent methionine and its two oxidative products, methionine sulfoxide and methionine sulfone.

#### Method 6

Cysteine-cystine oxidation is accomplished with dimethyl sulfoxide (DMSO).

Hydrolysis Solution: 6 N hydrochloric acid containing 0.1% to 1.0% of phenol, to which DMSO is added to obtain a final concentration of 2% (v/v).

Vapor Phase Hydrolysis— The protein/peptide hydrolysis is conducted at about  $110^{\circ}\text{C}$  for 24 hours. During the hydrolysis, the cysteine-cystine present in the sample is converted to cysteic acid by the DMSO present in the Hydrolysis Solution. As an approach to limit variability and to compensate for partial destruction, it is recommended to evaluate the cysteic acid recovery from oxidative hydrolyses of standard proteins containing 1 to 8 mol of cysteine. The response factors from protein/peptide hydrolysates are typically about 30% lower than those for nonhydrolyzed cysteic acid standards. Because histidine, methionine, tyrosine, and tryptophan are also modified, a complete compositional analysis is not obtained with this technique.

#### Method 7

Cysteine-cystine reduction and alkylation is accomplished by a vapor phase pyridylethylation reaction.

Reducing Solution— Transfer 83.3  $\mu\text{L}$  of pyridine, 16.7  $\mu\text{L}$  of 4-vinylpyridine, 16.7  $\mu\text{L}$  of tributylphosphine, and 83.3  $\mu\text{L}$  of water to a suitable container, and mix.

Procedure— Add the protein/peptide (between 1 and 100  $\mu\text{g}$ ) to a hydrolysis tube, and place in a larger tube. Transfer the Reducing Solution to the large tube, seal in vacuum (about 50  $\mu\text{m}$  of mercury or 6.7 Pa), and incubate at about  $100^{\circ}\text{C}$  for 5 minutes. Then remove the inner hydrolysis tube, and dry it in a vacuum desiccator for 15 minutes to remove residual reagents. The pyridylethylated protein/peptide can then be acid hydrolyzed using previously described procedures. The pyridylethylation reaction is performed simultaneously with a protein standard sample containing 1 to 8 mol of cysteine to improve accuracy in the pyridylethyl-cysteine recovery. Longer incubation times for the pyridylethylation reaction can cause modifications to the  $\alpha$ -amino terminal group and the  $\epsilon$ -amino group of lysine in the protein.

#### Method 8

Cysteine-cystine reduction and alkylation is accomplished by a liquid phase pyridylethylation reaction.

Stock Solutions— Prepare and filter three solutions: 1 M Tris hydrochloride (pH 8.5) containing 4 mM edetate disodium (Stock Solution 1), 8 M guanidine hydrochloride (Stock Solution 2), and 10% of 2-mercaptopropanoic acid in water (Stock Solution 3).

Reducing Solution— Prepare a mixture of Stock Solution 2 and Stock Solution 1 (3:1) to obtain a buffered solution of 6 M guanidine hydrochloride in 0.25 M Tris hydrochloride.

Procedure— Dissolve about 10  $\mu\text{g}$  of the test sample in 50  $\mu\text{L}$  of the Reducing Solution, and add about 2.5  $\mu\text{L}$  of Stock Solution 3. Store under nitrogen or argon for 2 hours at room temperature in the dark. To achieve the pyridylethylation reaction, add about 2  $\mu\text{L}$  of 4-vinylpyridine to the protein solution, and incubate for an additional 2 hours at room temperature in the dark. The protein/peptide is desalted by collecting the protein/peptide fraction from a reverse-phase HPLC separation. The collected sample can be dried in a vacuum centrifuge before acid hydrolysis.

#### Method 9

Cysteine-cystine reduction and alkylation is accomplished by a liquid phase carboxymethylation reaction.

Stock Solutions— Prepare as directed for Method 8.

Carboxymethylation Solution— Prepare a solution containing 100 mg of iodoacetamide per mL of alcohol.

Buffer Solution— Use the Reducing Solution, prepared as directed for Method 8.

Procedure— Dissolve the test sample in 50  $\mu\text{L}$  of the Buffer Solution, and add about 2.5  $\mu\text{L}$  of Stock Solution 3. Store under nitrogen or argon for 2 hours at room temperature in the dark. Add the Carboxymethylation Solution in a 1.5 fold ratio per total theoretical content of thiols, and incubate for an additional 30 minutes at room temperature in the dark. [note—If the thiol content of the protein is unknown, then add 5  $\mu\text{L}$  of 100 mM iodoacetamide for every 20 nmol of protein present.] The reaction is stopped by adding excess of 2-mercaptopropanoic acid. The protein/peptide is desalted by collecting the protein/peptide fraction from a reverse-phase HPLC separation. The collected sample can be dried in a vacuum



centrifuge before acid hydrolysis. The S-carboxyamidomethylcysteine formed will be converted to S-carboxymethyl-cysteine during acid hydrolysis.

#### Method 10

Cysteine-cystine is reacted with dithiodiglycolic acid or dithiodipropionic acid to produce a mixed disulfide. [note—The choice of dithiodiglycolic acid or dithiodipropionic acid depends on the required resolution of the amino acid analysis method.]

Reducing Solution: a solution containing 10 mg of dithiodiglycolic acid (or dithiodipropionic acid) per mL of 0.2 M sodium hydroxide.

Procedure— Transfer about 20 µg of the test sample to a hydrolysis tube, and add 5 µL of the Reducing Solution. Add 10 µL of isopropyl alcohol, and then remove all of the sample liquid by vacuum centrifugation. The sample is then hydrolyzed using Method 1. This method has the advantage that other amino acid residues are not derivatized by side reactions, and the sample does not need to be desalted prior to hydrolysis.

#### Method 11

Asparagine and glutamine are converted to aspartic acid and glutamic acid, respectively, during acid hydrolysis. Asparagine and aspartic acid residues are added and represented by Asx, while glutamine and glutamic acid residues are added and represented by Glx. Proteins/peptides can be reacted with bis(1,1-trifluoroacetoxy)iodobenzene (BTI) to convert the asparagine and glutamine residues to diaminopropionic acid and diaminobutyric acid residues, respectively, upon acid hydrolysis. These conversions allow the analyst to determine the asparagine and glutamine content of a protein/peptide in the presence of aspartic acid and glutamic acid residues.

Reducing Solutions— Prepare and filter three solutions: a solution of 10 mM trifluoroacetic acid (Solution 1), a solution of 5 M guanidine hydrochloride and 10 mM trifluoroacetic acid (Solution 2), and a freshly prepared solution of dimethylformamide containing 36 mg of BTI per mL (Solution 3).

Procedure— In a clean hydrolysis tube, transfer about 200 µg of the test sample, and add 2 mL of Solution 1 or Solution 2 and 2 mL of Solution 3. Seal the hydrolysis tube in vacuum. Heat the sample at 60° for 4 hours in the dark. The sample is then dialyzed with water to remove the excess reagents. Extract the dialyzed sample three times with equal volumes of n-butyl acetate, and then lyophilize. The protein can then be acid hydrolyzed using previously described procedures. The  $\alpha$ -,  $\beta$ -diaminopropionic and  $\alpha$ -,  $\gamma$ -diaminobutyric acid residues do not typically resolve from the lysine residues upon ion-exchange chromatography based on amino acid analysis. Therefore, when using ion-exchange as the mode of amino acid separation, the asparagine and glutamine contents are the quantitative difference in the aspartic acid and glutamic acid assayed contents with un-derivatized and BTI-derivatized acid hydrolysis. [note—The threonine, methionine, cysteine, tyrosine, and histidine assayed content can be altered by BTI derivatization; a hydrolysis without BTI will have to be performed if the analyst is interested in the protein/peptide content of these other amino acid residues.]

### METHODOLOGIES OF AMINO ACID ANALYSIS

Many amino acid analysis techniques exist, and the choice of any one technique often depends on the sensitivity required from the assay. In general, about one-half of the amino acid analysis techniques employed rely on the separation of the free amino acids by ion-exchange chromatography followed by postcolumn derivatization (e.g., with ninhydrin or o-phthalaldehyde). Postcolumn detection techniques can be used with samples that contain small amounts of buffer components, such as salts and urea, and generally require between 5 and 10 µg of protein sample per analysis. The remaining amino acid techniques typically involve precolumn derivatization of the free amino acids (e.g., phenyl isothiocyanate; 6-aminoquinolyl-N-hydroxysuccinimidyl carbonate; (dimethylamino)azobenzenesulfonyl chloride; 9-fluorenyl-methylchloroformate; and 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole) followed by reverse-phase HPLC. Precolumn derivatization techniques are very sensitive and usually require between 0.5 and 1.0 µg of protein sample per analysis but may be influenced by buffer salts in the samples. Precolumn derivatization techniques may also result in multiple derivatives of a given amino acid, which complicates the result interpretation. Postcolumn derivatization techniques are generally influenced less by performance variation of the assay than precolumn derivatization techniques.

The following Methods may be used for quantitative amino acid analysis. Instruments and reagents for these procedures are available commercially. Furthermore, many modifications of these methodologies exist with different reagent preparations, reaction procedures, and chromatographic systems. Specific parameters may vary according to the exact equipment and procedure used. Many laboratories will utilize more than one amino acid analysis technique to exploit the advantages offered by each. In each of these Methods, the analog signal is visualized by means of a data acquisition system, and the peak areas are integrated for quantification purposes.

#### Method 1—Postcolumn Ninhydrin Detection

Ion-exchange chromatography with postcolumn ninhydrin detection is one of the most common methods employed for quantitative amino acid analysis. As a rule, a Li-based cation-exchange system is employed for the analysis of the more complex physiological samples, and the faster Na-based cation-exchange system is used for the more simplistic amino acid mixtures obtained with protein hydrolysates (typically containing 17 amino acid components). Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and cation strength. A temperature gradient is often employed to enhance separation.

When the amino acid reacts with ninhydrin, the reactant has characteristic purple or yellow color. Amino acids, except imino acids, give a purple color, and show maximum absorption at 570 nm. The imino acids, such as proline, give a yellow color, and show maximum absorption at 440 nm. The postcolumn reaction between ninhydrin and amino acid eluted from the column is monitored at 440 nm and 570 nm, and the chromatogram obtained is used for the determination of amino acid composition.

The detection limit is considered to be 10 pmol for most of the amino acid derivatives, but 50 pmol for proline. Response linearity is obtained in the range of 20 to 500 pmol with correlation coefficients exceeding 0.999. To obtain good compositional data, samples larger than 1 µg before hydrolysis are best suited for this amino acid analysis of protein/peptide.

One method for postcolumn ninhydrin detection is shown below. Many other methods are also available, with instruments and reagents available commercially.

##### Mobile Phase Preparation—

Solution A— Transfer about 1.7 g of anhydrous sodium citrate and 1.5 mL of hydrochloric acid to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix. Adjust, if necessary, with hydrochloric acid to a pH of 3.0.

Solution B— Transfer about 1.7 g of anhydrous sodium citrate and 0.7 mL of hydrochloric acid to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix. Adjust, if necessary, with hydrochloric acid to a pH of 4.3.

Solution C— Prepare a solution containing 5% of sodium chloride, 1.9% of anhydrous sodium citrate, and 0.1% of phenol in water, and adjust to a pH of 6.

Column Regeneration Solution— Prepare a solution containing 0.8% of sodium hydroxide in water, and adjust to a pH of 13.

Mobile Phase— Use variable mixtures of Solution A, Solution B, and Solution C as directed for Chromatographic System.

Postcolumn Reagent— Transfer about 18 g of ninhydrin and 0.7 g of hydrindantin to 900 mL of a solution containing 76.7% of dimethyl sulfoxide, 0.7% of dihydrate lithium acetate, and 0.1% of acetic acid, and mix for at least 3 hours under inert gas, such as nitrogen. [note—This reagent is stable for 30 days if kept between 2° and 8° under inert gas.]

Buffer Solution— Prepare a solution containing 2% of anhydrous sodium citrate, 1% of hydrochloric acid, 0.5% of thiodiglycol, and 0.1% of benzoic acid in water, and adjust to a pH of 2.

Chromatographic System— The liquid chromatograph is equipped with a detector with appropriate interference filters at 440, 570, or 690 nm and a 4.0-mm  $\times$  120-mm column that contains 7.5-µm sulfonated styrene-divinylbenzene copolymer packing. The flow rate is about 14 mL per hour. The system is programmed as follows. Initially equilibrate the column with Solution A; at 25 minutes, the composition of the Mobile Phase is changed to 100% Solution B; and at 37 minutes, the composition is changed to 100% Solution C. At 75 minutes into the run, the last amino acid has been eluted from the column, and the column is regenerated with Column Regeneration Solution for 1 minute. The column is then equilibrated with Solution A for 11 minutes before the next injection. The column temperature is programmed as follows. The initial temperature is 48°; after 11.5 minutes, the temperature is increased to 65° at a rate of 3° per minute; at about 35 minutes, the temperature is increased to 77° at a rate of 3° per minute; and finally at about 52 minutes, the temperature is decreased to 48° at a rate of 3° per minute.

Procedure and Postcolumn Reaction— Reconstitute the lyophilized protein/peptide hydrolysate in the Buffer Solution, inject an appropriate amount into the chromatograph, and proceed as directed for Chromatographic System. As the amino acids are eluted from the column, they are mixed with the Postcolumn Reagent, which is delivered at a flow rate of 7



ML per hour, through a tee. After mixing, the column effluent and the Postcolumn Reagent pass through a tubular reactor at a temperature of 135°, where a characteristic purple or yellow color is developed. From the reactor, the liquid passes through a colorimeter with a 12-mm flow-through cuvette. The light emerging from the cuvette is split into three beams for analysis by the detector with interference filters at 440, 570, or 690 nm. The 690-nm signal may be electronically subtracted from the other signals for improved signal-to-noise ratios.

The 440-nm (imino acids) and the 570-nm (amino acids) signals may be added in order to simplify data handling.

#### Method 2—Postcolumn OPA Fluorometric Derivatization

Ion-exchange chromatography with postcolumn o-phthalaldehyde (OPA) fluorometric detection is used. The procedure employs an ion-exchange column for separation of free amino acids followed by postcolumn oxidation with sodium hypochlorite and derivatization using OPA and N-acetyl-l-cysteine. The sodium hypochlorite oxidation step allows secondary amines, such as proline, to react with the OPA reagent.

OPA reacts with primary amines in the presence of thiol compound to form highly fluorescent isoindole products. This reaction is utilized for the postcolumn derivatization in analysis of amino acids by ion-exchange chromatography. The rule of the separation is the same as Method I. Instruments and reagents for this form of amino acid analysis are available commercially. Many modifications of this method exist.

Although OPA does not react with secondary amines (imino acids, such as proline) to form fluorescent substances, the oxidation with sodium hypochlorite allows secondary amines to react with OPA. The procedure employs a strongly acidic cation-exchange column for separation of free amino acids followed by postcolumn oxidation with sodium hypochlorite and postcolumn derivatization using OPA and thiol compound, such as N-acetyl-l-cysteine and 2-mercaptoethanol. The derivatization of primary amino acids are not noticeably affected by the continuous supply of sodium hypochlorite.

Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes of pH and cation strength. After postcolumn derivatization of eluted amino acids with OPA, the reactant passes through the fluorometric detector. Fluorescence intensity of OPA-derivatized amino acids are monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

The detection limit is considered to be a few tens of pmol level for most of the amino acid derivatives. Response linearity is obtained in the range of a few pmol level to a few tens of nmol level. To obtain good compositional data, a sample greater than 500 ng before hydrolysis is best suited for the amino acid analysis of protein/peptide.

One method of postcolumn OPA fluorometric detection is shown below.

#### Mobile Phase Preparation—

Solution A— Prepare a solution of sodium hydroxide, citric acid, and alcohol in HPLC grade water having a 0.2 N sodium concentration and containing 7% of alcohol (w/v), adjusted to a pH of 3.2.

Solution B— Prepare a solution of sodium hydroxide and citric acid in HPLC grade water having a 0.6 N sodium concentration, adjusted to a pH of 10.0.

Solution C: 0.2 N sodium hydroxide.

Mobile Phase— Use variable mixtures of Solution A, Solution B, and Solution C as directed for Chromatographic System.

#### Postcolumn Reagent Preparation—

Alkaline Buffer— Prepare a solution containing 384 mM sodium carbonate, 216 mM boric acid, and 108 mM potassium sulfate, and adjust to a pH of 10.0.

Hypochlorite Reagent— To 1 L of Alkaline Buffer, add 0.4 mL of sodium hypochlorite solution (10% chlorine concentration). [note—The hypochlorite solution is stable for 2 weeks.]

OPA Reagent— Transfer 2 g of N-acetyl-l-cysteine and 1.6 g of OPA to a 15-mL volumetric flask, dissolve in and dilute with alcohol to volume, and mix. Transfer this solution and 4 mL of 10% aqueous polyethylene (23) lauryl ether to a 1-L volumetric flask, dilute with 980 mL of Alkaline Buffer, and mix.

Chromatographic System— The liquid chromatograph is equipped with a fluorometric detector set to an excitation wavelength of 348 nm and an emission wavelength of 450 nm and a 4.0-mm × 150-mm column that contains 7.5-μm packing L17. The flow rate is about 0.3 mL per minute, and the column temperature is set at 50°. The system is programmed as follows. The column is equilibrated with Solution A; over the next 20 minutes, the composition of the Mobile Phase is changed linearly to 85% Solution A and 15% Solution B; then there is a step change to 40% Solution A and 60% Solution B; over the next 18 minutes, the composition is changed linearly to 100% Solution B and held for 7 minutes; then there is a step change to 100% Solution C, and this is held for 6 minutes; then there is a step change to Solution A, and this composition is maintained for the next 8 minutes.

Procedure and Postcolumn Reaction— Inject about 1.0 nmol of each amino acid under test into the chromatograph, and proceed as directed for Chromatographic System. As the effluent leaves the column, it is mixed with the Hypochlorite Reagent. The mixture passes through the first postcolumn reactor which consists of stainless steel 0.5-mm × 2-m tubing. A second postcolumn reactor of similar design is placed immediately downstream from the first postcolumn reactor and is used for the OPA postcolumn reaction. The flow rates for both the Hypochlorite Reagent and the OPA Reagent are 0.2 mL per minute, resulting in a total flow rate (i.e., Hypochlorite Reagent, OPA Reagent, and column effluent) of 0.7 mL per minute exiting from the postcolumn reactors. Postcolumn reactions are conducted at 55°. This results in a residence time of about 33 seconds in the OPA postcolumn reactor. After postcolumn derivatization, the column effluent passes through the fluorometric detector.

#### Method 3—Precolumn Determination

Precolumn derivatization of amino acids with phenylisothiocyanate (PITC) followed by reverse-phase HPLC separation with UV detection is used.

PITC reacts with amino acids to form phenylthiocarbamyl (PTC) derivatives, which can be detected with high sensitivity at 254 nm. Therefore, precolumn derivatization of amino acids with PITC followed by a reverse-phase HPLC separation with UV detection is used to analyze the amino acid composition.

After the reagent is removed under vacuum, the derivatized amino acids can be stored dry and frozen for several weeks with no significant degradation. If the solution for injection is kept cold, no noticeable loss in chromatographic response occurs after three days.

Separation of the PTC-amino acids on a reverse-phase HPLC with ODS column is accomplished through a combination of changes in concentrations of acetonitrile and buffer ionic strength. PTC-amino acids eluted from the column are monitored at 254 nm.

The detection limit is considered to be 1 pmol for most of the amino acid derivatives. Response linearity is obtained in the range of 20 to 500 pmol with correlation coefficients exceeding 0.999. To obtain good compositional data, a sample larger than 500 ng of protein/peptide before hydrolysis is best suited for this amino analysis of proteins/peptides.

One method of precolumn PITC derivatization is described below.

#### Mobile Phase Preparation—

Solution A: 0.05 M ammonium acetate, adjusted with phosphoric acid to a pH of 6.8.

Solution B— Prepare 0.1 M ammonium acetate, adjust with phosphoric acid to a pH of 6.8, and then prepare a mixture of this solution and acetonitrile (1:1).

Solution C: a mixture of acetonitrile and water (70:30).

Mobile Phase— Use variable mixtures of Solution A, Solution B, and Solution C as directed for Chromatographic System.

#### Derivatization Reagent Preparation—

Coupling Buffer: a mixture of acetonitrile, pyridine, triethylamine, and water (10:5:2:3).

Sample Solvent: a mixture of water and acetonitrile (7:2).

Sample Derivatization Procedure— Dissolve the lyophilized test sample in 100 μL of the Coupling Buffer, and then dry in a vacuum centrifuge to remove any hydrochloride if a protein hydrolysis step was used. Dissolve the test sample in 100 μL of Coupling Buffer, add 5 μL of PITC, and incubate at room temperature for 5 minutes. The test sample is again dried in a vacuum centrifuge, and is dissolved in 250 μL of Sample Solvent.



...omatographic System— The liquid chromatograph is equipped with a 254-nm detector and a 4.6-mm × 250-mm column that contains 5-μm packing L1. The flow rate is about 1 mL per minute, and the column temperature is maintained at 52°. The system is programmed as follows. The column is equilibrated with Solution A; over the next 15 minutes, the composition of the Mobile Phase is changed linearly to 85% Solution A and 15% Solution B; over the next 15 minutes, the composition is changed linearly to 50% Solution A and 50% Solution B; then there is a step change to 100% Solution C, and this is held for 10 minutes; then there is a step change to 100% Solution A, and the column is allowed to equilibrate before the next injection.

Procedure— Inject about 1.0 nmol of each PITC-amino acid under test (10-μL sample in Sample Solvent) into the chromatograph, and proceed as directed for Chromatographic System.

#### Method 4—Precolumn AQC Derivatization

Precolumn derivatization of amino acids with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) followed by reverse-phase HPLC separation with fluorometric detection is used.

AQC reacts with amino acids to form stable, fluorescent unsymmetric urea derivatives (AQC-amino acids) which are readily amenable to analysis by reverse-phase HPLC. Therefore, precolumn derivatization of amino acids with AQC followed by reverse-phase HPLC separation is used to analyze the amino acid composition.

Separation of the AQC-amino acids on an ODS column is accomplished through a combination of changes in the concentrations of acetonitrile and salt. Selective fluorescence detection of the derivatives with an excitation wavelength at 250 nm and an emission wavelength at 395 nm allows for the direct injection of the reaction mixture with no significant interference from the only major fluorescent reagent byproduct, 6-aminoquinoline. Excess reagent is rapidly hydrolyzed ( $t_{1/2} < 15$  seconds) to yield 6-aminoquinoline-N-hydroxysuccinimide and carbon dioxide, and after 1 minute no further derivatization can take place.

Peak areas for AQC-amino acids are essentially unchanged for at least 1 week at room temperature, and the derivatives have more than sufficient stability to allow for overnight automated chromatographic analysis.

The detection limit is considered to be ranging from about 40 fmol to 320 fmol for each amino acid, except for Cys. The detection limit for Cys is approximately 800 fmol. Response linearity is obtained in the range of 2.5 μM to 200 μM with correlation coefficients exceeding 0.999. Good compositional data can be obtained from the analysis of derivatized protein hydrolysates containing as little as 30 ng of protein/peptide.

One method of precolumn AQC derivatization is shown below.

#### Mobile Phase Preparation—

Solution A— Prepare a solution having a composition of 140 mM sodium acetate and 17 mM triethylamine, and adjust with phosphoric acid to a pH of 5.02.

Solution B: a mixture of acetonitrile and water (60:40).

Mobile Phase— Use variable mixtures of Solution A and Solution B as directed for Chromatographic System.

Sample Derivatization Procedure— Dissolve about 2 μg of the test sample in 20 μL of 15 mM hydrochloric acid, and dilute with 0.2 M borate buffer (pH 8.8) to 80 μL. The derivatization is initiated by the addition of 20 μL of 10 mM AQC in acetonitrile, and allowed to proceed for 10 minutes at room temperature.

Chromatographic System— The liquid chromatograph is equipped with a fluorometric detector set at an excitation wavelength of 250 nm and an emission wavelength of 395 nm and a 3.9-mm × 150-mm column that contains 4-μm packing L1. The flow rate is about 1 mL per minute, and the column temperature is maintained at 37°. The system is programmed as follows. The column is equilibrated with Solution A; over the next 0.5 minute, the composition of the Mobile Phase is changed linearly to 98% Solution A and 2% Solution B; then over the next 14.5 minutes to 93% Solution A and 7% Solution B; then over the next 4 minutes to 87% Solution A and 13% Solution B; over the next 14 minutes to 68% Solution A and 32% Solution B; then there is a step change to 100% Solution B for a 5-minute wash; over the next 10 minutes, there is a step change to 100% Solution A; and the column is allowed to equilibrate before the next injection.

Procedure— Inject about 0.05 nmol of each AQC-amino acid under test into the chromatograph, and proceed as directed for Chromatographic System.

#### Method 5—Precolumn OPA Derivatization

Precolumn derivatization of amino acids with OPA followed by reverse-phase HPLC separation with fluorometric detection is used. This technique does not detect amino acids that exist as secondary amines (e.g., proline).

OPA in conjunction with a thiol reagent reacts with primary amine groups to form highly fluorescent isoindole products. 2-Mercaptoethanol and 3-mercaptopropionic acid can be used as thiol. OPA itself does not fluoresce and consequently produces no interfering peaks. In addition, its solubility and stability in aqueous solution, along with the rapid kinetics for the reactions, make it amenable to automated derivatization and analysis using an autosampler to mix the sample with the reagent. However, lack of reactivity with secondary amino acids has been a predominant drawback. This method does not detect amino acids that exist as secondary amines (e.g., proline). To compensate for this drawback, this technique may be combined with another technique described in Method 7 or Method 8.

Precolumn derivatization of amino acids with OPA is followed by reverse-phase HPLC separation. Because of the instability of the OPA-amino acid derivative, HPLC separation and analysis are performed immediately following derivatization. The liquid chromatograph is equipped with a fluorometric detector for the detection of derivatized amino acids.

Fluorescence intensity of the OPA-derivatized amino acids are monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

The detection limits as low as 50 fmol via fluorescence have been reported, although the practical limit of analysis remains at 1 pmol. One method of precolumn OPA derivatization is shown below.

#### Mobile Phase Preparation—

Solution A: a mixture of 100 mM sodium acetate (pH 7.2), methanol, and tetrahydrofuran (900:95:5).

Solution B: methanol.

Mobile Phase— Use variable mixtures of Solution A and Solution B as directed for Chromatographic System.

Derivatization Reagent— Dissolve 50 mg of OPA in 1.25 mL of methanol (protein sequencing grade). Add 50 μL of 2-mercaptoethanol and 11.2 mL of 0.4 M sodium borate (pH 9.5), and mix. [note—This reagent is stable for 1 week.]

Sample Derivatization Procedure— Transfer about 5 μL of the test sample to an appropriate container, add 5 μL of the Derivatization Reagent, and mix. After 1 minute, add not less than 20 μL of 0.1 M sodium acetate (pH 7.0). Use 20 μL of this solution for analysis. [note—Use of an internal standard (e.g., norleucine) is recommended for quantitative analysis because of potential reagent volume variations in the sample derivatization. The sample derivatization is performed in an automated on-line fashion. Because of the instability of the OPA-amino acid derivative, HPLC separation and analysis are performed immediately following derivatization.]

Chromatographic System— The liquid chromatograph is equipped with a fluorometric detector set at an excitation wavelength of 348 nm and an emission wavelength of 450 nm and a 4.6-mm × 75-mm column that contains 3-μm packing L3. The flow rate is about 1.7 mL per minute, and the column temperature is maintained at 37°. The system is programmed as follows. The column is equilibrated with 92% Solution A and 8% Solution B; over the next 2 minutes, the composition of the Mobile Phase is changed to 83% Solution A and 17% Solution B, and held for an additional 3 minutes; then changed to 54% Solution A and 46% Solution B over the next 5 minutes, and held for an additional 2 minutes; then changed to 34% Solution A and 66% Solution B over the next 2 minutes, and held for 1 minute; then over the next 0.3 minute changed to 20% Solution A and 80% Solution B, and held for an additional 2.6 minutes; and then finally over 0.6 minute changed to 92% Solution A and 8% Solution B, and held for an additional 0.6 minute.

Procedure— Inject about 0.02 nmol of each OPA-amino acid under test into the chromatograph, and proceed as directed for Chromatographic System.

#### Method 6—Postcolumn DABS-Cl Derivatization

Precolumn derivatization of amino acids with (dimethylamino)azobenzenesulfonyl chloride (DABS-Cl) followed by reverse-phase HPLC separation with visible light detection is used.



DABS-Cl is a chromophoric reagent employed for the labeling of amino acids. Amino acids labeled with DABS-Cl (DABS-amino acids) are highly stable and show the maximum absorption at 436 nm.

DABS-amino acids, all 19 naturally occurring amino acids derivatives, can be separated on an ODS column of a reverse-phase HPLC by employing gradient systems consisting of acetonitrile and aqueous buffer mixture. Separated DABS-amino acids eluted from the column are detected at 436 nm in the visible region.

This method can analyze the imino acids, such as proline, together with the amino acids, at the same degree of sensitivity. DABS-Cl derivatization method permits the simultaneous quantification of tryptophan residues by previous hydrolysis of the protein/peptide with sulfonic acids, such as mercaptoethanesulfonic acid, p-toluenesulfonic acid, or methanesulfonic acid, described for Method 2 in Protein Hydrolysis under Amino Acid Analysis. The other acid-labile residues, asparagine and glutamine, can also be analyzed by previous conversion into diaminopropionic acid and diaminobutyric acid, respectively, by treatment of protein/peptide with BTI, described for Method 11 in Protein Hydrolysis under Amino Acid Analysis.

The nonproteinogenic amino acid, norleucine, cannot be used as an internal standard in this method as this compound is eluted in a chromatographic region crowded with peaks of primary amino acids. Nitrotyrosine can be used as an internal standard because it is eluted in a clean region.

The detection limit of DABS-amino acid is about 1 pmol. As little as 2 to 5 pmol of an individual DABS-amino acid can be quantitatively analyzed with reliability, and only 10 ng to 30 ng of the dabsylated protein hydrolysate is required for each analysis.

One method for precolumn DABS-Cl derivatization is shown below.

Mobile Phase Preparation—

Solution A: 25 mM sodium acetate (pH 6.5) containing 4% of dimethylformamide.

Solution B: acetonitrile.

Mobile Phase— Use variable mixtures of Solution A and Solution B as directed for Chromatographic System.

Derivatization Reagent Preparation—

Sample Buffer: 50 mM sodium bicarbonate, adjusted to a pH of 8.1.

Derivatization Reagent— Dissolve 1.3 mg of DABS-Cl in 1 mL of acetonitrile. [note—This reagent is prepared fresh shortly before the derivatization step.]

Sample Dilution Buffer— Prepare a mixture of 50 mM sodium phosphate (pH 7.0) and alcohol (1:1).

Sample Derivatization Procedure— Dissolve the test sample in 20  $\mu$ L of Sample Buffer, add 40  $\mu$ L of Derivatization Reagent, and mix. The sample container is sealed with a silicon-rubber stopper, and heated to 70° for 10 minutes. During the sample heating, the mixture will become completely soluble. After the derivatization, dilute the test sample with an appropriate quantity of the Sample Dilution Buffer.

Chromatographic System— The liquid chromatograph is equipped with a 436-nm detector and a 4.6-mm  $\times$  250-mm column that contains packing L1. The flow rate is about 1 mL per minute, and the column temperature is maintained at 40°. The system is programmed as follows. The column is equilibrated with 85% Solution A and 15% Solution B; over the next 20 minutes, the composition of the Mobile Phase is changed to 60% Solution A and 40% Solution B; over the next 12 minutes, the composition is changed to 30% Solution A and 70% Solution B, and held for an additional 2 minutes.

Procedure— Inject about 0.05 nmol of the DABS-amino acids into the chromatograph, and proceed as directed for Chromatographic System.

Method 7—Precolumn FMOC-Cl Derivatization

Precolumn derivatization of amino acids with 9-fluorenylmethyl chloroformate (FMOC-Cl) followed by reverse-phase HPLC separation with fluorometric detection is used.

FMOC-Cl reacts with both primary and secondary amino acids to form highly fluorescent products. The reaction of FMOC-Cl with amino acid proceeds under mild conditions, in aqueous solution, and is completed in 30 seconds. The derivatives are stable, with only the histidine derivative showing any breakdown. Although FMOC-Cl is fluorescent itself, the reagent excess and fluorescent side-products can be eliminated without loss of FMOC-amino acids.

FMOC-amino acids are separated by reverse-phase HPLC using an ODS column. The separation is carried out by gradient elution varied linearly from a mixture of acetic acid buffer, methanol, and acetonitrile (50:40:10) to a mixture of acetonitrile and acetic acid buffer (50:50), and 20 amino acid derivatives that are separated in 20 minutes. Each derivative eluted from the column is monitored by a fluorometric detector set at an excitation wavelength of 260 nm and an emission wavelength of 313 nm.

The detection limit is in the low fmol range. A linearity range of 0.1  $\mu$ M to 50  $\mu$ M is obtained for most amino acids.

One method for precolumn FMOC-Cl derivatization is shown below.

Mobile Phase Preparation—

Acetic Acid Buffer— Transfer 3 mL of glacial acetic acid and 1 mL of triethylamine to a 1-L volumetric flask, and dilute with HPLC grade water to volume. Adjust with sodium hydroxide to a pH of 4.20.

Solution A: a mixture of Acetic Acid Buffer, methanol, and acetonitrile (50:40:10).

Solution B: a mixture of acetonitrile and Acetic Acid Buffer (50:50).

Mobile Phase— Use variable mixtures of Solution A and Solution B as directed for Chromatographic System.

Derivatization Reagent Preparation—

Borate Buffer— Prepare a 1 M boric acid solution, and adjust with sodium hydroxide to a pH of 6.2.

FMOC-Cl Reagent— Dissolve 155 mg of 9-fluorenylmethyl chloroformate in 40 mL of acetone, and mix.

Sample Derivatization Procedure— To 0.4 mL of the test sample add 0.1 mL of Borate Buffer and 0.5 mL of FMOC-Cl Reagent. After about 40 seconds, extract the mixture with 2 mL of pentane, and then extract again with fresh pentane. The aqueous solution with amino acid derivatives is then ready for injection.

Chromatographic System— The liquid chromatograph is equipped with a fluorometric detector set at an excitation wavelength of 260 nm and an emission wavelength of 313 nm and a 4.6-mm  $\times$  125-mm column that contains 3- $\mu$ m packing L1. The flow rate is about 1.3 mL per minute. The system is programmed as follows. The column is equilibrated with Solution A, and this composition is maintained for 3 minutes; over the next 9 minutes, it is changed to 100% Solution B; then over the next 0.5 minute, the flow rate is increased to 2 mL per minute, and held until the final FMOC-amino acid is eluted from the column. The total run time is about 20 minutes.

Procedure— Inject not less than 0.01 nmol of each FMOC-amino acid under test into the chromatograph, and proceed as directed for Chromatographic System. The FMOC-histidine derivative will generally give a lower response than the other derivatives.

Method 8—Precolumn NBD-F Derivatization

Precolumn derivatization of amino acids with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) followed by reverse-phase HPLC separation with fluorometric detection is used.

7-Fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) reacts with both primary and secondary amino acids to form highly fluorescent products. Amino acids are derivatized with NBD-F by heating to 60° for 5 minutes.

NBD-amino acid derivatives are separated on an ODS column of reverse-phase HPLC by employing a gradient elution system consisting of acetonitrile and aqueous buffer mixture, and 17 amino acid derivatives that are separated in 35 minutes. E-aminocaproic acid can be used as an internal standard because it is eluted in a clean chromatographic region. Each derivative eluted from the column is monitored by a fluorometric detector set at an excitation wavelength of 480 nm and an emission wavelength of 530 nm.



The sensitivity of this method is almost the same as that for the precolumn OPA derivatization method (Method 5), excluding proline to which OPA is not reactive and might be advantageous for NBD-F against OPA.

The detection limit for each amino acid is about 10 fmol. Profile analysis was achieved for about 1.5 mg of protein hydrolysates in the final precolumn labeling reaction mixture for HPLC.

One method for precolumn NBD-F derivatization is shown below.

Mobile Phase Preparation—

Solution A: a solution of 10mM sodium citrate containing 75 mM sodium perchlorate, adjusted with hydrochloric acid to a pH of 6.2.

Solution B: a mixture of acetonitrile and water (50:50).

Derivatization Reagent Preparation—

Sample Buffer: a 0.1 M boric acid solution, adjusted with sodium hydroxide to a pH of 9.2.

Derivatization Reagent— Dissolve 5 mg of NBD-F in 1.0 mL of alcohol, and mix.

Sample Derivatization Procedure— Dissolve the test sample in 20  $\mu$ L of Sample buffer, add 10  $\mu$ L of Derivatization Reagent, and mix. The sample container is heated at 60° for 5 minutes. After the derivatization, dilute the test sample with 300  $\mu$ L of Solution A.

Chromatographic System— The liquid chromatograph is equipped with a fluorometric detector set at an excitation wavelength of 480 nm and an emission wavelength of 530 nm and a 4.6-mm  $\times$  150-mm column that contains 5- $\mu$ m particle size ODS silica packing. The flow rate is about 1.0 mL per minute, and the column temperature is maintained at 40°. The system is programmed as follows. The column is equilibrated with 94% Solution A and 6% Solution B; over the next 16 minutes, the composition is changed linearly to 63% Solution A and 37% Solution B; over the next 5 minutes, the composition is changed linearly to 62% Solution A and 38% Solution B; over the next 9 minutes, the composition is changed linearly to 100% Solution B, and held for an additional 5 minutes; then finally over 2 minutes, the composition is changed linearly to 94% Solution A and 6% Solution B; and then the column is allowed to equilibrate before the next injection.

Procedure— Inject about 15 pmol of each NBD-amino acid under test into the chromatograph, and proceed as directed for Chromatographic System.

DATA CALCULATION AND ANALYSIS

When determining the amino acid content of a protein/peptide hydrolysate, it should be noted that the acid hydrolysis step destroys tryptophan and cysteine. Serine and threonine are partially destroyed by acid hydrolysis, while isoleucine and valine residues may be only partially cleaved. Methionine can undergo oxidation during acid hydrolysis, and some amino acids (e.g., glycine and serine) are common contaminants. Application of adequate vacuum (less than 200  $\mu$ m of mercury or 26.7 Pa) or introduction of inert gas (argon) in the headspace of the reaction vessel during vapor phase hydrolysis can reduce the level of oxidative destruction. Therefore, the quantitative results obtained for cysteine, tryptophan, threonine, isoleucine, valine, methionine, glycine, and serine from a protein/peptide hydrolysate may be variable and may warrant further investigation and consideration.

Calculations

Amino Acid Mole Percent— This is the number of specific amino acid residues per 100 residues in a protein. This result may be useful for evaluating amino acid analysis data when the molecular weight of the protein/peptide under investigation is unknown. This information can be used to corroborate the identity of a protein and has other applications. Carefully identify and integrate the peaks obtained as directed for each Procedure. Calculate the mole percent for each amino acid present in the test sample by the formula:

$$100rU / r$$

in which rU is the peak response, in nmol, of the amino acid under test; and r is the sum of peak responses, in nmol, for all amino acids present in the test sample. Comparison of the mole percent of the amino acids under test to data from known proteins can help establish or corroborate the identity of the sample protein.

Unknown Protein Samples— This data analysis technique can be used to estimate the protein concentration of an unknown protein sample using the amino acid analysis data.

Calculate the mass, in  $\mu$ g, of each recovered amino acid by the formula:

$$mMW / 1000$$

in which m is the recovered quantity, in nmol, of the amino acid under test; and MW is the average molecular weight, in mg, for that amino acid, corrected for the weight of the water molecule that was eliminated during peptide bond formation. The sum of the masses of the recovered amino acids will give an estimate of the total mass of the protein analyzed after appropriate correction for partially and completely destroyed amino acids. If the molecular weight of the unknown protein is available (i.e., by SDS-PAGE analysis or mass spectroscopy), the amino acid composition of the unknown protein can be predicted. Calculate the number of residues of each amino acid by the formula:

$$m / (1000M / MW)$$

in which m is the recovered quantity, in nmol, of the amino acid under test; M is the total mass, in  $\mu$ g, of the protein; and MW is the molecular weight, in mg, of the unknown protein.

Known Protein Samples— This data analysis technique can be used to investigate the amino acid composition and protein concentration of a protein sample of known molecular weight and amino acid composition using the amino acid analysis data. When the composition of the protein being analyzed is known, one can exploit the fact that some amino acids are recovered well, while other amino acid recoveries may be compromised because of complete or partial destruction (e.g., tryptophan, cysteine, threonine, serine, methionine), incomplete bond cleavage (i.e., for isoleucine and valine), and free amino acid contamination (i.e., by glycine and serine).

Because those amino acids that are recovered best represent the protein, these amino acids are chosen to quantify the amount of protein. Well-recovered amino acids are, typically, aspartate-asparagine, glutamate-glutamine, alanine, leucine, phenylalanine, lysine, and arginine. This list can be modified based on experience with one's own analysis system. Divide the quantity, in nmol, of each of the well-recovered amino acids by the expected number of residues for that amino acid to obtain the protein content based on each well-recovered amino acid. Average the protein content results calculated. The protein content determined for each of the well-recovered amino acids should be evenly distributed about the mean.

Discard protein content values for those amino acids that have an unacceptable deviation from the mean. Typically, a greater than 5% variation from the mean is considered unacceptable, but this is arbitrary. Recalculate the mean protein content from the remaining values to obtain the protein content of the sample. Divide the content of each amino acid by the calculated mean protein content to determine the amino acid composition of the sample by analysis.

Calculate the relative compositional error, in percentage, by the formula:

$$100m / mS$$

in which m is the experimentally determined quantity, in nmol per amino acid residue, of the amino acid under test; and mS is the known residue value for that amino acid. The average relative compositional error is the average of the absolute values of the relative compositional errors of the individual amino acids, typically excluding tryptophan and cysteine from this calculation. The average relative compositional error can provide important information on the stability of analysis run over time. The agreement in the amino acid composition between the protein sample and the known composition can be used to corroborate the identity and purity of the protein in the sample.

\* Suitable standards may be obtained from NIST (Gaithersburg, MD), Beckman Instruments (Fullerton, CA), Sigma Chemical (St. Louis, MO), Pierce (Rockford, IL), or Agilent (Palo Alto, CA).

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Alexey Khrenov, Ph.D.</a>	(BBPP05) Biologics and Biotechnology - Proteins and Polysaccharides

## 1053 BIOTECHNOLOGY-DERIVED ARTICLES—CAPILLARY ELECTROPHORESIS

This chapter provides guidance and procedures used for characterization of biotechnology-derived articles by capillary electrophoresis. This chapter is harmonized with the corresponding chapter in JP and EP. Other characterization tests, also harmonized, are shown in [Biotechnology-Derived Articles—Amino Acid Analysis](#) 1052, [Biotechnology-Derived Articles—Isoelectric Focusing](#) 1054, [Biotechnology-Derived Articles—Peptide Mapping](#) 1055, [Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis](#) 1056, and [Biotechnology-Derived Articles—Total Protein Assay](#) 1057.

## INTRODUCTION

Capillary electrophoresis is a physical method of analysis based on the migration, inside a capillary, of charged analytes dissolved in an electrolyte solution under the influence of a direct-current electric field. In this section we are describing four capillary electrophoresis methods: Free Solution Capillary Electrophoresis, Capillary Gel Electrophoresis, Capillary Isoelectric Focusing, and Micellar Electrophoresis.

## GENERAL PRINCIPLE

The migration velocity of the analyte under an electric field of intensity (E) is determined by the electrophoretic mobility of the analyte and the electroosmotic mobility of the buffer inside the capillary. The electrophoretic mobility of a solute ( $\mu_{ep}$ ) depends on the characteristics of the solute (electrical charge, molecular size, and shape) and the characteristics of the buffer in which the migration takes place (type and ionic strength of the electrolyte, pH, viscosity, and additives). The electrophoretic velocity ( $V_{ep}$ ) of a solute, assuming a spherical shape, is as follows:

$$V_{ep} = \mu_{ep} E = \left( \frac{q}{6\pi\eta r} \right) \left( \frac{V}{L} \right)$$

in which  $q$  is the effective charge of the particle;  $\eta$  is the viscosity of the buffer;  $r$  is the size of the solute ion;  $V$  is the applied voltage; and  $L$  is the total length of the capillary.

When an electric field is applied through the capillary filled with buffer, a flow of solvent, called electroosmotic flow, is generated inside the capillary. Its velocity depends on the electroosmotic mobility ( $\mu_{eo}$ ), which in turn depends on the charge density on the capillary internal wall and the buffer characteristics. The electroosmotic velocity ( $V_{eo}$ ) is as follows:

$$V_{eo} = \mu_{eo} E = \left( \frac{\epsilon\zeta}{\eta} \right) \left( \frac{V}{L} \right)$$

in which  $\epsilon$  is the dielectric constant of the buffer;  $\zeta$  is the zeta potential of the capillary surface; and the other terms are as defined above.

The electrophoretic and electroosmotic mobilities of the analyte may act in the same direction or in opposite directions, depending on the charge (positive or negative) of the solute, and the velocity of the solute ( $V$ ) is as follows:

$$V = V_{ep} \pm V_{eo}$$

The sum or the difference between the two velocities ( $V_{ep}$  and  $V_{eo}$ ) is used depending on whether the mobilities act in the same or opposite directions. Under conditions with a fast  $V_{eo}$ , with respect to the  $V_{ep}$  of the solutes, both negative and positive charged analytes can be separated in the same run. The time ( $t$ ) taken by the solute to migrate the distance ( $l$ ) from the injection end of the capillary to the detection point (capillary effective length) is as follows:

$$t = \frac{l}{V_{ep} \pm V_{eo}} = \frac{l(L)}{V(\mu_{ep} \pm \mu_{eo})}$$

in which the other terms are as defined above.

In general, the fused-silica capillaries used in electrophoresis bear negative charges on the inner wall, producing electroosmotic flow towards the cathode. The electroosmotic flow has to remain constant from run to run to obtain good reproducibility in the migration velocity of the solutes. For some applications, it might be necessary to reduce or suppress the electroosmotic flow by modifying the inner wall of the capillary or by changing the pH of the buffer solution.

When the sample is introduced in the capillary, each analyte ion of the sample migrates within the background electrolyte as an independent zone according to its electrophoretic mobility. The spreading of each solute band (zone dispersion) results from a different phenomena. Under ideal conditions, the sole contribution to the solute-zone broadening is molecular diffusion of the solute along the capillary (longitudinal diffusion). In this case, the efficiency of the zone is expressed as the number of theoretical plates ( $N$ ), as follows:

$$N = \frac{(\mu_{ep} \pm \mu_{eo})(Vl)}{2DL}$$

in which  $D$  is the molecular diffusion of the solute in the buffer; and the other terms are as defined above.

From a practical point of view, other phenomena, such as heat dissipation, sample adsorption onto the capillary wall, mismatched conductivity between sample and buffer, length of the injection plug, detector cell size, and unlevelled buffer reservoirs, can also significantly contribute to band dispersion. Separation between two bands (expressed by the resolution,  $RS$ ) can be achieved by modification of the electrophoretic mobility of the analytes, by the electroosmotic mobility induced by capillary, and by increasing the efficiency for the band of each analyte as follows:

$$R_s = \frac{\sqrt{N}(\mu_{epb} - \mu_{epa})}{4(\mu_{ep} \pm \mu_{eo})}$$

in which  $\mu_{epa}$  and  $\mu_{epb}$  are the electrophoretic mobilities of the two compounds to be separated;  $\mu_{ep}$  is the average electrophoretic mobility of the two solutes calculated as:

$$\mu_{ep} = \frac{1}{2} (\mu_{epb} + \mu_{epa})$$

and the other terms are as defined above.

#### APPARATUS

An apparatus for capillary electrophoresis is composed of a high voltage controllable power supply; two buffer reservoirs held at the same level and containing specified anodic and cathodic solutions; two electrodes assemblies (cathode and anode) immersed in the buffer reservoirs and connected to the power supply; a separation capillary usually made of fused-silica, sometimes with an optical viewing window aligned with the detector, depending on the detector, with the ends of the capillary placed in the buffer reservoirs and the capillary being filled with a solution specified in a given monograph; a suitable injection system; a detector capable of monitoring the amount of substance of interest passing through a segment of the separation capillary at a given time, generally based on absorption spectrophotometry (UV and visible), fluorimetry, conductimetric, amperometric, or mass spectrometric detection, depending on the specific applications, or even indirect detection to detect non-UV-absorbing and nonfluorescent compounds; and a thermostatic system capable of maintaining the temperature inside the capillary.

The method of injection of samples and its automation is critical for precise quantitative analysis. Methods of injection include gravity, pressure or vacuum, or electrokinetic injection. The amount of each sample component introduced electrokinetically depends on its electrophoretic mobility, thus possibly biasing the results.

It is expected that the capillary, the buffer solutions, the preconditioning method, the sample solution, and the migration conditions will be specified in the individual monograph. The electrolytic solution employed may be filtered to remove particles and degassed to avoid bubble formation that could interfere with the detection system. To achieve reproducible migration time of the solutes, it would be necessary to develop, for each analytical method, a rigorous rinsing routine after each injection.

#### FREE SOLUTION CAPILLARY ELECTROPHORESIS

In free solution capillary electrophoresis, analytes are separated in a capillary containing only buffer without any anticonvective medium. In this technique, separation takes place because the different components of the sample migrate as discrete bands with different velocities. The velocity of each band depends on the electrophoretic mobility of the solute and the electroosmotic flow on the capillary. Coated capillaries, with reduced electroosmotic flow, can be used to increase the separation capacity of those substances absorbing on fused-silica surfaces.

This mode of capillary electrophoresis is appropriate for the analysis of small (MW < 2000) and large (2000 < MW < 100,000) molecules. Due to the high efficiency achieved, molecules having only minute differences in their charge-to-mass ratio can be separated. This method also allows the separation of chiral compounds by adding chiral selectors to the separation buffer. The optimization of the separations requires consideration of a number of instrumental and electrolytic solution parameters.

##### Instrumental Parameters

**Voltage**— The separation time is universally proportional to applied voltage. However, an increase in the voltage used can cause excessive heat production, giving rise to temperature and viscosity gradients in the buffer inside the capillary, which causes band broadening and decreases resolution.

**Temperature**— The main effect of temperature is observed on buffer viscosity and electrical conductivity, thus affecting migration velocity. In some cases, an increase in capillary temperature can cause a conformational change of some proteins, modifying their migration time and the efficiency of the separation.

**Capillary**— The length and internal diameter of the capillary affects the analysis time, the efficiency of separations, and the load capacity. Increasing both effective length and total length can decrease the electric fields, at a constant voltage, which will increase migration time. For a given buffer and electric field, heat dissipation (thus, sample band broadening) depends on the internal diameter of the capillary. The latter also affects the detection limit, depending on the sample volume injected into the capillary and the detection system used.

The adsorption of sample components on the capillary wall limits efficiency; therefore, methods to avoid these interactions should be considered in the development of a separation method. This is critical in samples containing proteins. Strategies have been devised to avoid adsorption of proteins on the capillary wall. These strategies include both the use of extreme pH and the absorption of positively charged buffer additives that only require modification of the buffer composition. Other strategies include the coating of the internal wall of the capillary with a polymer covalently bonded to the silica that prevents interaction between the proteins and the negatively charged silica surface. Capillaries with coatings consisting of neutral-hydrophilic, cationic, and anionic polymers are commercially available.

##### Electrolytic Solution Parameters

**Buffer Type and Concentrations**— Suitable buffers for capillary electrophoresis have an appropriate buffer capacity in the pH range of choice and low mobility to minimize current generation.

To minimize peak shape distortion, it is important to match buffer-ion mobility to solute mobility whenever possible. The type of sample solvent used is important to achieve on-column sample focusing, which increases separation efficiency and improves detection. Also, an increase in buffer concentration at a given pH will decrease electroosmotic flow and solute velocity.

**Buffer pH**— The pH of the buffer can affect separation by modifying the charge of the analyte or other additives and by changing the electroosmotic flow. For protein and peptide separation, a change in the pH of the buffer from above the isoelectric point to below the isoelectric point changes the net charge of the solute from negative to positive. An increase in the buffer pH generally increases the electroosmotic flow.

**Organic Solvents**— Organic modifiers, such as methanol, acetonitrile, and others, are added to the aqueous buffer to increase the solubility of the solute or other additives and/or to affect the ionization degree of the sample components. The addition of these organic modifiers to the buffer generally causes a decrease in the electroosmotic flow.

**Additives for Chiral Separations**— To separate optical isomers, a chiral selector is added to the separation buffer. The most commonly used chiral selectors are cyclodextrins, although in some cases crown ethers, certain polysaccharides, or even proteins can be used. Because chiral recognition is governed by the different interactions between the chiral selector and each of the enantiomers, the resolution achieved for the chiral compounds depends largely on the type of chiral selector used. While developing a given separation it may be useful to test cyclodextrins having a different cavity size ( $\alpha$ ,  $\beta$ , or  $\gamma$ -cyclodextrin) or modified cyclodextrins with neutral (methyl, ethyl, hydroxyalkyl, etc.) or ionizable (aminomethyl, carboxymethyl, sulfobutylether, etc.) moieties. The resolution of chiral separations is also controlled by the concentration of the chiral selector, the composition and pH of the buffer, and the separation temperature. Organic additives, such as methanol or urea, can also affect the resolution of separation.

#### CAPILLARY GEL ELECTROPHORESIS

Separation takes place inside a capillary filled with a polymer acting as a molecular sieve. The smaller components in the sample move faster along the capillary than the larger ones. This method can be used for separation of biopolymers-proteins and DNA fragments, according to their molecular mass.

##### Characteristics of Chemical and Physical Gels

**Chemical Gels**— Chemical gels are prepared inside the capillary by reaction of monomers. One example of such a gel is a cross-linked polyacrylamide. This type of gel is bonded to



the fused-silica wall and cannot be removed without destroying the capillary. For protein analysis, the separation buffer usually contains sodium dodecyl sulfate, and the sample is denatured by heating in a mixture of sodium dodecyl sulfate and 2-mercaptoethanol or dithiothreitol before injection. Optimization of separation in a cross-linked gel is obtained by modifying the separation buffer (see Free Solution Capillary Electrophoresis) and by controlling the gel porosity during the gel preparation. For a cross-linked polyacrylamide gel, the porosity can be modified by changing the concentration of acrylamide and/or the ratio of the cross-linker. As a rule, a decrease in the porosity of the gel leads to a decrease in the mobility of the solutes. Due to the rigidity of this type of gel, only electrokinetic injection can be used.

**Physical Gels**— Physical gels are hydrophilic polymers (i.e., linear polyacrylamide, cellulose derivatives, dextran, etc.) which can be dissolved in aqueous separation buffers, giving rise to a separation medium that also acts as a molecular sieve. These polymeric separation media are easier to prepare than cross-linked polymers. They can be prepared in a vial and filled by pressure in a wall-coated capillary with no electroosmotic flow. Replacing the gel before every injection generally improves the separation reproducibility. The porosity of the physical gels can be increased by using polymers of higher molecular weight (at a given polymer concentration) or by decreasing the polymer concentration (for a given polymer molecular weight). A decrease in gel porosity leads to a decrease in the mobility of the solute for the same buffer. Both hydrodynamic and electromigration injection techniques can be used because the dissolution of these polymers in the buffer gives low viscosity solutions.

#### CAPILLARY ISOELECTRIC FOCUSING

The molecules migrate under the influence of the electric field, so long as they are charged, in a pH gradient generated by ampholytes having *pI* values in a wide range (poly-aminocarboxylic acids) dissolved in the separation buffer. The three basic steps in capillary isoelectric focusing are loading, focusing, and mobilization.

##### Loading—

**Loading in One Step**— The sample is mixed with ampholytes and introduced into the capillary by pressure or vacuum.

**Sequential Loading**— A leading buffer, then the ampholytes, then the sample mixed with ampholytes, again ampholytes alone, and finally the terminating buffer are introduced into the capillary. The volume of the sample must be small enough so as to not modify the pH gradient.

**Focusing**— When the voltage is applied, ampholytes migrate toward the cathode or the anode according to their net charge, creating the pH gradient from anode (lower pH) to cathode (higher pH). The components to be separated migrate until they reach a pH corresponding to their isoelectric point, and the current drops to very low values.

**Mobilization**— The bands of separated components migrate past the detector by one of the three following methods.

**Method 1**— During Focusing, under the influence of the electroosmotic flow when this flow is small enough to allow the focusing of the components.

**Method 2**— By application of positive pressure after Focusing.

**Method 3**— After Focusing, by adding salts in the cathode reservoir or the anode reservoir, depending on the direction chosen for mobilization, in order to alter the pH in the capillary when the voltage is applied. As the pH is changed, the proteins and ampholytes are mobilized in the direction of the reservoir, which contains added salts and pass the detector.

The separation achieved is expressed as  $\Delta pI$  and depends on the pH gradient ( $d\text{pH}$ ), the number of ampholytes having different *pI* values, the diffusion coefficient (D), the intensity of the electric field (E), and the variation of the electrophoretic mobility of the analyte with the pH, and is as follows:

$$\Delta pI = 3 \sqrt{\frac{D(d\text{pH} / dx)}{E(-d\mu / d\text{pH})}}$$

in which  $d\text{pH}/dx$  is the pH gradient; and  $-d\mu/d\text{pH}$  is the variation of the solution mobility with the pH in the region close to the *pI*.

**Optimization Parameters**— The major parameters that need to be considered in the development of separations are voltage, capillary, and solutes.

**Voltage**: use of high fields from 300 V/cm to 1,000 V/cm during Focusing.

**Capillary**— Depending on the Mobilization strategy selected (see above), the electroosmotic flow must be reduced or suppressed. Coated capillaries tend to reduce the electroosmotic flow.

**Solutions**— The anode buffer reservoir is filled with a solution of a lower pH than the *pI* of the most acidic ampholyte, and the cathode reservoir is filled with a solution with a higher pH than the *pI* of the most basic ampholyte. Phosphoric acid for the anode and sodium hydroxide for the cathode are frequently used.

Addition of a polymer, like methylcellulose, in the ampholyte solution tends to suppress convective forces (if any) and electroosmotic flow by increasing the viscosity. Commercial ampholytes covering many pH ranges are available and may also be mixed to obtain an expanded pH range. Broad pH ranges are used to estimate the isoelectric point, whereas narrower ranges are employed to improve accuracy. Calibration can be made by correlating migration time with the isoelectric point of a series of standard protein markers. During Focusing, precipitation of proteins at their isoelectric point can be prevented, if necessary, using buffer additives such as glycerol, surfactants, urea, or Zwitterionic buffers. However, depending on the concentration, urea can denature proteins.

#### MICELLAR ELECTROKINETIC CHROMATOGRAPHY (MEKC)

Separation takes place in an electrolytic solution that contains a surfactant, generally ionic, at a concentration above the critical micellar concentration. The solute molecules are distributed between the aqueous buffer and the pseudo-stationary phase composed by the micelles according to the solute's partition coefficient. The technique can be considered as a hybrid of electrophoresis and chromatography. It is an electrophoretic technique that can be used for the separation of both neutral and charged solutes maintaining the efficiency, speed, and instrumental suitability of capillary electrophoresis. One of the most widely used surfactants is sodium dodecyl sulfate, although other anionic and cationic surfactants, such as cetyl trimethyl ammonium salts, have also been used.

At neutral and alkaline pH, a strong electroosmotic flow is generated and moves the separation buffer ions in the direction of the cathode. If sodium dodecyl sulfate is used as surfactant, the electrophoretic migration of the anionic micelle is in the opposite direction, toward the anode. As a result, the overall micelle migration velocity is slowed compared to the bulk flow of the electrolytic solution. In the case of neutral solutes, because the analyte can partition between the micelle and the aqueous buffer and has no electrophoretic mobility, the analyte migration velocity will only depend on the partition coefficient between the micelle and the aqueous buffer. In the electropherogram, the peak corresponding to each uncharged solute is always between that of the electroosmotic flow marker and that of the micelle; and the time elapsed between these two peaks is called the separation window. For electrically charged solutes, the migration velocity depends on both the partition coefficient of the solute between the micelle and the aqueous buffer and on the electrophoretic mobility of the solute in the absence of micelles.

The separation mechanism is essentially chromatographic, and migration of the solute and resolution can be expressed in terms of the capacity factor of the solute ( $K'$ ), which is the ratio between the total number of moles of solute in the micelle to those in the mobile phase. For a neutral compound,  $K'$  is as follows:

$$K' = \frac{t_r - t_0}{t_0(1 - t_r/t_m)} = K \left( \frac{V_s}{V_m} \right)$$

in which  $t_r$  is the migration time of the solute;  $t_0$  is the analysis time of the unretained solute obtained by injecting an electroosmotic flow marker that does not enter the micelle (i.e., methanol);  $t_m$  is the micelle migration time measured by injecting a micelle marker, such as Sudan III, which migrates continuously associated in the micelle;  $K$  is the partition



coefficient of the solute; VS is the volume of the micelles phase; and VM is the volume of the mobile phase.

The resolution between two closely-migrating compounds (RS) is as follows:

$$R_s = \frac{\sqrt{N}}{4} \times \frac{\alpha-1}{\alpha} \times \frac{k_b}{k_b+1} \times \frac{1 - \left( \frac{t_0}{t_m} \right)}{1 + k_a \times \left( \frac{t_0}{t_m} \right)}$$

in which N is the number of theoretical plates for one of the compounds;  $\alpha$  is the selectivity obtained;  $k_a$  and  $k_b$  are retention factors for both components, respectively ( $k_b > k_a$ ); and the other terms are as defined above.

Similar, but not identical, equations give  $k$  and  $RS$  values for electrically charged compounds.

#### Optimization Parameters

The main parameters to be considered in the development of separations by MEKC are instrumental and electrolytic solution parameters.

##### Instrumental Parameters—

**Voltage**— Separation time is inversely proportional to applied voltage. An increase in voltage can cause excessive heat production that gives rise to temperature gradients and viscosity gradients of the buffer in the cross section of the capillary. This effect can be significant with high conductivity buffers, such as those containing micelles. Poor heat dissipation causes band broadening and decreases resolution.

**Temperature**— Variations in capillary temperature affect the partition coefficient of the solute between the buffer and the micelle, the critical micelle concentration, and the viscosity of the buffer. These parameters contribute to the migration time of the solutes.

**Capillary**— Length and internal diameter contribute to analysis time and efficiency of separations. Increasing both effective length and total length can decrease the electrical fields, working at constant voltage, and will increase migration time and improve the separation efficiency. The internal diameter controls heat dissipation, at a given buffer and electrical field, and provides a broadening of the sample band.

##### Electrolytic Solution Parameters—

**Surfactant Type and Concentration**— The type of surfactant, as the stationary phase in chromatography, affects the resolution because it modifies separation selectively. The  $\log K'$  of a neutral compound increases linearly with the concentration of detergent in the mobile phase. When  $K'$  approaches the value of

$$\sqrt{t_m / t_a}$$

resolution in MEKC reaches a maximum. Modifying the concentration of surfactant in the mobile phase changes the resolution.

**Buffer pH**— pH does not modify the partition coefficient of non-ionized solutes, but it can modify the electroosmotic flow in uncoated capillaries. A decrease in the buffer pH decreases the electroosmotic flow and, therefore, increases the resolution of the neutral solutes, giving rise to longer analysis time.

**Organic Solvents**— To improve separation of hydrophobic compounds, organic modifiers (methanol, propanol, acetonitrile, etc.) can be added to the separation electrolytic solution. The addition of these modifiers generally decreases migration time and selectivity of the separation. The addition of organic modifiers affects micelle formation, thus, a given surfactant concentration can be used only with a certain percentage of organic modifier before the micelleization equilibrium is eliminated or adversely affected, resulting in the absence of micelles and, therefore, the absence of the partition mechanism of MEKC. The elimination of micelles in the presence of a high content of organic solvent does not always mean that the separation will no longer be possible, because, in some cases, the hydrophobic interaction between the ionic surfactant monomer and the neutral solutes form solvophobic complexes that can be separated electrophoretically.

**Additives for Chiral Separations**— A chiral selector is included in the micellar system, either covalently bound to the surfactant or added to the micellar separation electrolyte. Micelles that have a moiety with chiral discrimination properties include salts, N-dodecanoyl-L-amino acids, bile salts, etc. Chiral resolution can also be achieved using chiral discriminators, such as cyclodextrins, added to the electrolytic solutions that contain micellicized achiral surfactants.

**Other Additives**— Selectivity can be modified by adding chemicals to the buffer. Addition of several types of cyclodextrins to the buffer is also used to reduce the interaction of hydrophobic solutes with the micelle, increasing the selectivity for this type of compound. The addition of substances able to modify solute-micelle interactions by adsorption on the latter has been used to improve the selectivity of the separations in MEKC. These additives may consist of a second surfactant (ionic or non-ionic), which gives rise to mixed micelles, metallic cations that dissolve in the micelle and give co-ordination complexes with the solutes.

#### Quantitative Analysis

Peak areas must be divided by the corresponding migration time to give the corrected area in order to compensate for the shift in migration time from run to run, thus reducing the variation of the response. Dividing the peak areas by migration time will also compensate for the different responses of sample constituents with different migration times. Where an internal standard is used, check that no peak of the substance to be examined is masked by that of the internal standard.

**Calculations**— From the values obtained, calculate the content of a component or components being determined. When indicated, the percentage of one (or more) components of the sample to be examined is calculated by determining the areas of the peak(s) as a percentage of the total corrected areas of all the peaks, excluding those due to solvents or any added reagents. The use of an automatic integration system (integrator or data acquisition and processing system) is recommended.

#### CAPILLARY ELECTROPHORESIS SYSTEM SUITABILITY

The choice of suitability parameters to be used will depend on the type of capillary electrophoresis that is performed. These parameters are the capacity factor ( $K'$ ) used only for Micellar Electrokinetic Chromatography, the number of theoretical plates ( $n$ ), the symmetry factor ( $AS$ ), and the resolution ( $RS$ ). Note that in previous sections, the theoretical expressions for  $n$  and  $RS$  have been described, but more practical equations that allow for the determination of these suitability parameters using the electrophoretograms are described below.

The number of theoretical plates ( $n$ ) may be calculated from the formula:

$$n = 5.54 (t / b0.5)^2$$

in which  $t$  is the distance, in mm, along the baseline between the point of injection and the perpendicular dropped from the maximum of the peak in question; and  $b0.5$  is the peak



width, in mm, at half-height.

The resolution (RS) may be calculated from the formula:

$$RS = 1.18(t_b - t_a) / b0.5b + b0.5a$$

in which  $t_b$  and  $t_a$  are the distances, in mm, along the baseline between the point of injection and the perpendicular dropped from the maxima of two adjacent peaks ( $t_b > t_a$ ); and  $b0.5b$  and  $b0.5a$  are the peak widths, in mm, at half-height.

The resolution (RS) may also be calculated by measuring the height of the valley (c) between two partly resolved peaks in a standard preparation, the height of the smaller peak (d), and by specifying  $(c/d) \leq x$ , in which  $x$  is the limit indicated in the individual monograph.

The symmetry factor of a peak (AS) may be calculated using the formula:

$$AS = b0.05/2A$$

in which  $b0.05$  is the width of the peak at one-twentieth of the peak height; and  $A$  is the distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

Other system suitability parameters include tests for area repeatability (i.e., standard deviation of areas or of area/migration time) and tests for migration time repeatability (i.e., standard deviation of migration time). For migration time repeatability, it will be necessary to provide for a test to measure the suitability of the capillary washing procedures. To avoid the lack of repeatability of the migration time, an alternative practice is to use a migration time relative to an internal standard.

A test for the verification of the signal-to-noise ratio for a standard preparation or the determination of the limit of quantitation is a useful system suitability parameter. The detection limit and quantitation limit correspond to a signal-to-noise ratio greater than 3 and 10, respectively. The signal-to-noise ratio (S/N) is calculated as follows:

$$S/N = 2H/hn$$

in which  $H$  is the height of the peak corresponding to the component concerned in the electrophoretogram obtained with the specified reference solution; and  $hn$  is the absolute value of the largest noise fluctuation from the baseline in an electrophoretogram obtained after injection of a blank and observed over a distance equal to twenty times the width at the half-height of the peak in the electrophoretogram obtained with the reference solution, and situated equally around the place where this peak would be found.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Alexey Khrenov, Ph.D</a> Senior Scientific Associate 1-301-816-8345	(BBPP05) Biologics and Biotechnology - Proteins and Polysaccharides

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#### 1054 BIOTECHNOLOGY-DERIVED ARTICLES—ISOELECTRIC FOCUSING

This chapter provides guidance and procedures used for the characterization of biotechnology-derived articles by isoelectric focusing. This chapter is harmonized with the corresponding chapters in JP and EP. Other characterization tests, also harmonized, are shown in the USP general information chapters [Biotechnology-Derived Articles—Amino Acid Analysis](#) [1052](#), [Biotechnology-Derived Articles—Capillary Electrophoresis](#) [1053](#), [Biotechnology-Derived Articles—Peptide Mapping](#) [1055](#), [Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis](#) [1056](#), and [Biotechnology-Derived Articles—Total Protein Assay](#) [1057](#).

#### INTRODUCTION

Isoelectric focusing (IEF) is a method of electrophoresis that separates proteins according to their isoelectric points. Separation is carried out in a slab of polyacrylamide or agarose gel that contains a mixture of amphoteric electrolytes (ampholytes). When subjected to an electrical field, the ampholytes migrate in the gel to create a pH gradient. In some cases, gels containing an immobilized pH gradient, prepared by incorporating weak acids and bases to specific regions of the gel network during the preparation of the gel, are used. When the applied proteins reach the gel fraction that has a pH that is the same as their isoelectric point, their charge is neutralized and migration ceases. Gradients can be made over various ranges of pH, according to the mixture of ampholytes chosen.

#### GENERAL PRINCIPLES

When a protein is at the position of its isoelectric point, it has no net charge and cannot be moved in a gel matrix by the electric field. It may, however, move from that position by diffusion. The pH gradient forces a protein to remain in its isoelectric point position, thus concentrating it; this concentration effect is called “focusing”. Increasing the applied voltage or reducing the sample load results in improved resolution of bands. The applied voltage is limited by the heat generated because the heat must be dissipated. The use of thin gels and an efficient cooling plate controlled by a thermostatic circulator prevents the burning of the gel while allowing sharp focusing. The separation is estimated by determining the minimum pl difference, which is necessary to separate two neighboring bands, as follows:

$$\Delta pI = 3 \sqrt{\frac{D(dpH/dx)}{E(-d\mu/dpH)}}$$

in which  $D$  is the diffusion coefficient of the protein;  $dpH/dx$  is the pH gradient;  $E$  is the intensity of the electric field, in volts per centimeter; and  $-d\mu/dpH$  is the variation of the solute mobility with the pH in the region close to the pl. Because  $D$  and  $-d\mu/dpH$  for a given protein cannot be altered, the separation can be improved by using a narrower pH range and by increasing the intensity of the electric field.

From an operational point, special attention must be paid to sample characteristics and/or preparation. Salt in a sample can be problematic, and it is best to prepare the sample, if possible, in deionized water or 2% ampholytes using dialysis or gel filtration if necessary. Potentials of 2500 volts have been used and are considered optimal under a given set of conditions. Up to 30 watts of constant power can be applied and will generally give complete separation in 1.5 to 3.0 hours. The time required for completion of focusing in thin-layer polyacrylamide gels is determined by placing a colored protein (e.g., hemoglobin) at different positions on the gel surface and by applying the electric field: the steady state is reached when all applications give an identical band pattern. In some procedures the completion of the focusing is indicated by the time elapsed after the sample application.

Resolution between protein bands on an IEF gel prepared with carrier ampholytes can be quite good. Better resolution may be achieved by using immobilized pH gradients where the buffering species, which are analogous to carrier ampholytes, are copolymerized within the gel matrix. Proteins exhibiting pl values differing by as little as 0.02 pH units may be resolved using a gel prepared with carrier ampholytes, whereas immobilized pH gradients can resolve protein differing by approximately 0.001 pH units.

The IEF gel can be used as an identity test when migration on the gel is compared to a standard preparation and IEF calibration proteins; the IEF gel can be used as a limit test when the density of a band on IEF is compared subjectively with the density of bands appearing in a standard preparation, or it can be used as a semiquantitative test when the density is measured using a densitometer or similar instrumentation to determine the relative concentration of protein in the bands.

#### APPARATUS

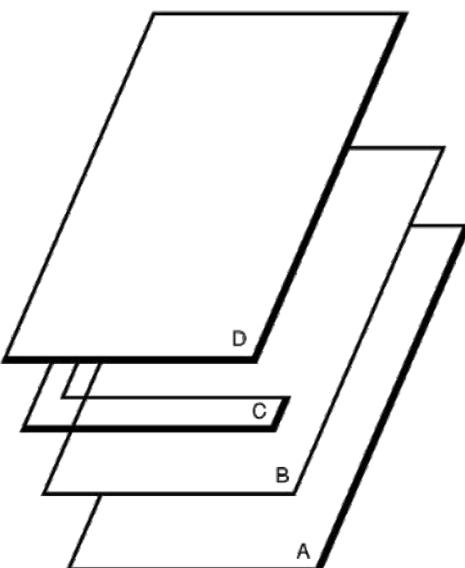
An apparatus for isoelectric focusing consists of a controllable direct current generator, of stabilized output; a rigid plastic isoelectric focusing chamber that contains a cooled plate of suitable material to support the gel; and a plastic cover with platinum electrodes that are connected to the gel by means of paper wicks of suitable width, length, and thickness, impregnated with solutions of anodic and cathodic electrolytes.

#### PROCEDURE

Unless otherwise indicated in a given monograph, the following procedure in thick polyacrylamide slab gels is to be used.

##### Preparation of the Gels

Assembly— Composed of a glass plate (A) on which a polyester film (B) is placed to facilitate handling of the gel, one or more spacers (C), a second glass plate (D), and clamps to hold the structure together (see [Figure 1](#)).



**Figure 1. Mould**

7.5% Polyacrylamide Gel— Dissolve 29.1 g of acrylamide and 0.9 g of methylenebisacrylamide in 100 mL of water. To 2.5 volumes of this solution, add the mixture of ampholytes specified in the individual monograph, and dilute up to 10 volumes with water. Mix carefully, and degas the solution.

Preparation of the Assembly— Place the polyester film on the lower glass plate, apply the spacer, place the second glass plate, and fit the clamps. Before use, place the mixture on a magnetic stirrer, and add 0.25 volumes of a 10% solution of ammonium persulfate and 0.25 volumes of tetramethylenediamine. Immediately fill the space between the glass plates of the assembly with the gel.

Fixing Solution for Isoelectric Focusing Polyacrylamide Gel— Mix 35 g of sulfosalicylic acid and 100 g of trichloroacetic acid in 1000 mL of water.

Coomassie Staining Solution and Destaining Solution— Use the same solutions indicated in general information chapter [Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis](#) (1056).

Procedure— Dismantle the assembly, and using the polyester film, transfer the gel onto the cooled support wetted with a few mL of a suitable liquid, taking care to avoid forming air bubbles. Prepare the test solutions and reference solutions as specified in the individual monograph. Place strips of paper for sample application, about 10 mm × 5 mm in size, on the gel, and impregnate each with the prescribed amount of the test and reference solutions. If the protein concentration of the solution is too low, several strips may be superimposed (up to four). Also apply the prescribed quantity of a solution of proteins with known isoelectric points as pH markers to calibrate the gel. In some procedures, the gel has precast slots where a solution of the sample is applied instead of using impregnated paper strips. Cut two strips of paper to the length of the gel, and impregnate them with the electrolyte solutions: acid for the anode and alkaline for the cathode. The compositions of the anode and cathode solutions are given in the individual monograph. Apply these paper wicks to each side of the gel several mm from the edge. Fit the cover so that the electrodes are in contact with the wicks (with respect to the anodic and cathodic poles). Proceed with the isoelectric focusing by applying the electrical parameters described in the individual monograph. Switch off the current when the migration of the mixture of standard proteins has stabilized. Using forces, remove the sample application strips and the two electrode wicks. Immerse the gel in Fixing Solution for Isoelectric Focusing Polyacrylamide Gel. Incubate with gentle shaking at room temperature for 30 minutes. Drain off the solution, and add 200 mL of Destaining Solution. Incubate with shaking for 1 hour. Drain the gel, and add Coomassie Staining Solution. Incubate for 30 minutes. Destain the gel by passive diffusion with Destaining Solution until the bands are well visualized against a clear background. Locate the position and intensity of the bands in the electropherogram, as prescribed in the individual monograph.

Alternative Procedure— When a monograph references the general method for isoelectric focusing above, variations in methodology or procedure may be used, subject to validation. These variations include the use of commercially available precast gels; the use of immobilized pH gradients; the use of rod gels, and the use of cassettes of different dimensions, including ultrathin (0.2 mm) gels; variations in the sample application procedure, including different sample volumes or the use of sample application masks or wicks other than paper; the use of alternate running conditions, including variations in the electric field depending on gel dimensions and equipment, and the use of fixed migration times rather than subjective interpretation of band stability; the inclusion of a prefocusing step; the use of automated instrumentation; and the use of agarose gels.

##### Validation of Procedure

Where alternative methods to the general method are employed, they must be validated. The following criteria may be used to validate the separation: formation of a stable pH gradient of desired characteristics, evaluated using colored pH markers of known isoelectric points; comparison with the electropherogram provided with the chemical reference substance for the preparation to be examined; and any other validation criteria as prescribed in the individual monograph.

##### SPECIFIED VARIATIONS TO THE GENERAL METHOD

Variations to the general method required for the analysis of specific substances may be specified in detail in individual monographs. Variations may include the addition of urea in the running gel (a 3 M concentration is often satisfactory to keep the protein in solution, but up to 8 M can be used). Some proteins precipitate at their isoelectric point. In this case, urea is included in the gel formulation to keep the protein in solution. If urea is used, only fresh solutions should be used to prevent carbamylation of the protein. Other variations include the use of alternative staining methods and the use of gel additives such as nonionic detergents (e.g., octylglucoside) or zwitterionic detergents (e.g., CHAPS or CHAPSO) to prevent proteins from aggregating or precipitating.



note—The following are general precautionary items that can be used to improve the method.

- Samples can be applied to any area on the gel, but in general, they should be applied to areas where they are expected to focus. To protect the proteins from extreme pH environments, samples should not be applied close to either electrode. During method development, the analyst can try applying the protein in three positions on the gel (i.e., middle and both ends); the pattern of a protein applied at opposite ends of the gel may not be identical.
- A phenomenon known as cathodic drift, where the pH gradient decays over time, may occur if a gel is focused too long. Although not well understood, electroendoosmosis and absorption of carbon dioxide may be factors that lead to cathodic drift. Cathodic drift is observed as focused protein migrating off the cathode end of the gel. Immobilized pH gradients may be used to address this problem.
- Efficient cooling (approximately 4°) of the bed that the gel lies on during focusing is important. High field strengths used during isoelectric focusing can lead to overheating and affect the quality of the focused gel.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Alexey Khrenov, Ph.D</a> Senior Scientific Associate 1-301-816-8345	(BBPP05) Biologics and Biotechnology - Proteins and Polysaccharides

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#### 1055 BIOTECHNOLOGY-DERIVED ARTICLES—PEPTIDE MAPPING

This chapter provides guidance and procedures used for characterization of biotechnology-derived articles by peptide mapping. This chapter is harmonized with the corresponding chapter in JP and EP. Other characterization tests, also harmonized, are shown in [Biotechnology-Derived Articles—Amino Acid Analysis](#) 1052, [Biotechnology-Derived Articles—Capillary Electrophoresis](#) 1053, [Biotechnology-Derived Articles—Isoelectric Focusing](#) 1054, [Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis](#) 1056, and [Biotechnology-Derived Articles—Total Protein Assay](#) 1057.

#### INTRODUCTION

Peptide mapping is an identity test for proteins, especially those obtained by rDNA technology. It involves the chemical or enzymatic treatment of a protein, resulting in the formation of peptide fragments, followed by separation and identification of the resultant fragments in a reproducible manner. It is a powerful test that is capable of identifying single amino acid changes resulting from events such as errors in the reading of complementary DNA (cDNA) sequences or point mutations. Peptide mapping is a comparative procedure because the information obtained, compared to a reference standard or reference material similarly treated, confirms the primary structure of the protein, is capable of detecting whether alterations in structure have occurred, and demonstrates process consistency and genetic stability. Each protein presents unique characteristics that must be well understood so that the scientific and analytical approaches permit validated development of a peptide map that provides sufficient specificity.

This section provides detailed assistance in the application of peptide mapping and its validation to characterize the desired protein product, to evaluate the stability of the expression construct of cells used for recombinant DNA products, to evaluate the consistency of the overall process, and to assess product stability, as well as to ensure the identity of the protein product or to detect the presence of protein variant. The validation scheme presented differentiates between qualification of the method at an early stage in the regulatory process, the Investigational New Drug (IND) level, and full validation in support of New Drug Application (NDA), Product License Application (PLA), or Marketing Authorization Application (MAA).

The validation concepts described are consistent with the general information chapter [Validation of Compendial Procedures](#) 1225 and with the International Conference on Harmonization (ICH) document on Analytical Methods Validation.

#### THE PEPTIDE MAP

Peptide mapping is not a general method, but involves developing specific maps for each unique protein. Although the technology is evolving rapidly, there are certain methods that are generally accepted. Variations of these methods will be indicated, when appropriate, in specific monographs.

A peptide map may be viewed as a fingerprint of a protein and is the end product of several chemical processes that provide a comprehensive understanding of the protein being analyzed. Four major steps are necessary for the development of the procedure: isolation and purification of the protein, if the protein is part of a formulation; selective cleavage of the peptide bonds; chromatographic separation of the peptides; and analysis and identification of the peptides. A test sample is digested and assayed in parallel with a reference standard or reference material. Complete cleavage is more likely to occur when enzymes such as endoproteases (e.g., trypsin) are used instead of chemical cleavage reagents. A map should contain enough peptides to be meaningful. On the other hand, if there are too many fragments, the map might lose its specificity because many proteins will then have the same profiles.

#### Isolation and Purification

Isolation and purification are necessary for analysis of bulk drugs or dosage forms containing interfering excipients and carrier proteins and, when required, will be specified in the monograph. Quantitative recovery of protein from the dosage form should be validated.

#### Selective Cleavage of Peptide Bonds

The selection of the approach used for the cleavage of peptide bonds will depend on the protein under test. This selection process involves determination of the type of cleavage to be employed—enzymatic or chemical—and the type of cleavage agent within the chosen category. Several cleavage agents and their specificity are shown in [Table 1](#).

Table 1. Examples of Cleavage Agents

Type	Agent	Specificity
Enzymatic	Trypsin, EC 3.4.21.4	C-terminal side of Arg and Lys
	Chymotrypsin, EC 3.4.21.1	C-terminal side of hydrophobic residues (e.g., Leu, Met, Ala, aromatics)
	Pepsin A (Pepsin), EC 3.4.23.1	Nonspecific digest
	Lysyl endopeptidase (Lys-C endopeptidase), EC 3.4.21.50	C-terminal side of Lys
	Glutamyl endopeptidase (Glu-C endopeptidase; V8 protease); (from <i>S. aureus</i> strain V8), EC 3.4.21.19	C-terminal side of Glu and Asp
Chemical	Peptidyl-Asp metalloendopeptidase (Asp-N endopeptidase), EC 3.4.24.33	N-terminal side of Asp
	Clostrypain (Arg-C endopeptidase), EC 3.4.22.8	C-terminal side of Arg
	Cyanogen bromide	C-terminal side of Met
	2-Nitro-5-thiocyanobenzoic acid	N-terminal side of Cys
	O-Iodosobenzoic acid	C-terminal side of Trp and Tyr
	Dilute acid	Asp and Pro
	BNPS-skatole	Trp



This list is not all-inclusive and will be expanded as other cleavage agents are identified.

Pretreatment of Sample— Depending on the size or the configuration of the protein, different approaches in the pretreatment of samples can be used. For monoclonal antibodies, the heavy and light chains will need to be separated before mapping. If trypsin is used as a cleavage agent for proteins with a molecular mass greater than 100,000 Da, lysine residues must be protected by citraconylation or maleylation; otherwise, too many peptides will be generated.

Pretreatment of the Cleavage Agent— Pretreatment of cleavage agents, especially enzymatic agents, might be necessary for purification purposes to ensure reproducibility of the map. For example, trypsin used as a cleavage agent will have to be treated with tosyl-l-phenylalanine chloromethyl ketone to inactivate chymotrypsin. Other methods, such as purification of trypsin by HPLC or immobilization of enzyme on a gel support, have been successfully used when only a small amount of protein is available.

Pretreatment of the Protein— It might be necessary under certain conditions to concentrate the sample, or to separate the protein from added substances and stabilizers used in the formulation of the product if these interfere with the mapping procedure. Physical procedures used for pretreatment can include ultrafiltration, column chromatography, and lyophilization.

Other pretreatments such as the addition of chaotropic agents (e.g., urea) can be used to unfold the protein prior to mapping. It is often necessary to reduce and alkylate the disulfide bonds prior to digestion in order to allow the enzyme to have full access to cleavage sites and permit some unfolding of the protein.

Digestion with trypsin can introduce ambiguities in the tryptic map as a result of side reactions occurring during the digestion reaction, such as nonspecific cleavage, deamidation, disulfide isomerization, oxidation of methionine residues, or formation of pyroglutamyl groups created from the deamidation of glutamine at the N-terminal side of a peptide.

Furthermore, peaks may be produced by autohydrolysis of trypsin. Their intensities depend on the ratio of trypsin to protein. To avoid autohydrolysis, solutions of proteases may be prepared at a pH that is not optimal (e.g., at pH 5 for trypsin), which would mean that the enzyme would not become active until diluted with the digest buffer.

Establishment of Optimal Digestion Conditions— Factors that affect the completeness and effectiveness of digestion of proteins are those that could affect any chemical or enzymatic reactions.

pH— The digestion mixture pH is empirically determined to ensure the optimal performance of the given cleavage agent. For example, a highly acidic environment (e.g., pH 2, formic acid) is necessary when using cyanogen bromide as a cleavage agent; however, a slightly alkaline environment (pH 8) is optimal when using trypsin as a cleavage agent. As a general rule, the pH of the reaction milieu should not alter the chemical integrity of the protein during the digestion and should not change during the course of the fragmentation reaction.

Temperature— A temperature between 25° and 37° is adequate for most digestions. The temperature used is intended to minimize chemical side reactions. The type of protein under test will dictate the temperature of the reaction milieu because some proteins are more susceptible to denaturation as the temperature of the reaction increases. For example, digestion of recombinant bovine somatotropin is conducted at 4° because at higher temperatures it will precipitate during digestion.

Time— If a sufficient amount of sample is available, a time course study is considered in order to determine the optimum time to obtain a reproducible map and avoid incomplete digestion. Time of digestion varies from 2 to 30 hours. The reaction is stopped by the addition of an acid that does not interfere with the tryptic map, or by freezing.

Amount of Cleavage Agent— Although excessive amounts of cleavage agent are used to accomplish a reasonably rapid digestion time (i.e., 6 to 20 hours), the amount of cleavage agent is minimized to avoid its contribution to the chromatographic map pattern. A protein-to-protease ratio between 20:1 and 200:1 is generally used. It is recommended that the cleavage agent be added in two or more stages to optimize cleavage. Nonetheless, the final reaction volume remains small enough to facilitate the next step in peptide mapping—the separation step. To sort out digestion artifacts that might be interfering with the subsequent analysis, a blank determination is performed using a digestion control with all the reagents except the test protein.

#### Chromatographic Separation

Many techniques are used to separate peptides for mapping. The selection of a technique depends on the protein being mapped. Techniques that have been successfully used for the separation of peptides are shown in [Table 2](#).

Table 2. Techniques Used for the Separation of Peptides

Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC)
Ion-Exchange Chromatography (IEC)
Hydrophobic Interaction Chromatography (HIC)
Polyacrylamide Gel Electrophoresis (PAGE), nondenaturating
Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)
Capillary Electrophoresis (CE)
Paper Chromatography
High-Voltage Paper Electrophoresis (HVPE)

In this section, a most widely used reverse-phase HPLC (RP-HPLC) method is described as one of the procedures of chromatographic separation.

The purity of solvents and mobile phases is a critical factor in HPLC separation. HPLC-grade solvents and water that are commercially available are recommended for RP-HPLC. Dissolved gases present a problem in gradient systems where the solubility of the gas in a solvent may be less in a mixture than in a single solvent. Vacuum degassing and agitation by sonication are often used as useful degassing procedures. The solid particles in the solvents are drawn into the HPLC system; they can damage the sealing of pump valves or clog the top of the chromatographic column. Both pre- and post-pump filtration are also recommended.

Chromatographic Column— The selection of a chromatographic column is empirically determined for each protein. Columns with 100 Å or 300 Å pore size and silica support can give optimal separation. For smaller peptides, column packings of octylsilane chemically bonded to totally porous silica articles 3 to 10 µm in diameter (L7) and of octadecylsilane chemically bonded to porous silica or ceramic microparticles 3 to 10 µm in diameter (L1) are more efficient than the butyl silane chemically bonded to totally porous silica particles 5 to 10 µm in diameter (L26).

Solvent— The most commonly used solvent is water with acetonitrile as the organic modifier to which less than 0.1% of trifluoroacetic acid is added. If necessary, add isopropyl alcohol or n-propyl alcohol to solubilize the digest components, provided that the addition does not unduly increase the viscosity of the components.

Mobile Phase— Buffered mobile phases containing phosphate are used to provide some flexibility in the selection of pH conditions, since shifts of pH in the 3.0 to 5.0 range enhance the separation of peptides containing acidic residues (e.g., glutamic and aspartic acids). Sodium or potassium phosphates, ammonium acetate, and phosphoric acid, with a pH between 2 and 7 (or higher for polymer-based supports), have also been used with acetonitrile gradients. Acetonitrile-containing trifluoroacetic acid is also used quite often.

Gradient Selection— Gradients can be linear, nonlinear, or include step functions. A shallow gradient is recommended in order to separate complex mixtures. Gradients are optimized to provide clear resolution of one or two peaks that will become "marker" peaks for the test.

Isocratic Selection— Isocratic HPLC systems using a single mobile phase are used on the basis of their convenience of use and improved detector responses. Optimal composition of a mobile phase to obtain clear resolution of each peak is sometimes difficult to establish. Mobile phases for which slight changes in component ratios or in pH significantly affect retention times of peaks in peptide maps should not be used in isocratic HPLC systems.

Other Parameters— Temperature control of the column is usually necessary to achieve good reproducibility. The flow rates for the mobile phases range from 0.1 to 2.0 mL per minute, and the detection of peptides is performed with a UV detector at 200 to 230 nm. Other methods of detection have been used (e.g., postcolumn derivatization), but they are not as robust or as versatile as UV detection.

System Suitability— The section System Suitability under [Chromatography](#) provides an experimental means for measuring the overall performance of the test method. The acceptance criteria for system suitability depend on the identification of critical test parameters that affect data interpretation and acceptance. These critical parameters are also criteria



...el monitor peptide digestion and peptide analysis. An indicator that the desired digestion endpoint was achieved is the comparison with a reference standard or reference material, which is treated exactly as the article under test. The use of a USP Reference Standard in parallel with the protein under test is critical in the development and establishment of system suitability limits. In addition, a specimen chromatogram should be included with the USP Reference Standard or reference material for comparison purposes. Other indicators may include visual inspection of protein or peptide solubility, the absence of intact protein, or measurement of responses of a digestion-dependent peptide. The critical system suitability parameters for peptide analysis will depend on the particular mode of peptide separation and detection, and on the data analysis requirements.

When peptide mapping is used as an identification test, the system suitability requirements for the identified peptides cover selectivity and precision. In this case, as well as when identification of variant proteins is done, the identification of the primary structure of the peptide fragments in the peptide map provides both a verification of the known primary structure and the identification of protein variants by comparison with the peptide map of the USP Reference Standard or reference material for the specified protein. The use of a digested USP Reference Standard or reference material for a given protein in the determination of peptide resolution is the method of choice. For an analysis of a variant protein, a characterized mixture of a variant and a reference standard can be used, especially if the variant peptide is located in a less-resolved region of the map. The index of pattern consistency can be simply the number of major peptides detected. Peptide pattern consistency can be best defined by the resolution of peptide peaks. Chromatographic parameters—such as peak-to-peak resolution, maximum peak width, peak tailing factors, and column efficiency—may be used to define peptide resolution. Depending on the protein under test and the method of separation used, single peptide or multiple peptide resolution requirements may be necessary.

The replicate analysis of the digest of the USP Reference Standard or reference material for the protein under test yields measures of precision and quantitative recovery. Recovery of the identified peptides is generally ascertained by the use of internal or external peptide standards. The precision is expressed as the relative standard deviation (RSD). Differences in the recovery and precision of the identified peptides are expected; therefore, the system suitability limits will have to be established for both the recovery and the precision of the identified peptides. These limits are unique for a given protein and will be specified in the individual monograph.

Visual comparison of the relative retention times, the peak responses, the number of peaks, and the overall elution pattern is completed initially. It is then complemented and supported by mathematical analysis of the peak response ratios and by the chromatographic profile of a 1:1 (v/v) mixture of sample and USP Reference Standard or reference material digest. If all peaks in the sample digest and in the USP Reference Standard or reference material digest have the same relative retention times and peak response ratios, then the identity of the sample under test is confirmed.

If peaks that initially eluted with significantly different relative retention times are then observed as single peaks in the 1:1 mixture, the initial difference would be an indication of system variability. However, if separate peaks are observed in the 1:1 mixture, this would be evidence of the nonequivalence of the peptides in each peak. If a peak in the 1:1 mixture is significantly broader than the corresponding peak in the sample and USP Reference Standard or reference material digest, it may indicate the presence of different peptides. The use of computer-aided pattern recognition software for the analysis of peptide mapping data has been proposed and applied, but issues related to the validation of the computer software preclude its use in a compendial test in the near future. Other automated approaches have been used that employ mathematical formulas, models, and pattern recognition. Such approaches, for example, the automated identification of compounds by IR spectroscopy and the application of diode-array UV spectral analysis for identification of peptides, have been proposed. These methods have limitations due to inadequate resolutions, co-elution of fragments, or absolute peak response differences between USP Reference Standard or reference material and sample fragments.

The numerical comparison of the retention times and peak areas or peak heights can be done for a selected group of relevant peaks that have been correctly identified in the peptide maps. Peak areas can be calculated using one peak showing relatively small variation as an internal reference, keeping in mind that peak area integration is sensitive to baseline variation and likely to introduce error in the analysis. Alternatively, the percentage of each peptide peak height relative to the sum of all peak heights can be calculated for the sample under test. The percentage is then compared to that of the corresponding peak of the USP Reference Standard or reference material. The possibility of autohydrolysis of trypsin is monitored by producing a blank peptide map that is the peptide map obtained when a blank solution is treated with trypsin.

The minimum requirement for the qualification of peptide mapping is an approved test procedure that includes system suitability as a test control. In general, for an IND, qualification of peptide mapping for a protein is sufficient. As the regulatory approval process for the protein progresses, additional qualifications of the test can include a partial validation of the analytical procedure to provide assurance that the method will perform as intended in the development of a peptide map for the specified protein.

#### Analysis and Identification of Peptides

This section gives guidance on the use of peptide mapping during development in support of regulatory applications.

The use of a peptide map as a qualitative tool does not require the complete characterization of the individual peptide peaks. However, validation of peptide mapping in support of regulatory applications requires rigorous characterization of each of the individual peaks in the peptide map. Methods to characterize peaks range from N-terminal sequencing of each peak followed by amino acid analysis to the use of mass spectroscopy (MS).

For characterization purposes, when N-terminal sequencing and amino acid analysis are used, the analytical separation is scaled up. Because scale-up might affect the resolution of peptide peaks, it is necessary, using empirical data, to assure that there is no loss of resolution due to scale-up. Eluates corresponding to specific peptide peaks are collected, vacuum-concentrated, and chromatographed again, if necessary. Amino acid analysis of fragments may be limited by the peptide size. If the N-terminus is blocked, it may need to be cleared before sequencing. C-terminal sequencing of proteins or a combination of carboxypeptidase digestion and matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) MS can also be used for characterization purposes.

The use of MS for characterization of peptide fragments is by direct infusion of isolated peptides or by the use of on-line LC-MS for structure analysis. In general, it includes electrospray and MALDI-TOF analyzers as well as fast atom bombardment (FAB). Tandem MS has also been used to sequence a modified protein and to determine the type of amino acid modification that has occurred. The comparison of mass spectra of the digests before and after reduction provides a method to assign the disulfide bonds to the various sulphydryl-containing peptides.

If regions of the primary structure are not clearly demonstrated by the peptide map, it might be necessary to develop a secondary peptide map. The goal of a validated method of characterization of a protein through peptide mapping is to reconcile and account for at least 95% of the theoretical composition of the protein structure.

#### THE USE OF PEPTIDE MAPPING FOR GENETIC STABILITY EVALUATION

A validated peptide map can be used to assess the integrity of the predicted primary sequence of a protein product (i.e., its genetic stability). It can also be used to determine lot-to-lot consistency of the biotechnology-derived product process. Furthermore, the performance of the protein expression of the production system is best assessed by peptide mapping of the expressed protein. Peptide maps of protein produced at various times of the protein expression process, including a point well beyond the normal protein expression time, compared with those of a USP Reference Standard or reference material, will evaluate the genetic stability of the expression system as a function of time.

Variant protein sequences can arise from a genetic variation at the DNA level (point mutation) or as an error in the translation process. A validated peptide map is the best approach to the detection of protein variants. However, the limitations of the peptide mapping itself must be taken into consideration. The detection of a structured variant is possible only if the corresponding peptide variant is easily isolated and characterized. To establish genetic stability will require the use of a battery of biochemical methods, provided that the variants have properties different from those of the "normal" protein.

#### VALIDATION

##### Critical Factors

Validation of peptide mapping requires that a protocol be designed, outlining in detail the experiment to be conducted and the criteria for acceptance of the map. Criteria for acceptance of mapping include detection limit, specificity, linearity, range, accuracy, precision, and reagent stability. Reproducibility of the peptide map is a critical element in the utilization of such a map as an identity test and for confirming genetic stability. Those technical aspects of peptide mapping that influence the reproducibility of the map will be discussed.

The setting of limits, with respect to quantification (peak area or height) and identification (retention times) for the selected group of relevant peaks is based on empirical observations. These limits detect significant differences between the sample and USP Reference Standard or reference material within a series of analyses.



Another critical issue is the recovery of peptides and its impact on peak area determination and reproducibility and on the establishment of acceptance criteria. The recovery criteria address all aspects of test methodology, from digestion to chromatographic conditions. Determination of peptide recovery includes quantitative amino acid analysis, spike addition, radiolabeling, and UV summation. An overall recovery of about 80% is considered satisfactory. Recovery of individual peptides is more problematic and is handled on a case-by-case basis. The critical factors considered in the validation of a peptide map are as follows.

**Written Test Procedures**— These procedures include a detailed description of the analytical method in which reagents, equipment, sample preparation, method of analysis, and analysis of the data are defined.

**Validation Protocol**— A protocol is prepared that contains a procedure for test validation.

**Acceptance Criteria**— The criteria can be minimal at the early stages, but need to be better defined as validation studies progress.

**Reporting of Results**— Results from the validation study are documented with respect to the analytical parameters listed in the validation protocol.

**Revalidation of the Test Procedure**— If the method used requires alteration that could affect the analytical parameter previously assessed in the validation of the procedure, the test procedure must be revalidated. Significant changes in the processing of the article, in laboratories performing the analysis, in formulation of the bulk or the finished products, and in any other significant parameter will require revalidation of the methods.

#### Requirements

#### Precision—

**Intratest Precision**— This is a measure of the reproducibility of peptide mapping. The two critical steps in peptide mapping are fragmentation (i.e., digestion) and separation of peptides. An acceptable precision occurs where the absolute retention times and the relative peak areas are constant from run to run, and the average variation in retention time is small relative to that of a selected internal reference peak. The reproducibility of the map can be enhanced if a temperature-controlled column oven is used, if an extensive equilibration of the system is performed prior to the start of the test, if a blank (control digest mixture without protein) is run first to minimize “first run effects,” and if a USP Reference Standard or a reference material digest is interspersed periodically with test samples to evaluate chromatographic drift.

The criteria for validation of the fragmentation step are similar to those described below for separation of peptides, but they are met for consecutive tests of a series of separately prepared digests of the protein under test.

The criteria for validation of the separation of peptides step include the following:

1. The average standard deviation of the absolute retention times of all major peaks for a set of consecutive tests of the same digest does not exceed a specified acceptance criterion.
2. The average standard deviation of absolute peak area for all fully resolved major peaks does not exceed a specified percentage.

**Intertest Precision**— This is a measure of the reproducibility of the peptide mapping when the test is performed on different days, by different analysts, in different laboratories, with reagents or enzymes from different suppliers or different lots from the same supplier, with different instruments, on columns of different makes or columns of the same make from different lots, and on individual columns of the same make from the same lot. Although it would be desirable, from a scientific perspective, to validate all of these variables in terms of their impacts on precision, a practical approach is to validate the test using those variables most likely to be encountered under operational conditions. Additional variables can be included when needed.

The experimental design allows the analyst to make comparisons using peak retention times and areas that are expressed relative to a highly reproducible internal reference peak within the same chromatogram. The relative peak area is expressed as the ratio of the peak area to that of the internal reference peak. The relative retention time can be expressed as the difference between the absolute retention time and that of the reference peak. The use of relative values eliminates the need to make separate corrections for differences due to injector-to-injector volumes, units of measure for peak areas, column dimensions, and instrument dead volumes. The variability in the retention times and peak areas for the Intertest Precision experiments is expected to be slightly higher than the variability observed for Intratest Precision.

**Robustness**— Factors such as composition of the Mobile Phase, protease quality or chemical reagent purity, column variation and age, and digest stability are likely to affect the overall performance of the test and its reproducibility. Tolerances for each of the key parameters are evaluated and baseline limits established in case the test is used for routine lot release purposes.

**Mobile Phase**— The composition of the Mobile Phase is optimized to obtain the maximum resolution of peptides throughout the elution profile. A balance between optimal resolution and overall reproducibility is desired. A lower pH might improve peak separation but might shorten the life of the column, resulting in lack of reproducibility. Peptide maps at a pH above and below the pH of the procedure are compared to the peptide map obtained at the pH of the procedure and checked for significant differences; they are also reviewed with respect to the acceptance criteria established in the validation protocol.

**Protease Quality or Chemical Reagent Purity**— A sample of the USP Reference Standard or reference material for the protein under test is prepared and digested with different lots of cleavage agent. The chromatograms for each digest are compared in terms of peak areas, shape, and number. The same procedure can be applied to other critical chemicals or pretreatment procedures used during sample preparation, such as reducing and carboxymethylation reagents.

**Column Considerations**— Column-to-column variability, even within a single lot, can affect the performance of the column in the development of peptide maps. Column size may also lead to significant differences. A USP Reference Standard or reference material of the protein under test is digested and the digest is chromatographed on different lots of column from a single manufacturer. The maps are then evaluated in terms of the overall elution profile, retention times, selectivity resolution, and recovery. To evaluate the overall lifetime of the column in terms of robustness, perform a peptide mapping test on different columns and vary significantly the number of injections (e.g., from 10 injections to 250 injections). The resulting maps can then be compared for significant differences in peak broadening, peak area, and overall resolution. As a column ages, an increase in back pressure might be observed that might affect the peptide maps.

A sensible precaution in the use of peptide mapping columns is to select alternative columns in case the original columns become unavailable or are discontinued. Perform a peptide mapping test using equivalent columns from different manufacturers, and examine the maps. Differences in particle shape and size, pore size and volume, carbon load, and end-capping can lead to significant differences in retention times, elution profile selectivity, resolution, and recovery. Slight modifications in the gradient profile may be required to achieve equivalency of mapping when using columns from different manufacturers. [note—The equivalency between instrumentation used for the validation of the test and for routine quality control testing should be considered. It might be preferable to use the same HPLC system for all applications. Otherwise, equivalency of the systems is determined, which may require some changes in the chromatographic test conditions.]

**Digest Stability**— The length of time a digest can be kept before it is chromatographed, as well as the conditions under which the digest is stored before chromatography, is assessed. Several aliquots from a single digest are stored at different storage conditions and chromatographed. These maps are then evaluated for significant differences.

**Reproducibility**— Determination of various parameters indicated above is repeated using the same USP Reference Standard or reference material and test sample in at least two different laboratories by two analysts equipped with similar HPLC systems. The generated peptide maps are evaluated for significant differences.

**Auxiliary Information**— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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## 1056 BIOTECHNOLOGY-DERIVED ARTICLES—POLYACRYLAMIDE GEL ELECTROPHORESIS

This chapter provides guidance and procedures used for characterization of biotechnology-derived articles by polyacrylamide gel electrophoresis. This chapter is harmonized with the corresponding chapter in JP and EP. Other characterization tests, also harmonized, are shown in [Biotechnology-Derived Articles—Amino Acid Analysis](#) 1052, [Biotechnology-Derived Articles—Capillary Electrophoresis](#) 1053, [Biotechnology-Derived Articles—Isoelectric Focusing](#) 1054, [Biotechnology-Derived Articles—Peptide Mapping](#) 1055, and [Biotechnology-Derived Articles—Total Protein Assay](#) 1057.

### INTRODUCTION

Polyacrylamide gel electrophoresis (PAGE) is used for the qualitative characterization of proteins in biological preparations, for control of purity, and for quantitative determinations.

This procedure is limited to the analysis of proteins with a weight range of 14,000 to 100,000 Da. It is possible to extend the weight range of an electrophoresis gel by various techniques (e.g., gradient gels or particular buffer systems), but such techniques are not discussed in this chapter. Analytical gel electrophoresis is an appropriate method with which to identify and to assess the homogeneity of proteins in drug substances. These methods are routinely used for the estimation of protein subunit molecular weights and for the determination of the subunit compositions of purified proteins.

Ready-to-use gels and reagents are commercially available and can be used instead of those described in this chapter, provided that they give equivalent results and that they meet the validation requirements.

### GENERAL PRINCIPLE OF ELECTROPHORESIS

Under the influence of an electrical field, charged particles migrate in the direction of the electrode bearing the opposite polarity. In gel electrophoresis, the movements of the particles are retarded by interactions with the surrounding gel matrix, which acts as a molecular sieve. The opposing interactions of the electrical force and molecular sieving result in differential migration rates according to the sizes, shapes, and charges of particles. Because of their different physicochemical properties, different macromolecules of a mixture migrate at different speeds during electrophoresis and thus are separated into discrete fractions. Electrophoretic separations can be conducted in systems without support phases (e.g., free solution separation in capillary electrophoresis) and in stabilizing media, such as thin-layer plates, films, or gels.

### CHARACTERISTICS OF POLYACRYLAMIDE GELS FOR PROTEIN ELECTROPHORESIS

The sieving properties of polyacrylamide gels are established by the three-dimensional network of fibers and pores that is formed as the bifunctional bisacrylamide cross-links adjacent to polyacrylamide chains. Polymerization is catalyzed by a free-radical-generating system composed of ammonium persulfate and N,N,N',N'-tetramethylethylenediamine (TEMED).

As the acrylamide concentration of a gel increases, its effective pore size decreases. The effective pore size of a gel is operationally defined by its sieving properties, that is, by the resistance it imparts to the migration of macromolecules. There are limits to the acrylamide concentrations that can be used. At high acrylamide concentrations, gels break much more easily and are difficult to handle. As the pore size of a gel decreases, the migration rate of a protein through the gel decreases. By adjusting the pore size of a gel, through manipulating the acrylamide concentration, the resolution of the method can be optimized for a given protein product. Thus, a given gel is physically characterized by its respective composition of acrylamide and bisacrylamide.

In addition to the composition of the gel, the state of the protein is an important component of electrophoretic mobility. In the case of proteins, electrophoretic mobility is dependent on the pK value of the charged groups and the size of the molecule. It is influenced by the type, the concentration, and the pH of the buffer; by the temperature and the field strength; and by the nature of the support material.

#### Denaturation with Sodium Dodecyl Sulfate

Denaturing PAGE using sodium dodecyl sulfate (SDS) is the most common mode of electrophoresis used in assessing the pharmaceutical quality of protein products. Typically, analytical electrophoresis of proteins is carried out under conditions that ensure dissociation of the proteins into their individual polypeptide subunits and that minimize aggregation of these subunits. The strongly anionic detergent SDS is used in combination with heat to dissociate the proteins before they are loaded on the gel. The denatured polypeptides bind SDS, become negatively charged, and exhibit a consistent charge-to-weight ratio regardless of protein type. Because the amount of SDS bound is almost always proportional to the molecular weight of the polypeptide and is typically independent of its sequence, SDS-polypeptide complexes migrate through polyacrylamide gels in reasonable accordance with the size of the polypeptide.

The electrophoretic mobilities of the resultant detergent-polypeptide complexes all assume the same functional relationship to the molecular weights of the polypeptides. Migration of SDS derivatives is toward the anode in a predictable manner, with low molecular weight complexes migrating faster than larger ones. This means that the molecular weight of a protein can be estimated from its relative mobility in calibrated SDS PAGE and that a single band in such a gel is a criterion of purity.

Modifications to the polypeptide backbone, such as N- or O-linked glycosylation, however, have a significant impact on the apparent molecular weight of a protein. This is due to the fact that SDS does not bind to a carbohydrate moiety in a manner similar to that of the polypeptide. Thus, a consistent charge-to-weight ratio is not maintained. The apparent molecular weight of proteins having undergone post-translational modifications is not a true reflection of the weight of the polypeptide chain.

#### Reducing Conditions

Polypeptide subunits and their three-dimensional structure can be maintained in proteins by the presence of disulfide bonds. A goal of SDS PAGE analysis under reducing conditions is to disrupt this structure by reducing disulfide bonds. Complete denaturation and dissociation of proteins by treatment with 2-mercaptoethanol or dithiothreitol (DTT) will result in the unfolding of the polypeptide backbone and subsequent complexation with SDS. Under these conditions, the molecular weight of the polypeptide subunits can be calculated by linear regression in the presence of suitable molecular weight standards.

#### Nonreducing Conditions

For some analyses, complete dissociation of protein to peptide subunits is not desirable. In the absence of treatment with reducing agents, disulfide covalent bonds remain intact, preserving the oligomeric form of the protein. Oligomeric SDS-protein complexes migrate more slowly than their SDS-polypeptide subunits. In addition, nonreduced proteins may not be completely saturated with SDS and hence may not bind the detergent in a constant weight ratio. This makes molecular weight determinations of these molecules less straightforward than analyses of fully denatured polypeptides, because, for valid comparisons, it is necessary that both standards and unknown proteins be in similar configurations. However, the staining of a single band in such a gel is a criterion of purity.

#### Characteristics of a Discontinuous Buffer System

The most popular electrophoretic method for the characterization of a complex mixture of proteins involves the use of a discontinuous buffer system consisting of two contiguous, but distinct, gels: a resolving or separating (lower) gel and a stacking (upper) gel. The two gels are cast with different porosities, pHs, and ionic strengths. In addition, different mobile ions are used in the gel and electrode buffers. The buffer discontinuity concentrates large volumes of sample in the stacking gel, resulting in improved resolution. When power is applied, a voltage drop develops across the sample solution that drives the proteins into the stacking gel. Glycinate ions from the electrode buffer follow the proteins into the stacking gel. A moving boundary region is rapidly formed with the highly mobile chloride ions in the front and the relatively slow glycinate ions in the rear. A localized high-voltage gradient forms between the leading and trailing ion fronts, causing the SDS-protein complexes to form into a thin zone (stack) and migrate between the chloride and glycinate phases. Within a broad limit, regardless of the height of the applied sample, all SDS proteins condense into a very narrow region and enter the resolving gel as a well-defined, thin zone of high protein density. The large-pore stacking gel does not retard the migration of most proteins and serves mainly as an anticonvective medium. At the interface between the stacking and resolving gels, the proteins experience a sharp retardation due to the restrictive pore size of the resolving gel. Once in the resolving gel, proteins continue to be slowed by the sieving of the matrix. The glycinate ions overtake the proteins, which then move in a space of uniform pH formed by tris(hydroxymethyl)aminomethane (Tris) and glycine. Molecular sieving causes the SDS-polypeptide complexes to separate on the basis of their molecular weights.



## Preparation of Gels

In a discontinuous buffer SDS-polyacrylamide gel, it is important to pour the resolving gel, let the gel set, and then pour the stacking gel, because the composition of acrylamide–bisacrylamide, buffer, and pH are different.

## Gel Stock Solutions—

30% Acrylamide–Bisacrylamide Solution— Prepare a solution containing 290 g of acrylamide and 10 g of methylene bisacrylamide per L of warm water, and filter. [note—Acrylamide and methylene bisacrylamide are slowly converted during storage to acrylic acid and bisacrylic acid, respectively. This deamidation reaction is catalyzed by light and alkali. The pH of the solution must be 7.0 or lower. Store the solution in dark bottles at room temperature. Fresh solutions are prepared every month.]

Ammonium Persulfate Solution— Prepare a small quantity of solution having a concentration of 100 g of ammonium persulfate per L, and store at 4°. [note—Ammonium persulfate provides the free radicals that drive polymerization of acrylamide and bisacrylamide. Ammonium persulfate decomposes slowly; therefore, prepare fresh solutions weekly.]

TEMED— Use an electrophoresis-grade reagent. [note—TEMED accelerates the polymerization of acrylamide and bisacrylamide by catalyzing the formation of free radicals from ammonium persulfate. Because TEMED works only as a free base, polymerization is inhibited at low pH.]

SDS Solution— Use an electrophoresis-grade reagent. Prepare a solution having a concentration of about 100 g of SDS per L, and store at room temperature.

1.5 M Buffer Solution— Transfer about 90.8 g of Tris to a 500-mL flask, dissolve in 400 mL of water, adjust with hydrochloric acid to a pH of 8.8, dilute with water to volume, and mix.

1 M Buffer Solution— Transfer about 60.6 g of Tris to a 500-mL flask, add 400 mL of water, adjust with hydrochloric acid to a pH of 6.8, dilute with water to volume, and mix.

Plate Preparation— Clean two glass plates (10 cm × 8 cm), the polytef comb, the two spacers, and the silicone rubber tubing (0.6 mm × 35 cm) with mild detergent, rinse thoroughly with water, and blot dry.

Lubricate the spacers and the tubing with nonsilicone grease. Apply the spacers along each of the two short sides of the glass plate 2 mm away from the edges and 2 mm away from the long side corresponding to the bottom of the gel.

Begin to lay the tubing on the glass plate by using one spacer as a guide. Carefully twist the tubing at the bottom of the spacer, and follow the long side of the glass plate. While holding the tubing with one finger along the long side, twist the tubing again, and lay it on the second short side of the glass plate, using the spacer as a guide.

Place the second glass plate in perfect alignment with the first, and hold the gel mold together by hand pressure. Apply two clamps on each of the two short sides of the mold. Carefully apply four clamps on the longer side of the mold, thus forming the bottom of the mold. Verify that the tubing is running along the edge of the glass plates and has not been extruded while placing the clamps. The mold is now ready for the pouring of the gel.

Resolving Gel— In a conical flask, prepare the appropriate volume of solution, containing the desired concentration of acrylamide, as shown in [Table 1](#). Mix the components in the order shown. Before adding the Ammonium Persulfate Solution and the TEMED, pour the solution into a disposable filtration unit equipped with a nitrocellulose filter having a 0.45-μm porosity, and apply vacuum. Allow the solution to degas by swirling the filtration unit, and disconnect the vacuum when no more bubbles are formed in the solution. Add appropriate amounts of Ammonium Persulfate Solution and TEMED, as shown in [Table 1](#); swirl; and pour immediately into the gap between the two glass plates of the mold. Leave sufficient space for the stacking gel (the length of the teeth of the comb plus 1 cm). Using a pipet, carefully overlay the solution with water-saturated isobutyl alcohol. Leave the gel in a vertical position at room temperature for polymerization.

Table 1. Preparation of Resolving Gel

Solution Component	Component Volume (mL) per Gel Mold Volume Below							
	5 mL	10 mL	15 mL	20 mL	25 mL	30 mL	40 mL	50 mL
6% Acrylamide								
Water	2.6	5.3	7.9	10.6	13.2	15.9	21.2	26.5
30% Acrylamide–Bisacrylamide Solution	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.0
1.5 M Buffer Solution	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
SDS Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
Ammonium Persulfate Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.004	0.008	0.012	0.016	0.02	0.024	0.032	0.04
8% Acrylamide								
Water	2.3	4.6	6.9	9.3	11.5	13.9	18.5	23.2
30% Acrylamide–Bisacrylamide Solution	1.3	2.7	4.0	5.3	6.7	8.0	10.7	13.3
1.5 M Buffer Solution	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
SDS Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
Ammonium Persulfate Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.003	0.006	0.009	0.012	0.015	0.018	0.024	0.03
10% Acrylamide								
Water	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
30% Acrylamide–Bisacrylamide Solution	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
1.5 M Buffer Solution	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
SDS Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
Ammonium Persulfate Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
12% Acrylamide								
Water	1.6	3.3	4.9	6.6	8.2	9.9	13.2	16.5
30% Acrylamide–Bisacrylamide Solution	2.0	4.0	6.0	8.0	10.0	12.0	16.0	20.0
1.5 M Buffer Solution	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
SDS Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
Ammonium Persulfate Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
14% Acrylamide								
Water	1.4	2.7	3.9	5.3	6.6	8.0	10.6	13.8
30% Acrylamide–Bisacrylamide Solution	2.3	4.6	7.0	9.3	11.6	13.9	18.6	23.2
1.5 M Buffer Solution	1.2	2.5	3.6	5.0	6.3	7.5	10.0	12.5



SDS Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
Ammonium Persulfate Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
15% Acrylamide								
Water	1.1	2.3	3.4	4.6	5.7	6.9	9.2	11.5
30% Acrylamide–Bisacrylamide Solution	2.5	5.0	7.5	10.0	12.5	15.0	20.0	25.0
1.5 M Buffer Solution	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
SDS Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
Ammonium Persulfate Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

After polymerization is complete (about 30 minutes later), pour off the overlay, and wash the top of the gel several times with water to remove the isobutyl alcohol overlay and any unpolymerized acrylamide. Drain as much fluid as possible from the top of the gel, then remove any remaining water with the edge of a paper towel.

Stacking Gel—In a conical flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide, as shown in [Table 2](#).

Table 2. Preparation of Stacking Gel

Solution Component	Component Volume (mL) per Gel Mold Volume Below							
	1 mL	2 mL	3 mL	4 mL	5 mL	6 mL	8 mL	10 mL
Water	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
30% Acrylamide–Bisacrylamide Solution	0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1.0 M Buffer Solution	0.13	0.25	0.38	0.5	0.63	0.75	1.0	1.25
SDS Solution	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
Ammonium Persulfate Solution	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

Mix the components in the order shown. Before adding the Ammonium Persulfate Solution and the TEMED, pour the solution into a disposable filtration unit equipped with a nitrocellulose filter having a 0.45- $\mu$ m porosity, and apply vacuum. Allow the solution to degas by swirling the filtration unit, and disconnect the vacuum when no more bubbles are formed in the solution. Add appropriate amounts of Ammonium Persulfate Solution and TEMED as shown in [Table 2](#), swirl, and pour immediately into the gap between the two glass plates of the mold directly onto the surface of the polymerized Resolving Gel. Immediately insert a clean polytef comb into the stacking gel solution, being careful to avoid trapping air bubbles. Add more stacking gel solution to fill the spaces of the comb completely. Leave the gel in a vertical position, and allow it to polymerize at room temperature. After polymerization is complete (about 30 minutes later), carefully remove the polytef comb, and proceed as directed below.

#### Electrophoretic Separation

Sample Buffer 1—Dissolve 1.89 g of Tris, 5.0 g of SDS, 50 mg of bromophenol blue, and 25.0 mL of glycerol in 100 mL of water. Adjust with hydrochloric acid to a pH of 6.8, and dilute with water to 125 mL. Before use, dilute with an equal volume of water or sample, and mix.

Sample Buffer 2 (for reducing conditions)—Prepare as directed in Sample Buffer 1 except to add 12.5 mL of 2-mercaptoethanol before adjusting the pH. Alternatively, prepare as directed for Sample Buffer 1 except to start with about 1.93 g of Tris and add a suitable quantity of DTT to obtain a final 100  $\mu$ M DTT concentration.

Running Buffer—Dissolve 151.4 g of Tris, 721.0 g of aminoacetic acid (glycine), and 50.0 g of SDS in water; dilute with water to 5000 mL; and mix to obtain a stock solution. Immediately before use, dilute this stock solution with water to 10 times its volume, mix, and adjust to a pH between 8.1 and 8.8.

Procedure—Rinse the wells immediately with water or with the Running Buffer to remove any unpolymerized acrylamide. (If necessary, straighten the teeth of the Stacking Gel with a blunt hypodermic needle attached to a syringe.) Remove the clamps on one short side, carefully pull out the tubing, and replace the clamps. Proceed similarly on the other short side. Remove the tubing from the bottom part of the gel.

Mount the completed gel in the electrophoresis apparatus. Add the electrophoresis buffers to the top and bottom reservoirs. Remove any bubbles that become trapped at the bottom of the gel between the glass plates. [note—Removal is best done with a bent hypodermic needle attached to a syringe. Never prerun the gel before loading the samples, because that will destroy the discontinuity of the buffer systems. Before loading the sample, carefully rinse the slot with Running Buffer.]

Prepare the test and standard solutions in the recommended Sample Buffer, and treat as directed in the individual monograph. Apply the appropriate volume of each solution to the Stacking Gel wells.

Start the electrophoresis under the conditions recommended by the manufacturer of the equipment. Electrophoresis running time and current or voltage may need to be varied in order to achieve optimum separation. Check that the dye front is moving into the Resolving Gel. When the dye has reached the bottom of the gel, stop the electrophoresis. Remove the gel assembly from the apparatus, and separate the glass plates. Remove the spacers, cut off and discard the Stacking Gel, and immediately proceed with staining.

#### Detection of Proteins in Gels

Coomassie staining is the most common protein staining method, with a detection level on the order of 1 to 10  $\mu$ g of protein per band. Silver staining is the most sensitive method for staining proteins in gels, because a band containing 10 to 100 ng can be detected; but the method is more cumbersome and less rugged. All of the steps in gel staining are performed at room temperature with gentle agitation (e.g., on a rocking platform shaker or equivalent). Gloves must be worn when staining the gels to prevent fingerprint residue staining.

#### Reagents—

Coomassie Staining Solution—Prepare a solution of Coomassie brilliant blue R-250 having a concentration of 1.25 g per L in a mixture of water, methanol, and glacial acetic acid (5:4:1). Filter, and store at room temperature.

Destaining Solution—Prepare a mixture of water, methanol, and glacial acetic acid (5:4:1).

Fixing Solution 1—Prepare a mixture of water, methanol, and trichloroacetic acid (5:4:1).

Fixing Solution 2—Transfer 250 mL of methanol to a 500-mL volumetric flask, add 0.27 mL of formaldehyde, dilute with water to volume, and mix.

Silver Nitrate Reagent—To a mixture of 40 mL of 1 M sodium hydroxide and 3 mL of ammonium hydroxide, add, dropwise and with stirring, 8 mL of a 200 g per L solution of silver nitrate; dilute with water to 200 mL, and mix.

Developing Solution—Transfer 2.5 mL of a citric acid solution (2 in 100) and 0.27 mL of formaldehyde to a 500.0-mL volumetric flask, dilute with water to volume, and mix.

Stopping Solution—Prepare a 10% (v/v) solution of acetic acid.

Coomassie Staining—Immerse the gel in an excess of Coomassie Staining Solution, and incubate for at least 1 hour. Remove the Coomassie Staining Solution. Destain the gel with an excess of Destaining Solution. Change the Destaining Solution several times, until the stained protein bands are clearly distinguishable on a clear background. The more thoroughly the gel is destained, the smaller the amount of protein that can be detected. Destaining can be accelerated by including a few g of anion-exchange resin or a small sponge in the Destaining Solution. [note—The acid–alcohol solutions used in this procedure do not completely fix proteins in the gel. This can lead to losses of some low molecular weight proteins during the staining and destaining of thin gels. Permanent fixation is obtainable by incubating the gel in Fixing Solution 1 for 1 hour before it is immersed in the Coomassie Staining



#### Solution.]

Silver Staining— Immerse the gel in an excess of Fixing Solution 2, and incubate for 1 hour. Remove Fixing Solution 2, add fresh Fixing Solution 2, and incubate for at least 1 hour, or overnight if convenient. Discard Fixing Solution 2, and wash the gel in an excess of water for 1 hour. Soak the gel for 15 minutes in a 1% solution of glutaraldehyde (v/v). Wash the gel twice, for 15 minutes each time, with an excess of water. Soak the gel in fresh Silver Nitrate Reagent for 15 minutes in darkness. Wash the gel three times, for 5 minutes each time, with an excess of water. Immerse the gel for about 1 minute in Developing Solution until satisfactory staining has been obtained. Stop the development by incubation in the Stopping Solution for 15 minutes, then rinse the gel with water, and proceed with drying as indicated below.

#### Drying of Gels

For Coomassie staining, after the destaining step, incubate the gel in a glycerol solution (1 in 10) for at least 2 hours. For silver staining, add to the final rinsing step a 5-minute incubation in a glycerol solution (1 in 50).

Immerse two sheets of porous cellophane in water, and incubate for 5 to 10 minutes. Place one of the sheets on a drying frame. Carefully lift the gel, and place it on the cellophane sheet. Remove any trapped air bubbles, and pour a few mL of water around the edges of the gel. Place the second sheet on top, and remove any trapped air bubbles. Complete the assembly of the drying frame. Place in a drying oven, leave at room temperature until dry, or use a commercial gel dryer.

#### Molecular Weight Determination

Molecular weights of proteins are determined by comparison of their mobilities with those of several marker proteins of known molecular weight. Mixtures of proteins with precisely known molecular weights blended for uniform staining are available for calibrating gels. They are available in various molecular weight ranges. Concentrated stock solutions of proteins of known molecular weight are diluted in a sample buffer and loaded on the same gel as the protein sample to be tested.

Immediately after the gel has been run, the position of the bromophenol blue tracking dye is marked to identify the leading edge of the electrophoretic ion front. This can be done by cutting notches in the edges of the gel or by inserting a needle soaked in India ink into the gel at the dye front. After staining, measure the migration distances of each protein band (markers and unknowns) from the top of the Resolving Gel. Divide the migration distance of each protein by the distance traveled by the tracking dye. The normalized migration distances so obtained are called the relative mobilities of the proteins (relative to the dye front) and conventionally denoted as RF. Construct a (semilogarithmic) plot of the logarithm of the molecular weights (MR) of the protein standards as functions of the RF values. [note—The graphs are slightly sigmoid.] From the graph so obtained, estimate the unknown molecular weights by linear regression analysis or interpolation, as long as unknown samples are positioned along the linear part of the graph.

If the proteins of the molecular weight marker are not distributed along 80% of the length of the gel and over the required separation range (i.e., the range covering the product and its dimer or the products and its related impurities), and the separation obtained for the relevant protein bands does not show a linear relationship between the logarithm of the molecular weight and the RF, the test is not valid.

#### Quantification of Impurities

Where the impurity limit is specified in the individual monograph, a reference solution corresponding to that level of impurity is prepared by diluting the test solution. For example, where the limit is 5.0%, a reference solution is a 1 in 20 dilution of the test solution. No impurity—any band other than the main band—in the electropherogram obtained from the test solution is more intense than the main band obtained with the reference solution.

Under validated conditions and when using the Coomassie staining procedure, impurities may be quantified by normalization to the main band, using an integrating densitometer. In this case, the responses must be validated for linearity.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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#### 1057 BIOTECHNOLOGY-DERIVED ARTICLES—TOTAL PROTEIN ASSAY

This chapter provides guidance and procedures used for characterization of biotechnology-derived articles. This chapter is harmonized with the corresponding chapter in JP and EP.

Other characterization tests, also harmonized, are provided in [Biotechnology-Derived Articles—Amino Acid Analysis](#) (1052), [Biotechnology-Derived Articles—Capillary Electrophoresis](#) (1053), [Biotechnology-Derived Articles—Isoelectric Focusing](#) (1054), [Biotechnology-Derived Articles—Peptide Mapping](#) (1055), and [Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis](#) (1056).

#### INTRODUCTION

The following procedures are provided as illustrations of the determination of total protein content in pharmacopeial preparations. Other techniques, such as HPLC, are also acceptable if total protein recovery is demonstrated. Many of the total protein assay methods described below can be performed successfully using kits from commercial sources. [note—Where water is required, use distilled water.]

#### Method 1

Protein in solution absorbs UV light at a wavelength of 280 nm due to the presence of aromatic amino acids, mainly tyrosine and tryptophan. This property is the basis of Method 1. Protein determination at 280 nm is mainly a function of the tyrosine and tryptophan content of the protein. If the buffer used to dissolve the protein has a high absorbance relative to that of water, there is an interfering substance in the buffer. This interference can be compensated for when the spectrophotometer is adjusted to zero buffer absorbance. The results may be compromised if the interference results in a large absorbance that challenges the limit of sensitivity of the spectrophotometer. Furthermore, at low concentrations protein can be absorbed onto the cuvette, thereby reducing the content in solution. This can be prevented by preparing samples at higher concentrations or by using a nonionic detergent in the preparation. [note—Keep the Test Solution, the Standard Solution, and the buffer at the same temperature during testing.]

Test Solution— Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration of 0.2 to 2 mg per mL.

Standard Solution— Unless otherwise specified in the individual monograph, prepare a solution of USP Reference Standard or reference material for the protein under test in the same buffer and at the same concentration as the Test Solution.

Procedure— Concomitantly determine the absorbances of the Standard Solution and the Test Solution in quartz cells at a wavelength of 280 nm with a suitable spectrophotometer (see [Spectrophotometry and Light-Scattering](#) (851)), using the buffer as the blank. To obtain accurate results, the response should be linear in the range of protein concentrations to be assayed.

Light-Scattering— The accuracy of the UV spectroscopic determination of protein can be decreased by the scattering of light by the test specimen. If the proteins in solution exist as particles comparable in size to the wavelength of the measuring light (250 to 300 nm), scattering of the light beam results in an apparent increase in absorbance of the test specimen. To calculate the absorbance at 280 nm due to light-scattering, determine the absorbances of the Test Solution at wavelengths of 320, 325, 330, 335, 340, 345, and 350 nm. Using the linear regression method, plot the log of the observed absorbance versus the log of the wavelength, and determine the standard curve best fitting the plotted points. From the graph so obtained, extrapolate the absorbance value due to light-scattering at 280 nm. Subtract the absorbance due to light-scattering from the total absorbance at 280 nm to obtain the absorbance value of the protein in solution. Filtration with a filter having a 0.2-μm porosity or clarification by centrifugation may be performed to reduce the effect of light-scattering, especially if the solution is noticeably turbid.



Calculations— Calculate the concentration, CU, of protein in the test specimen by the formula:

$$CS(AU / AS)$$

in which CS is the concentration of the Standard Solution; and AU and AS are the corrected absorbances of the Test Solution and the Standard Solution, respectively (see [Spectrophotometry and Light-Scattering \(851\)](#)).

#### Method 2

This method, commonly referred to as the Lowry assay, is based on the reduction by protein of the phosphomolybdc–tungstic mixed acid chromogen in the Folin-Ciocalteu's phenol reagent, resulting in an absorbance maximum at 750 nm. The Folin-Ciocalteu's phenol reagent reacts primarily with tyrosine residues in the protein, which can lead to variation in the response of the assay to different proteins. Because the method is sensitive to interfering substances, a procedure for precipitation of the protein from the test specimen may be used.

Where separation of interfering substances from the protein in the test specimen is necessary, proceed as directed below for Interfering Substances prior to preparation of the Test Solution. The effect of interfering substances can be minimized by dilution, provided the concentration of the protein under test remains sufficient for accurate measurement.

**Standard Solutions**— Unless otherwise specified in the individual monograph, dissolve the USP Reference Standard or reference material for the protein under test in the buffer used to prepare the Test Solution. Dilute portions of this solution with the same buffer to obtain not fewer than five Standard Solutions having concentrations between 5 and 100 µg of protein per mL, the concentrations being evenly spaced.

**Test Solution**— Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the Standard Solutions. An appropriate buffer will produce a pH in the range of 10.0 to 10.5.

**Blank**— Use the buffer used for the Test Solution and the Standard Solutions.

#### Reagents and Solutions—

**Copper Sulfate Reagent**— Dissolve 100 mg of cupric sulfate and 200 mg of sodium tartrate in water, dilute with water to 50 mL, and mix. Dissolve 10 g of sodium carbonate in water to a final volume of 50 mL, and mix. Slowly pour the sodium carbonate solution into the copper sulfate solution with mixing. Prepare this solution fresh daily.

**SDS Solution**— Dissolve 5 g of sodium dodecyl sulfate in water, and dilute with water to 100 mL.

**Sodium Hydroxide Solution**— Dissolve 3.2 g of sodium hydroxide in water, dilute with water to 100 mL, and mix.

**Alkaline Copper Reagent**— Prepare a mixture of Copper Sulfate Reagent, SDS Solution, and Sodium Hydroxide Solution (1:2:1). This reagent may be stored at room temperature for up to 2 weeks.

**Diluted Folin-Ciocalteu's Phenol Reagent**— Mix 10 mL of Folin-Ciocalteu's phenol TS with 50 mL of water. Store in an amber bottle, at room temperature.

**Procedure**— To 1 mL of each Standard Solution, the Test Solution, and the Blank, add 1 mL of Alkaline Copper Reagent, and mix. Allow to stand at room temperature for 10 minutes. Add 0.5 mL of the Diluted Folin-Ciocalteu's Phenol Reagent to each solution, mix each tube immediately, and allow to stand at room temperature for 30 minutes. Determine the absorbances of the solutions from the Standard Solutions and the Test Solution at the wavelength of maximum absorbance at 750 nm with a suitable spectrophotometer (see [Spectrophotometry and Light-Scattering \(851\)](#)), using the solution from the Blank to set the instrument to zero.

**Calculations**— [note—The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] Using the linear regression method, plot the absorbances of the solutions from the Standard Solutions versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the absorbance of the Test Solution, determine the concentration of protein in the Test Solution.

#### interfering substances

In the following procedure, deoxycholate–trichloroacetic acid is added to a test specimen to remove interfering substances by precipitation of proteins before testing. This technique also can be used to concentrate proteins from a dilute solution.

**Sodium Deoxycholate Reagent**— Prepare a solution of sodium deoxycholate in water having a concentration of 150 mg in 100 mL.

**Trichloroacetic Acid Reagent**— Prepare a solution of trichloroacetic acid in water having a concentration of 72 g in 100 mL.

**Procedure**— Add 0.1 mL of Sodium Deoxycholate Reagent to 1 mL of a solution of the protein under test. Mix on a vortex mixer, and allow to stand at room temperature for 10 minutes. Add 0.1 mL of Trichloroacetic Acid Reagent, and mix on a vortex mixer. Centrifuge at 3000 × g for 30 minutes, decant the liquid, and remove any residual liquid with a pipet. Redissolve the protein pellet in 1 mL of Alkaline Copper Reagent. Proceed as directed for the Test Solution.

**note**—Color development reaches a maximum in 20 to 30 minutes during incubation at room temperature, after which there is a gradual loss of color. Most interfering substances cause a lower color yield; however, some detergents cause a slight increase in color. A high salt concentration may cause a precipitate to form. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.

#### Method 3

This method, commonly referred to as the Bradford assay, is based on the absorption shift from 470 nm to 595 nm observed when the brilliant blue G dye binds to protein. The brilliant blue G dye binds most readily to arginyl and lysyl residues in the protein, which can lead to variation in the response of the assay to different proteins.

**Standard Solutions**— Unless otherwise specified in the individual monograph, dissolve the USP Reference Standard or reference material for the protein under test in the buffer used to prepare the Test Solution. Dilute portions of this solution with the same buffer to obtain not fewer than five Standard Solutions having concentrations between 100 µg and 1 mg of protein per mL, the concentrations being evenly spaced.

**Test Solution**— Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the Standard Solutions.

**Blank**— Use the buffer used to prepare the Test Solution and the Standard Solutions.

**Coomassie Reagent**— Dissolve 100 mg of brilliant blue G<sup>+</sup> in 50 mL of alcohol. [note—Not all dyes have the same brilliant blue G content, and different products may give different results.] Add 100 mL of phosphoric acid, dilute with water to 1 L, and mix. Pass the solution through filter paper (Whatman No. 1 or equivalent), and store the filtered reagent in an amber bottle at room temperature. [note—Slow precipitation of the dye will occur during storage of the reagent. Filter the reagent before use.]

**Procedure**— Add 5 mL of the Coomassie Reagent to 100 µL of each Standard Solution, the Test Solution, and the Blank, and mix by inversion. Avoid foaming, which will lead to poor reproducibility. Determine the absorbances of the solutions from the Standard Solutions and the Test Solution at 595 nm with a suitable spectrophotometer (see [Spectrophotometry and Light-Scattering \(851\)](#)), using the Blank to set the instrument to zero. [note—Do not use quartz (silica) spectrophotometer cells: the dye binds to this material. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.]

There are relatively few interfering substances, but detergents and ampholytes in the test specimen should be avoided. Highly alkaline specimens may interfere with the acidic reagent.

**Calculations**— [note—The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] Using the linear regression method, plot the absorbances of the solutions from the Standard Solutions versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the absorbance of the Test Solution, determine the concentration of protein in the Test Solution.

#### Method 4

This method, commonly referred to as the bicinchoninic acid or BCA assay, is based on reduction of the cupric (Cu<sup>2+</sup>) ion to cuprous (Cu<sup>1+</sup>) ion by protein. The bicinchoninic acid reagent is used to detect the cuprous ion. The method has few interfering substances. When interfering substances are present, their effect may be minimized by dilution, provided that the concentration of the protein under test remains sufficient for accurate measurement.



Standard Solutions— Unless otherwise specified in the individual monograph, dissolve the USP Reference Standard or reference material for the protein under test in the buffer used to prepare the Test Solution. Dilute portions of this solution with the same buffer to obtain not fewer than five Standard Solutions having concentrations between 10 and 1200 µg of protein per mL, the concentrations being evenly spaced.

Test Solution— Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the Standard Solutions.

Blank— Use the buffer used to prepare the Test Solution and the Standard Solutions.

#### Reagents—

BCA Reagent— Dissolve about 10 g of bicinchoninic acid, 20 g of sodium carbonate monohydrate, 1.6 g of sodium tartrate, 4 g of sodium hydroxide, and 9.5 g of sodium bicarbonate in water. Adjust, if necessary, with sodium hydroxide or sodium bicarbonate to a pH of 11.25. Dilute with water to 1 L, and mix.

Copper Sulfate Reagent— Dissolve about 2 g of cupric sulfate in water to a final volume of 50 mL.

Copper-BCA Reagent— Mix 1 mL of Copper Sulfate Reagent and 50 mL of BCA Reagent.

Procedure— Mix 0.1 mL of each Standard Solution, the Test Solution, and the Blank with 2 mL of the Copper-BCA Reagent. Incubate the solutions at 37° for 30 minutes, note the time, and allow to come to room temperature. Within 60 minutes following the incubation time, determine the absorbances of the solutions from the Standard Solutions and the Test Solution in quartz cells at 562 nm with a suitable spectrophotometer (see [Spectrophotometry and Light-Scattering \(851\)](#)), using the Blank to set the instrument to zero. The color intensity continues to increase gradually after the solutions are cooled to room temperature. If substances that will cause interference in the test are present, proceed as directed for Interfering Substances under Method 2. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.

Calculations— [note—The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] Using the linear regression method, plot the absorbances of the solutions from the Standard Solutions versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the absorbance of the Test Solution, determine the concentration of protein in the Test Solution.

#### Method 5

This method, commonly referred to as the Biuret assay, is based on the interaction of cupric (Cu<sup>2+</sup>) ion with protein in an alkaline solution and the resultant development of absorbance at 545 nm.

Standard Solutions— Unless otherwise specified in the individual monograph, prepare a solution of Albumin Human for which the protein content has been previously determined by nitrogen analysis (using the nitrogen-to-protein conversion factor of 6.25) or of the USP Reference Standard or reference material for the protein under test in sodium chloride solution (9 in 1000). Dilute portions of this solution with sodium chloride solution (9 in 1000) to obtain not fewer than three Standard Solutions having concentrations between 0.5 and 10 mg per mL, the concentrations being evenly spaced. [note—Low responses may be observed if the sample under test has a significantly different level of proline than that of Albumin Human. A different standard protein may be employed in such cases.]

Test Solution— Prepare a solution of the test protein in sodium chloride solution (9 in 1000) having a concentration within the range of the concentrations of the Standard Solutions.

Blank— Use sodium chloride solution (9 in 1000).

Biuret Reagent— Dissolve about 3.46 g of cupric sulfate in 10 mL of hot water, and allow to cool (Solution 1). Dissolve about 34.6 g of sodium citrate dihydrate and 20.0 g of sodium carbonate in 80 mL of hot water, and allow to cool (Solution 2). Mix Solution 1 and Solution 2, and dilute with water to 200 mL. This Biuret Reagent is stable at room temperature for 6 months. Do not use the reagent if it develops turbidity or contains any precipitate.

Procedure— To one volume of a solution of the Test Solution add an equal volume of sodium hydroxide solution (6 in 100), and mix. Immediately add a volume of Biuret Reagent equivalent to 0.4 volume of the Test Solution, and mix. Allow to stand at a temperature between 15° and 25° for not less than 15 minutes. Within 90 minutes after the addition of the Biuret Reagent, determine the absorbances of the Standard Solutions and the solution from the Test Solution at the wavelength of maximum absorbance at 545 nm with a suitable spectrophotometer (see [Spectrophotometry and Light-Scattering \(851\)](#)), using the Blank to set the instrument to zero. [note—Any solution that develops turbidity or a precipitate is not acceptable for calculation of protein concentration.]

Calculations— Using the least-squares linear regression method, plot the absorbances of the Standard Solutions versus the protein concentrations, determine the standard curve best fitting the plotted points, and calculate the correlation coefficient for the line. [note—Within the given range of the standards, the relationship of absorbance to protein concentration is approximately linear.] A suitable system is one that yields a line having a correlation coefficient of not less than 0.99. From the standard curve and the absorbance of the Test Solution, determine the concentration of protein in the test specimen, making any necessary correction.

Interfering Substances— To minimize the effect of interfering substances, the protein can be precipitated from the initial test specimen as follows. Add 0.1 volume of 50% trichloroacetic acid to 1 volume of a solution of the test specimen, withdraw the supernatant layer, and dissolve the precipitate in a small volume of 0.5 N sodium hydroxide. Use the solution so obtained to prepare the Test Solution.

Comments— This test shows minimal difference between equivalent IgG and albumin samples. Addition of the sodium hydroxide and the Biuret Reagent as a combined reagent, insufficient mixing after the addition of the sodium hydroxide, or an extended time between the addition of the sodium hydroxide solution and the addition of the Biuret Reagent will give IgG samples a higher response than albumin samples. The trichloroacetic acid method used to minimize the effects of interfering substances can also be used to determine the protein content in test specimens at concentrations below 500 µg per mL.

#### Method 6

This fluorometric method is based on the derivatization of the protein with o-phthalaldehyde (OPA), which reacts with the primary amines of the protein (i.e., NH<sub>2</sub>-terminal amino acid and the ε-amino group of the lysine residues). The sensitivity of the test can be increased by hydrolyzing the protein before testing. Hydrolysis makes the ε-amino group of the constituent amino acids of the protein available for reaction with the o-phthalaldehyde reagent. The method requires very small quantities of the protein.

Primary amines, such as tris(hydroxymethyl)aminomethane and amino acid buffers, react with o-phthalaldehyde and must be avoided or removed. Ammonia at high concentrations will react with o-phthalaldehyde as well. The fluorescence obtained when amine reacts with o-phthalaldehyde can be unstable. The use of automated procedures to standardize this procedure may improve the accuracy and precision of the test.

Standard Solutions— Unless otherwise specified in the individual monograph, dissolve the USP Reference Standard or reference material for the protein under test in the buffer used to prepare the Test Solution. Dilute portions of this solution with the same buffer to obtain not fewer than five Standard Solutions having concentrations between 10 and 200 µg of protein per mL, the concentrations being evenly spaced.

Test Solution— Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the Standard Solutions.

Blank— Use the buffer used to prepare the Test Solution and the Standard Solutions.

#### Reagents—

Borate Buffer— Dissolve about 61.83 g of boric acid in water, and adjust with potassium hydroxide to a pH of 10.4. Dilute with water to 1 L, and mix.

Stock OPA Reagent— Dissolve about 120 mg of o-phthalaldehyde in 1.5 mL of methanol, add 100 mL of Borate Buffer, and mix. Add 0.6 mL of polyoxyethylene (23) lauryl ether, and mix. This solution is stable at room temperature for at least 3 weeks.

OPA Reagent— To 5 mL of Stock OPA Reagent add 15 µL of 2-mercaptoethanol. Prepare at least 30 minutes prior to use. This reagent is stable for one day.

Procedure— Adjust each of the Standard Solutions and the Test Solution to a pH between 8 and 10.5. Mix 10 µL of the Test Solution and each of the Standard Solutions with 100 µL of



A Reagent, and allow to stand at room temperature for 15 minutes. Add 3 mL of 0.5 N sodium hydroxide, and mix. Using a suitable fluorometer (see [Spectrophotometry and Light Scattering](#) 851), determine the fluorescent intensities of solutions from the Standard Solutions and the Test Solution at an excitation wavelength of 340 nm and an emission wavelength between 440 and 455 nm. [note—The fluorescence of an individual specimen is read only once because irradiation decreases the fluorescent intensity.]

**Calculations**— The relationship of fluorescence to protein concentration is linear. Using the linear regression method, plot the fluorescent intensities of the solutions from the Standard Solutions versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the fluorescent intensity of the Test Solution, determine the concentration of protein in the test specimen.

#### Method 7

This method is based on nitrogen analysis as a means of protein determination. Interference caused by the presence of other nitrogen-containing substances in the test specimen can affect the determination of protein by this method. Nitrogen analysis techniques destroy the protein under test but are not limited to protein presentation in an aqueous environment.

**Procedure 1**— Determine the nitrogen content of the protein under test as directed under [Nitrogen Determination](#) 461. Commercial instrumentation is available for the Kjeldahl nitrogen assay.

**Procedure 2**— Commercial instrumentation is available for nitrogen analysis. Most nitrogen analysis instruments use pyrolysis (i.e., combustion of the sample in oxygen at temperatures approaching 1000°), which produces nitric oxide (NO) and similar oxides of nitrogen (NOx) from the nitrogen present in the test protein. Some instruments convert the nitric oxides to nitrogen gas, which is quantified with a thermal conductivity detector. Other instruments mix nitric oxide (NO) with ozone (O3) to produce excited nitrogen dioxide (NO2), which emits light when it decays and can be quantified with a chemiluminescence detector. A protein reference material or reference standard that is relatively pure and is similar in composition to the test proteins is used to optimize the injection and pyrolysis parameters and to evaluate consistency in the analysis.

**Calculations**— The protein concentration is calculated by dividing the nitrogen content of the sample by the known nitrogen content of the protein. The known nitrogen content of the protein can be determined from the chemical composition of the protein or by comparison with the nitrogen content of the USP Reference Standard or reference material.

\* Dye purity is important in the reagent preparation. Serva Blue G (Crescent Chemical Company, Hauppauge, NY) is an acceptable grade.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

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## 1058 ANALYTICAL INSTRUMENT QUALIFICATION

### INTRODUCTION

A large variety of laboratory equipment, instruments, and computerized analytical systems, ranging from simple nitrogen evaporators to complex multiple-function technologies (see [Instrument Categories](#)), are used in the pharmaceutical industry to acquire data to help ensure that products are suitable for their intended use. An analyst's objective is to consistently obtain reliable and valid data suitable for the intended purpose. Depending on the applications, users validate their procedures, calibrate their instruments, and perform additional instrument checks, such as system suitability tests and analysis of in-process quality control check samples to help ensure that the acquired data are reliable. With the increasing sophistication and automation of analytical instruments, an increasing demand has been placed on users to qualify their instruments.

Unlike method validation and system suitability activities, analytical instrument qualification (AIQ) currently has no specific guidance or procedures. Competing opinions exist regarding instrument qualification and validation procedures and the roles and responsibilities of those who perform them. Consequently, various approaches have been used for instrument qualification, approaches that require varying amounts of resources and generate widely differing amounts of documentation. This chapter provides a scientific approach to AIQ and considers AIQ as one of the major components required for generating reliable and consistent data. Note that the amount of rigor applied to the qualification process will depend on the complexity and intended use of the instrumentation. This approach emphasizes AIQ's place in the overall process of obtaining reliable data from analytical instruments.

#### Validation versus Qualification

In this chapter, the term validation is used for manufacturing processes, analytical procedures, and software procedures and the term qualification is used for instruments. Thus, the phrase "analytical instrument qualification" (AIQ) is used for the process of ensuring that an instrument is suitable for its intended application.

### COMPONENTS OF DATA QUALITY

There are four critical components involved in the generation of reliable and consistent data (quality data). [Figure 1](#) shows these components as layered activities within a quality triangle. Each layer adds to the overall quality. Analytical instrument qualification forms the base for generating quality data. The other components essential for generating quality data are analytical method validation, system suitability tests, and quality control check samples. These quality components are described below.



Figure 1. Components of data quality.

#### Analytical Instrument Qualification

AIQ is the collection of documented evidence that an instrument performs suitably for its intended purpose. Use of a qualified instrument in analyses contributes to confidence in the validity of generated data.

#### Analytical Method Validation

Analytical method validation is the collection of documented evidence that an analytical procedure is suitable for its intended use. Use of a validated procedure with qualified analytical instruments provides confidence that the procedure will generate test data of acceptable quality. Additional guidance on validation of compendial procedures may be found in the general information chapter [Validation of Compendial Procedures](#) (1225).

#### System Suitability Tests

System suitability tests verify that the system will perform in accordance with the criteria set forth in the procedure. These tests are performed along with the sample analyses to ensure that the system's performance is acceptable at the time of the test. USP general chapter [Chromatography](#) (621) presents a more detailed discussion of system suitability tests as related to chromatographic systems.

#### Quality Control Check Samples

Many analysts carry out their tests on instruments standardized using reference materials and/or calibration standards. Some analyses also require the inclusion of quality control check samples to provide an in-process or ongoing assurance of the test's suitable performance. In this manner, AIQ and analytical method validation contribute to the quality of analysis before analysts conduct the tests. System suitability tests and quality control checks help ensure the quality of analytical results immediately before or during sample analysis.

### ANALYTICAL INSTRUMENT QUALIFICATION PROCESS

The following sections address in detail the AIQ process. The other three components of building quality into analytical data—analytical method validation, system suitability tests, and quality control check samples—are not within the scope of this chapter.

#### Qualification Phases

Instrument qualification is not a single continuous process, but instead results from several discrete activities. For convenience, these activities can be grouped into four phases: design qualification (DQ), installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ).

Some AIQ activities cover more than one qualification phase, and analysts potentially could perform them during more than one of the phases (see [Table 1](#)). However, in many instances there is need for specific order to the AIQ activities; for example, installation qualification must occur first in order to initiate other qualification activities. The AIQ activities will be defined and documented.

Table 1. Timing, Applicability, and Activities for Each Phase of Analytical Instrument Qualification\*

Design Qualification	Installation Qualification	Operational Qualification	Performance Qualification
Timing and Applicability			
Prior to purchase of a new model of instrument	At installation of each instrument (new, old, or existing unqualified)	After installation or major repair of each instrument	Periodically at specified intervals for each instrument
Activities			
Assurance of manufacturer's DQ	Description	↔ Fixed parameters	Preventive maintenance and repairs
Assurance of adequate support availability from manufacturer	Instrument delivery		Establish practices to address operation, calibration, maintenance, and change control
Instrument's fitness for use in laboratory	Utilities/facility	↔ Environment	
	Assembly and installation		
	Network and data storage	↔ Secure data storage, backup, and archive	
	Installation verification	↔ Instrument function tests	↔ Performance checks

\* Activities under each phase are usually performed as given in the table. However, in some cases, it may be more appropriate to perform or combine a given activity with another phase. Such activities spanning more than one qualification phase are shown as connected by double arrows. If an activity listed under a given phase is performed under another phase, it is not necessary to repeat the activity under the phase where the activity is listed. Performing the activity is far more important than the phase under which the activity is performed.

#### Design Qualification

Design qualification (DQ) is the documented collection of activities that define the functional and operational specifications of the instrument and criteria for selection of the vendor, based on the intended purpose of the instrument. Design qualification (DQ) may be performed not only by the instrument developer or manufacturer but also may be performed by the user. The manufacturer is generally responsible for robust design and maintaining information describing how the analytical instrument is manufactured (design specifications, functional requirements, etc.) and tested before shipment to users. Nonetheless, the user should ensure that commercial off-the-shelf (COTS) instruments are suitable for their intended application and that the manufacturer has adopted a quality system that provides for reliable equipment. Users should also determine the manufacturer's capability for support installation, services, and training. This determination might be aided by the user's previous interaction with the manufacturer.

#### Installation Qualification

Installation qualification (IQ) is the documented collection of activities necessary to establish that an instrument is delivered as designed and specified, and is properly installed in the selected environment, and that this environment is suitable for the instrument. IQ applies to an instrument that is new or was pre-owned, or to any instrument that exists on site but has not been previously qualified. Relevant parts of IQ would also apply to a qualified instrument that has been transported to another location or is being reinstalled for other reasons, such as prolonged storage. The activities and documentation typically associated with IQ are as follows.

**Description**— Provide a description of the instrument or the collection of instrument components, including its manufacturer, model, serial number, software version, and location. Use drawings and flow charts where appropriate.

**Instrument Delivery**— Ensure that the instrument, software, manuals, supplies, and any other instrument accessories arrive as specified in the purchase order and that they are undamaged. For a pre-owned or existing instrument, manuals and documentation should be obtained.

**Utilities/Facility/Environment**— Verify that the installation site satisfactorily meets manufacturer-specified environmental requirements.

**Assembly and Installation**— Assemble and install the instrument, and perform any preliminary diagnostics and testing. Assembly and installation may be done by the manufacturer, vendor, specialized engineers, or qualified in-house personnel. Manufacturer-established installation tests and guides provide a valuable baseline reference for determining instrument acceptance. Any abnormal event observed during assembly and installation merits documenting. Installation packages purchased from the manufacturer or the vendor may, however, need to be supplemented with user-specific criteria.

**Network and Data Storage**— Some analytical systems require users to provide network connections and data storage capabilities at the installation site. When required, connect the



instrument to the network, and check its functionality.

Installation Verification— Perform the initial diagnostics and testing of the instrument after installation.

#### Operational Qualification

After a successful IQ, the instrument is ready for OQ testing. Operational qualification (OQ) is the documented collection of activities necessary to demonstrate that an instrument will function according to its operational specification in the selected environment. Testing activities in the OQ phase may consist of these test parameters.

Fixed Parameters— These tests measure the instrument's nonchanging parameters such as length, height, weight, voltage inputs, acceptable pressures, and loads. If the manufacturer-supplied specifications for these parameters satisfy the user, the test requirements may be waived. However, if the user wants to confirm the parameters, testing can be performed at the user's site. Fixed parameters do not change over the life of the instrument, and therefore never need redetermination. [note—These tests could also be performed during the IQ phase (see [Table 1](#)); if so, fixed parameters need not be redetermined as part of OQ testing.]

Secure Data Storage, Backup, and Archiving— When applicable, test secure data handling such as storage, backup, audit trails, and archiving at the user's site according to written procedures.

Instrument Function Tests— Instrument functions required by the user should be tested to verify that the instrument operates as intended by the manufacturer. Manufacturer-supplied information is useful in identifying specifications for these parameters and in designing tests to evaluate the identified parameters. Users, or their qualified designees, should perform these tests to verify that the instrument meets manufacturer or user specifications in the user's environment.

The extent of OQ testing that an instrument undergoes depends on its intended applications. Therefore, no specific OQ tests for any instrument or application are offered in this chapter.

Routine analytical tests do not constitute OQ testing. OQ tests are specifically designed to verify the instrument's operation according to specifications in the user's environment, and repeating the testing at regular intervals may not be required. However, when the instrument undergoes major repairs or modifications, relevant OQ and/or PQ tests should be repeated to verify whether the instrument continues to operate satisfactorily. If an instrument is moved to another location, an assessment should be made of what, if any, OQ test should be repeated.

OQ tests can be modular or holistic. Modular testing of individual components of a system may facilitate interchanging of such components without requalification. Holistic tests, which involve the entire system, are also acceptable.

#### Performance Qualification

Performance qualification (PQ) is the documented collection of activities necessary to demonstrate that an instrument consistently performs according to the specifications defined by the user, and is appropriate for the intended use. After IQ and OQ have been performed, the instrument's continued suitability for its intended use is demonstrated through performance qualification. The PQ phase may include the following parameters.

Performance Checks— Set up a test or series of tests to verify the acceptable performance of the instrument for its intended use. PQ tests are usually based on the instrument's typical on-site applications and may consist of analyzing known components or standards. The tests should be based on good science and reflect the general intended use of the instrument. Some system suitability tests or quality control checks that are performed concurrently with the test samples can be used to demonstrate that the instrument is performing suitably. PQ tests may resemble those performed during OQ, but the specifications for their results may be set differently if required. Nevertheless, user specifications for PQ tests should demonstrate trouble-free instrument operation for the intended applications. As is the case with OQ testing, PQ tests may be modular or holistic.

Testing frequency depends on the ruggedness of the instrument and the criticality of the tests performed. Testing may be unscheduled—for example, each time the instrument is used. It may also be scheduled for regular intervals. Experience with the instrument can influence this decision. It may be useful to repeat the same PQ tests each time the instrument is used so that a history of the instrument's performance can be compiled. Alternatively, the instrument may be incorporated into an integrated support system to assure that it remains continually qualified. Some system suitability tests or quality control checks that are performed concurrently with the test samples also imply that the instrument is performing suitably.

Preventive Maintenance and Repairs— When an instrument fails to meet PQ test specifications, it requires maintenance or repair. A periodic preventive maintenance may also be recommended for many instruments. The relevant PQ test(s) should be repeated after the needed maintenance or repair to ensure that the instrument remains qualified.

Practices for Operation, Calibration, Maintenance, and Change Control— Establish practices to maintain and calibrate the instrument. Each maintenance and calibration activity should be documented.

#### ROLES AND RESPONSIBILITIES

##### Users

Users are ultimately responsible for instrument operations and data quality. The user's group encompasses analysts, their supervisors, instrument specialists, and organization management. Users should be adequately trained in the instrument's use, and their training records should be maintained as required by the regulations.

Users should also be responsible for qualifying their instruments because their training and expertise in the use of instruments make them the best-qualified group to design the instrument test(s) and specification(s) necessary for successful AIQ. Consultants, equipment manufacturer or vendors, validation specialists, and quality assurance (QA) personnel can advise and assist as needed, but the final responsibility for qualifying instruments lies with the users. The users must also maintain the instrument in a qualified state by routinely performing PQ.

##### Quality Unit

The role of the Quality Unit in AIQ remains the same as for any other regulated activity. Quality personnel are responsible for assuring that the AIQ process meets compliance requirements, that processes are being followed, and that the intended use of the equipment is supported by valid and documented data.

##### Manufacturers

Manufacturers and developers are responsible for DQ when designing the instrument. They are also responsible for validation of relevant processes used in manufacturing and assembly of the instrument. Manufacturers should test the assembled instruments before shipping them to users.

Finally, it is desirable that manufacturers and vendors should notify all known users about hardware defects discovered after a product's release; offer user training, service, repair, and installation support; and invite user audits as necessary.

#### SOFTWARE VALIDATION

Software used for analytical work can be classified into three categories: firmware; instrument control, data acquisition, and processing software; and stand-alone software. Although software validation is not the primary focus of this chapter, the following sections describe in which cases this activity is under the scope of the analytical instrument qualification.

##### Firmware

Computerized analytical instruments contain integrated chips with low-level software (firmware). Such instruments will not function without properly operating firmware, and users generally cannot alter firmware design or function. Firmware is therefore considered a component of the instrument itself. Indeed, the qualification of hardware is not possible without operating it via its firmware. Thus, when the hardware (that is, the analytical instrument) is qualified at the user's site, the integrated firmware is also essentially qualified. No separate on-site qualification of the firmware is needed. Whenever possible, the firmware version should be recorded as part of the IQ activities. Any changes made to firmware versions should be tracked through change control of the instrument (see Change Control, below).

##### Instrument Control, Data Acquisition, and Processing Software



Software for instrument control, data acquisition, and processing for many of today's computerized instruments is loaded on a computer connected to the instrument. Operation of the instrument is then controlled via the software, leaving fewer operating controls on the instrument. Also, the software is needed for data acquisition and postacquisition calculations.

Thus, both hardware and software, their functions inextricably intertwined, are critical to providing analytical results.

The manufacturer should perform DQ, validate this software, and provide users with a summary of validation. At the user site, holistic qualification, which involves the entire instrument and software system, is more efficient than modular validation of the software alone. Thus, the user qualifies the instrument control, data acquisition, and processing software by qualifying the instrument according to the AIQ process.

#### Stand-Alone Software

An authoritative guide for validating stand-alone software, such as LIMS, is available.<sup>1</sup> The validation process is administered by the software developer, who also specifies the development model appropriate for the software. Validation takes place in a series of activities planned and executed through various stages of the development cycle.

#### CHANGE CONTROL

Changes to instruments, including software, become inevitable as manufacturers add new features and correct known defects. However, implementing all such changes may not always benefit users. Users should therefore adopt changes they deem useful or necessary and should also assess the effects of changes to determine what, if any, requalification is required. The change control process enables them to do this.

Change control may follow the DQ/IQ/OQ/PQ classification process. For DQ, evaluate the changed parameters, and determine whether need for the change warrants implementing it. If implementation of the change is needed, install the changes to the system during IQ. Evaluate which of the existing OQ and PQ tests need revision, deletion, or addition as a result of the installed change. Where the change calls for additions, deletions, or revisions to the OQ or PQ tests, follow the procedure outlined below.

**Operational Qualification—** Revise OQ tests as necessitated by the change. Perform the relevant tests affected by the change. This ensures the instrument's effective operation after the change is installed.

**Performance Qualification—** Revise PQ tests as necessitated by the change. Perform the PQ testing after installation of the change if similar testing is not already performed during OQ. In the future, perform the revised PQ testing.

For changes to firmware and to software for instrument control, data acquisition, and processing, change control is performed through DQ/IQ/OQ/PQ of the affected instrument. Change control for stand-alone software requires user-site testing of changed functionality.

#### AIQ DOCUMENTATION

Documents obtained during instrument qualification should be retained in an accessible manner. Where multiple instruments of one kind exist, documents common to all instruments and documents specific to an instrument may be stored separately. During change control, additional documents may supplement those obtained during the qualification process, and both sets of documents should be retained and maintained in a suitable manner that allows for appropriate protection and access.

#### INSTRUMENT CATEGORIES

Modern laboratories typically include a suite of instruments and equipment varying from simple nitrogen evaporators to complex automated instruments. Therefore, applying a single set of principles to qualifying such dissimilar instruments would be scientifically inappropriate. Users are most capable of establishing the level of qualification needed for an instrument. On the basis of the level needed, it is convenient to categorize instruments into three groups: A, B, and C, as defined below. Examples of instruments in each group are provided. Note that the list of instruments provided here is for illustration only and is not meant to be exhaustive. It does not provide the exact category for an instrument at a user site. That category should be determined by users for their specific instruments or applications.

The exact grouping of an instrument must be determined by users for their specific requirements. Depending on individual user requirements, the same instrument may appropriately fall into one group for one user and another group for another user. Therefore, a careful selection of groups by users is highly encouraged.

##### Group A

Group A includes standard equipment with no measurement capability or usual requirement for calibration, where the manufacturer's specification of basic functionality is accepted as user requirements. Conformance of Group A equipment with user requirements may be verified and documented through visual observation of its operation. Examples of equipment in this group are nitrogen evaporators, magnetic stirrers, vortex mixers, and centrifuges.

##### Group B

Group B includes standard equipment and instruments providing measured values as well as equipment controlling physical parameters (such as temperature, pressure, or flow) that need calibration, where the user requirements are typically the same as the manufacturer's specification of functionality and operational limits. Conformance of Group B instruments or equipment to user requirements is determined according to the standard operating procedures for the instrument or equipment, and documented during IQ and OQ. Examples of instruments in this group are balances, melting point apparatus, light microscopes, pH meters, variable pipets, refractometers, thermometers, titrators, and viscosimeters. Examples of equipment in this group are muffle furnaces, ovens, refrigerator-freezers, water baths, pumps, and dilutors.

##### Group C

Group C includes instruments and computerized analytical systems, where user requirements for functionality, operational, and performance limits are specific for the analytical application. Conformance of Group C instruments to user requirements is determined by specific function tests and performance tests. Installing these instruments can be a complicated undertaking and may require the assistance of specialists. A full qualification process, as outlined in this document, should apply to these instruments. Examples of instruments in this group include the following:

- atomic absorption spectrometers
- differential scanning calorimeters
  - dissolution apparatus
  - electron microscopes
  - flame absorption spectrometers
- high-pressure liquid chromatographs
  - mass spectrometers
  - microplate readers
  - thermal gravimetric analyzers
- X-ray fluorescence spectrometers
- X-ray powder diffractometers
  - densitometers
  - diode-array detectors
  - elemental analyzers
  - gas chromatographs
    - IR spectrometers
    - near-IR spectrometers
    - Raman spectrometers

- UV/Vis spectrometers
- inductively coupled plasma-emission spectrometers

1 General Principles of Software Validation: Final Guidance for Industry and FDA Staff, U.S. Department of Health and Human Services, Food and Drug Administration, Rockville, MD, January 11, 2002. <http://www.fda.gov/cdrh/comp/guidance/938.html> (accessed September 2004).

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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#### 1061 COLOR—INSTRUMENTAL MEASUREMENT

The observed color (see [631 Color and Achromicity](#)) of an object depends on the spectral energy of the illumination, the absorbing characteristics of the object, and the visual sensitivity of the observer over the visible range. Similarly, it is essential that any instrumental method that is widely applicable take these same factors into account.

Instrumental methods for measurement of color provide more objective data than the subjective viewing of colors by a small number of individuals. With adequate maintenance and calibration, instrumental methods can provide accurate and precise measurements of color and color differences that do not drift with time. The basis of any instrumental measurement of color is that the human eye has been shown to detect color via three "receptors." Hence, all colors can be broken down into a mixture of three radiant stimuli that are suitably chosen to excite all three receptors in the eye. Although no single set of real light sources can be used to match all colors (i.e., for any three lights chosen, some colors require a negative amount of one or more of the lights), three arbitrary stimuli have been defined, with which it is possible to define all real colors. Through extensive color-matching experiments with human subjects having normal color vision, distributing coefficients have been measured for each visible wavelength (400 nm to 700 nm) giving the relative amount of stimulation of each receptor caused by light of that wavelength. These distribution coefficients  $x$ ,  $y$ ,  $z$ , are shown below. Similarly, for any color the amount of stimulation of each receptor in the eye is defined by the set of Tristimulus values ( $X$ ,  $Y$ , and  $Z$ ) for that color.

The relationships between the distribution coefficient (see [accompanying figure](#)) and the tristimulus values are given in the equations

$$X = \int_0^{\infty} f_{\lambda} \bar{x}_{\lambda} P_{\lambda} d\lambda / Y',$$

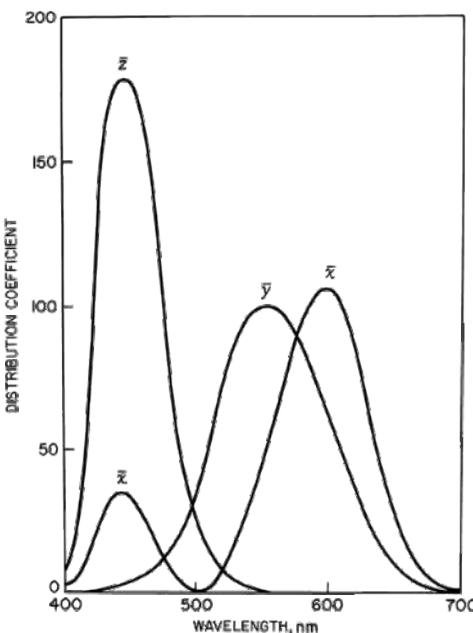
$$Y = \int_0^{\infty} f_{\lambda} \bar{y}_{\lambda} P_{\lambda} d\lambda / Y', \text{ and}$$

$$Z = \int_0^{\infty} f_{\lambda} \bar{z}_{\lambda} P_{\lambda} d\lambda / Y',$$

in which

$$Y' = \int_0^{\infty} f_{\lambda} P_{\lambda} d\lambda$$

is the spectral power of the illuminant, and  $f_{\lambda}$  is either the spectral reflectance ( $P_{\lambda}$ ) or spectral transmittance ( $T_{\lambda}$ ) of the material.



Distribution Coefficients from 400 to 700 nm

Once the tristimulus values of a color have been determined, they may be used to calculate the coordinates of the color in an idealized three-dimensional color space referred to as a visually uniform color space. Many sets of color equations have been developed in an attempt to define such a space. The equations given in this chapter represent a compromise between simplicity of calculation and conformance with ideality.

The coordinates of a color in a visually uniform color space may be used to calculate the deviation of a color from a chosen reference point. Where the instrumental method is used to determine the result of a test requiring color comparison of a test preparation with that of a standard or matching fluid, the parameter to be compared is the difference, in visually uniform color space, between the color of the blank and the color of the test specimen or standard.



#### Procedure

The considerations discussed under [Spectrophotometry and Light-Scattering](#) (851) apply to instrumental color measurement as well. In the spectrophotometric method, reflectance or transmittance values are obtained at discrete wavelengths throughout the visible spectrum, a band width of 10 nm or less being used. These values are then used to calculate the tristimulus values through the use of weighting factors.<sup>1</sup> In the colorimetric method, the weighting is performed through the use of filters.

In the measurement of the spectral reflectance of opaque solids, the angle of viewing is separated from the angle of illumination in such a manner that only rays reflected diffusely from the test specimen enter the receptor. Specular reflection and stray light are excluded.

For the measurement of the spectral transmittance of clear liquids, the specimen is irradiated from within 5 degrees of the normal to its surface, and the transmitted energy measured is that confined within 5 degrees from the normal. The color of solutions changes with the thickness of the layer measured. Unless special considerations dictate otherwise, a layer 1 cm thick should be used.

The methods described here are not applicable to hazy liquids or translucent solids.

#### calibration

For purposes of calibration, one of the following reference materials may be used, as required by instrument geometry. For transmittance measurements, purified water may be used as a white standard and assigned a transmittance of 1.000 at all wavelengths. Then the tristimulus values X, Y, and Z for CIE source C are 98.0, 100.0, and 118.1, respectively. For reflectance measurements, opaque porcelain plaques, whose calibration base is the perfect diffuse reflector and whose reflectance characteristics have been determined for the appropriate instrumental geometry, may be used.<sup>2</sup> If the geometry of sample presentation precludes the use of such plaques, pressed barium sulfate, white reflectance standard grade, may be used.<sup>3</sup>

After calibration with the above-mentioned materials, it is desirable whenever possible to measure a reference material as close to the color of the sample as possible. If a sample of the material being tested is not suitable for use as a long-term standard, color chips are available<sup>4</sup> which span the entire visually uniform color space in small increments. The use of such a reference standard is encouraged as a means of monitoring instrument performance even for absolute color determinations.

#### spectrophotometric method

Determine the reflectance or transmittance from 380 to 770 nm at intervals of 10 nm. Express the result as a percentage, the maximum being 100.0. Calculate the tristimulus values X, Y, and Z as follows.

Reflecting Materials— For reflecting materials the quantities X, Y, and Z are

$$\begin{aligned} X &= \sum_{380}^{770} \rho_\lambda \bar{x}_\lambda P_\lambda \Delta\lambda / Y', \\ Y &= \sum_{380}^{770} \rho_\lambda \bar{y}_\lambda P_\lambda \Delta\lambda / Y', \text{ and} \\ Z &= \sum_{380}^{770} \rho_\lambda \bar{z}_\lambda P_\lambda \Delta\lambda / Y' \end{aligned}$$

in which

$$Y' = \sum_{380}^{770} \bar{y}_\lambda P_\lambda \Delta\lambda, \rho_\lambda$$

is the spectral reflectance of the material,  $\bar{x}_\lambda P_\lambda$ ,  $\bar{y}_\lambda P_\lambda$ , and  $\bar{z}_\lambda P_\lambda$  are known values associated with each Standard Source, 1,2 and  $\Delta\lambda$  is expressed in nm.

Transmitting Materials— For transmitting materials, the quantities X, Y, and Z are calculated as above,  $\tau_\lambda$  (spectral transmittance) being substituted for  $\rho_\lambda$ .

#### colorimetric method

Operate a suitable colorimeter<sup>5</sup> to obtain values equivalent to the tristimulus values, X, Y, and Z. The accuracy with which the results obtained from the filter colorimeter match the tristimulus values may be indicated by determining the tristimulus values of plaques of strongly saturated colors and comparing these values with those computed from spectral measurements on a spectrophotometer.

#### Interpretation

##### color coordinates

The Color Coordinates, L\*, a\*, and b\* are defined by

$$\begin{aligned} L^* &= 116 (Y/Y_o)^{1/3} - 16, \\ a^* &= 500 [(X/X_o)^{1/3} - (Y/Y_o)^{1/3}], \text{ and} \\ b^* &= 200 [(Y/Y_o)^{1/3} - (Z/Z_o)^{1/3}] \end{aligned}$$

in which X<sub>o</sub>, Y<sub>o</sub>, and Z<sub>o</sub> are the tristimulus values of the nominally white or colorless standard, and Y/Y<sub>o</sub> > 0.01. Usually they are equal to the tristimulus values of the standard illuminant, with Y<sub>o</sub> set equal to 100.0. In this case X<sub>o</sub> = 98.0 and Z<sub>o</sub> = 118.1.

#### color difference

The total Color Difference ΔE\* is

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

in which ΔL\*, Δa\*, and Δb\* are the differences in color coordinates of the specimens being compared.

Instrumental variables can influence results. Although reliable comparisons can be made between similar colors measured concomitantly, results obtained on different instruments or under different operating conditions should be compared with caution. If it is necessary to compare data obtained from different instruments or taken at different times, etc., it is very helpful to have concomitant data obtained on a standard reference material such as color chips for opaque materials. Comparison of the readings on the reference material helps to identify variations caused by instrument performance.

1 Typical weighting factors are given by ASTM Z58.7.1-1951 as reported in the Journal of the Optical Society of America, Vol. 41, 1951, pages 431-439.

2 Suitable items are available from BYK-Gardner USA, 2431 Linden Lane, Silver Spring, MD 20910, or from Hunter Associates Laboratory, Inc., 11491 Sunset Hills Road, Reston, VA 22090.

3 Suitable material is available from Eastman Kodak Company, Rochester, NY 14650, as "White Reflectance Standard."

4 Centroid Color Charts may be obtained from suppliers of instruments for measurement of color.

5 A suitable tristimulus colorimeter is available from BYK-Gardner USA, 2431 Linden Lane, Silver Spring, MD 20910, or from Hunter Associates Laboratory, Inc., 11491 Sunset Hills Road, Reston, VA 22090.

Auxiliary Information— Please [check your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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1065 ION CHROMATOGRAPHY

## INTRODUCTION

Ion chromatography (IC) is a high-performance liquid chromatography (HPLC) instrumental technique used in USP test procedures such as identification tests and assays to measure inorganic anions and cations, organic acids, carbohydrates, sugar alcohols, aminoglycosides, amino acids, proteins, glycoproteins, and potentially other analytes.

As dictated by the nature of the analyte, IC has been applied to all aspects of the manufacturing and disposition of pharmaceutical products, including characterization of active ingredients, excipients, degradation products, impurities, and process streams. The following sample types are among those that have been analyzed: raw materials, intermediates (including media and culture broths), bulk active ingredients, diluents, formulated products, production equipment cleaning solutions, and waste streams. The technique is especially valuable for ionic or ionizable (in the mobile phase) analytes that have little or no native UV absorbance. The ability to couple the ion-exchange separation with numerous detection strategies, e.g., pulsed amperometric detection (PAD), expands IC applications to instances where analyte-specific detection strategies can provide the required degree of sensitivity or specificity. Utilization of such strategies allows IC applications to be implemented on appropriately configured HPLC systems. Additionally, ion-exclusion separations and pulsed amperometric detection expand the range of application of IC to aliphatic organic acids as well as to nonionic analytes of significant pharmaceutical interest including alcohols, alditols, carbohydrates, and amino acids. The wide dynamic range of the methodology makes it applicable for the quantification of trace contaminants as well as major product components.

Because IC typically uses dilute acids, alkalis, or salt solutions as the mobile phase, and does not use an organic solvent, IC does not require the purchase of costly organic solvents and hazardous disposal of the waste effluent. The effluent can be disposed of after appropriate neutralization (to ~pH 7) and, when necessary, after dilution with water.

IC allows separation using ion exchange, ion exclusion, or ion-pair approaches. IC separations are based on differences in charge density of the analyte species, which in turn depend on the valence and size of the individual ionic species to be measured. Separations are also performed on the basis of differences in the hydrophobic character of the ionic species. IC is typically performed at ambient temperature. As with other forms of HPLC, IC separations are based on varying capacity factors and typically follow the Knox equation. Ion chromatography is a technique complimentary to the more commonly used reversed-phase and normal-phase HPLC and to atomic absorption and ion-coupled plasma (plasma spectrochemistry) techniques in pharmaceutical analysis.

## APPARATUS

IC instruments closely resemble conventional HPLC instruments. Typical components include an autosampler, a high-pressure pump, an injection valve with a sample loop of suitable size (typically 10 to 250  $\mu$ L), a guard column, an analytical column, an optional suppressor or other forms of a post-column reaction system, a flow-through detector, and a data system ranging in complexity from an integrator to a computerized data system (Figure 1).

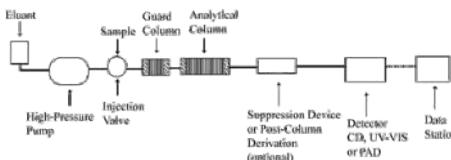


Figure 1. Components of a typical IC system illustrated schematically; CD = conductivity detector and PAD = pulsed amperometric detector.

Because mobile phases generally consist of dilute acids, alkalis, or salt solutions, the components in contact with the mobile phase and the sample are typically made from inert materials, such as polyetheretherketone. Conventional HPLC systems also may be used provided that their components are compatible with the mobile phase and injected sample solutions. A metal-free system should be used for trace metal analysis. Following suitable preparation, the sample is introduced via the injection valve. After the optional chemical suppression or other post-column reaction on the column effluent, the analyte species are detected using conductivity, amperometry, UV/VIS, or other detection modes. Because IC uses a predominantly ionic mobile phase, a suppressor is often necessary prior to conductometric detection, although nonsuppressed conductometric detection has been successfully used in pharmaceutical analysis.

### Stationary and Mobile Phases

As IC has developed and matured as an instrumental technique, the number of ion-exchange materials developed for IC has increased, facilitated by the understanding of the processes taking place at the surface of the stationary phase. In contrast to the silica-based column packing prevalent in classical HPLC, organic polymers are predominately used as support materials for IC. Such materials have a higher stability with respect to extremes in pH and in many cases are compatible with organic solvents. Typically, separation of anions requires the use of polymer-based anion exchangers and dilute bases as mobile phases. However, for cation separations, the stability over the entire pH range that is typical of organic polymers is not necessary, because dilute acids serve as mobile phases. Therefore, silica-based cation exchangers that exhibit a significantly higher chromatographic efficiency are commonly used for the separation of cations.

Depending on the separation mode (ion exchange, ion exclusion, or ion-pair), different types of stationary phases are used. For ion exchange, the stationary phase is either an anion or a cation exchanger. Typically, a strong cation exchanger is used for the ion-exclusion separation of organic acids, and a reversed-phase stationary phase is used when ion-pair is the separation mode. The ion-exchange capacity of a resin is defined as the number of ion-exchange sites per weight equivalent of the column packing and is typically expressed in terms of mEq per g of resin. With ion exchange, the retention times for the analyte ions increase with increasing ion-exchange capacity of the resin. This effect can be partly compensated for by using mobile phases of higher ionic strength. Styrene/divinylbenzene copolymers, polymethacrylate, and polyvinyl resins are the substrate materials used in the manufacturing process of the polymer-based ion exchangers. Organic polymers are functionalized directly at their surface, with the exception of latex-based ion exchangers, where the totally porous latex particle acts as an ion-exchange material. Surface-functionalized, "pellicular" substrates show a much higher chromatographic efficiency compared with the fully functionalized resins.

With ion exchange, a mobile phase consisting of mono- or divalent ionic species, alone or mixed at an optimum ratio, is used to accomplish the separation. In ion-exclusion methods,



particularly for organic acids, the mobile phase consists of mineral acids to maintain organic acids in their undissociated forms. Often, the nature of the analyte dictates the mobile phase and the detection mode used. Typical mobile phases used in IC are described below in the section on detectors.

#### Detectors

Conductivity detection is by far the most commonly employed mode of detection in IC. Although the original IC development work included the use of low-capacity ion-exchange resins for efficient chromatographic separation and conductometric detection of ions in a chemically suppressed mobile phase, the advances in column technologies as well as instrumentation development allow the use of high-capacity ion exchange today.

In suppressed IC, the background conductance of the ionic mobile phase is significantly reduced as it flows through the suppression device. For example, dilute NaOH, about 10 to 50 mM, used as the mobile phase in IC of anions is converted to H<sub>2</sub>O (poor conductivity) when the column effluent containing NaOH flows through a suppressor device present in an acidic form. The analyte ionic species in the column effluent are converted from their sodium or other metal salt forms to highly conducting acid forms (due to higher equivalent conductance of hydrogen ions compared to other cations). Analogous reactions occur in the hydroxide form suppressor in IC of cations, wherein the acidic mobile phase is converted to water, and the analyte cations are converted to highly conducting hydroxide forms (due to higher equivalent conductance of hydroxide ions compared to other anions).

The reduced background conductance and the enhanced signal due to the ionic species result in an enhanced signal-to-noise ratio for the conductometric detection of ions in suppressed IC. This results in reduced background noise and increasing sensitivity and reproducibility of the analysis. The commonly used chemical suppression devices fall into three broad categories. In the first type, the reactions occur across an ion-exchange membrane with the regenerant ions furnished by either a chemical or as products of electrolysis of water. In the second type, the suppression reactions occur in a packed bed of high-exchange capacity resin material, with regeneration either by a chemical or by electrolysis of water. In the third type, although not commonly used, the suppression reactions occur as the eluent stream mixes with the flowing stream of high-capacity resin material.

For pharmaceutical analyses, suppressed conductometric detection may be used for detection of trace ions in high purity waters. The commonly used mobile phases for the separation of anions by suppressed IC include hydroxide ions or a mixture of bicarbonate and carbonate ions. The common mobile phases for separation of cations usually consist of mineral acids or methanesulfonic acid.

Ion-chromatographic analyses also can be performed without chemical suppression, in which case the analytical column effluent flows directly to a conductivity detector. The typical eluents used in nonsuppressed IC are phthalic acid and p-hydroxybenzoic acid for the determination of anions and methanesulfonic acid for the determination of cations. The equivalent conductance values of chloride, sulfate, and other common anions are significantly greater than that of the eluent anion, and therefore, a positive peak is detected as the anions are carried through the detector. The equivalent conductance values of sodium, potassium, calcium, magnesium, and other common cations are significantly lower than that of the cation (H<sup>+</sup>) in the eluent. In this instance, a negative peak is detected as the cations are carried through the detector.

Nonsuppressed IC is easier to perform, and it is a useful technique for determining ions of weak acids such as cyanide and sulfide, which are nonconductive after chemical suppression but show a higher baseline noise. Pharmaceutical analyses can be performed in the nonsuppressed mode because the quantification limits are usually in the upper mg per L to low percentage levels. While suppressor-based methodologies must often be implemented on the instrument systems specifically designed for this purpose, IC may be performed without the suppressor on an existing HPLC. This is possible because the commonly used eluents in IC include dilute bases or acids that are compatible for use on existing HPLC instruments. When this approach is considered, analysts are encouraged to consult the instrument manufacturer for applicability of the instrument for the IC analysis.

#### other detectors

Other commonly used detection modes in IC include pulsed amperometry, direct UV detection, or post-column derivatization followed by UV/VIS detection.

**Pulsed Amperometric Detection Mode (PAD)**— PAD uses a specialized mode of the conventional amperometric technique. This type of detector is commonly used for the detection of electroactive species, e.g., organic compounds such as carbohydrates, sugar alcohols, amino acids, and organic sulfur species. In PAD, analytes are detected by an oxidative desorption process at the surface of an electrode located in the column effluent stream. Following the detection process, a series of potentials are applied for fixed time periods to clean the electrode surface. Unlike conventional amperometry that suffers from electrode surface fouling, a rapidly repeating sequence of different working potentials, referred to as waveform, helps the removal of the products of redox reactions from the electrode surface.

**Direct and Indirect UV Detection**— Direct UV Detection is used for inorganic and organic ions that possess a UV chromophore. These include organic acids, bromide, iodide, nitrate, nitrite, thiosulfate, and cyano-metal complexes. Analogous to the inverse conductometric detection of cations, UV detection may also be performed indirectly. This method is called indirect photometric chromatography (IPC).

**Photometric Detection**— Photometric detection involves chelation of the metal ions in column effluent with a color-forming reagent prior to detection with a visible wavelength. A classic example is the separation of metal ions in which the column effluent is chelated with 4-(2-pyridylazo)-resorcinol followed by detection at 510 to 530 nm.

#### SAMPLE PREPARATION

Typically sample preparation for IC includes dilution or filtering through a 0.45-μm filter, or both. Under certain circumstances, samples may require removal of undesirable species through solid-phase extraction (SPE) techniques. For example, a highly alkaline sample can be neutralized by having it pass through an SPE cartridge packed with cation-exchange material in the acidic form.

#### PROCEDURE

Conductometric detection requires high purity water (generally, resistivity greater than 18 megohm-cm) and high-purity chemicals for the preparation of the mobile phase. For ion-pair separation with UV detection, water and mobile phase components of low UV absorbance should be used.

For ion exchange, the retention time of ions increases with a decrease in the ionic strength and valency (charge) of the mobile phase components. For example, at equimolar concentrations of sodium hydroxide or sodium carbonate mobile phase, capacity factors (k') for anions are smaller with sodium hydroxide as the mobile phase than with sodium carbonate as the mobile phase. Some mobile phases, such as sodium hydroxide, can absorb ambient carbon dioxide, resulting in its composition change and often in baseline artifacts. In this instance, care should be taken to prevent absorption of carbon dioxide by the sodium hydroxide mobile phase.

For ion exclusion, capacity factors of organic acids increase with an increase in ionic strength or concentration of mineral acids but decrease with the increase of the column temperature. Because permeation volume remains constant, these effects are usually small. Addition of a solvent such as acetonitrile shortens the retention of organic acids.

Like other HPLC techniques, IC systems are calibrated by plotting peak responses in comparison with known concentrations of a reference standard, using either an external or internal standardization procedure.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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#### 1070 EMERGENCY MEDICAL SERVICES VEHICLES AND AMBULANCES—STORAGE OF PREPARATIONS

The storage and handling of pharmaceuticals in emergency vehicles and ambulances should be done so that the attributes of the official articles are preserved. There are a number of practices that need consideration when an effective plan is formulated, evaluated, put in place, and periodically re-evaluated. Those practices are listed here.



Monitoring devices should be in place to record weekly temperatures, and allow the calculation of mean kinetic temperature (MKT) for conformance to controlled room temperature storage for those vehicles utilized continuously. Measurement should also be made during a typical challenging 24-hour period, and the derived temperature should be used for the calculation of MKT and storage temperature of the sample.

#### PHARMACEUTICAL STORAGE CABINET MONITORING; LOCATION OF PARKED VEHICLES

Ambulances and other emergency medical response vehicles that routinely carry Pharmacopeial articles should be monitored to verify that temperature profiles and onboard pharmaceutical storage cabinets or cold chests are within established limits. Suitable monitoring devices are to be placed in the pharmaceutical cabinet of each vehicle that records highest and lowest temperatures, at the least, of each hot summer and cold winter day. To avoid temperature extremes, ambulance personnel should consider parking in the shade or in air-conditioned garages in the summer or in heated garages in the winter.

#### STOCK ROTATION

A program of regular stock rotation should be in place for articles with low rates of turnover. Rotation is understood as transfer of the articles with suitable marking of stock items to an appropriate climate controlled facility or storage cabinet such as in an ambulance bay. Off-vehicle storage of each article is subject to the storage requirement in the approved labeling or the pertinent USP monograph.

#### PORTABLE CARRYING CASE STORAGE AND MONITORING

The portable bag or carrying case in which drugs are kept is to be insulated, and when not in use, should be kept in a pharmaceutical storage cabinet or at controlled room temperature within facilities. Storage in portable bags or cases only, rather than in onboard cabinets, should be considered to facilitate stock rotation where indicated. The use of time-temperature indicators is recommended to monitor cumulative insult to the contents of all compartments.

#### ADDITIONAL REQUIREMENTS FOR SOME ARTICLES

All articles are to be protected from excessive heat (40°). If the article requires storage in a cold or dry place or at controlled room temperature, then suitable measures are to be taken to maintain it within the defined limits (see Preservation, Packaging, Storage, and Labeling under General Notices and Requirements.) Articles that have the most stringent storage requirements determine the storage of mixed loads.

#### STORAGE AND HANDLING OF SENSITIVE PREPARATIONS

Environmentally sensitive preparations are not to be stored in emergency response vehicles unless the onboard cabinet in which the medications is stored is climate controlled or a time-temperature indicator is attached to each package. If environmentally sensitive preparations must be kept in the emergency medical services vehicle, then the supply of medications should be rotated with reserve stock on a schedule based on local climate, but not longer than every 3 days.

#### USE OF TIME-TEMPERATURE INDICATORS

Attach time-temperature indicators to individual thermally sensitive preparations where time outside of the onboard cabinet can exceed 4 days total. Onboard cabinets must be insulated and while medications are inside, active heating and cooling devices should be used in accord with the local climate and as specified for the preparations.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

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1072 DISINFECTANTS AND ANTISEPTICS

#### INTRODUCTION

A sound cleaning and sanitization program is needed for controlled environments used in the manufacture of Pharmacopeial articles to prevent the microbial contamination of these articles. Sterile drug products may be contaminated via their pharmaceutical ingredients, process water, packaging components, manufacturing environment, processing equipment, and manufacturing operators. Current Good Manufacturing Practices (cGMPs) emphasize the size, design, construction, and location of buildings and construction materials, and the appropriate material flow to facilitate cleaning, maintenance, and proper operations for the manufacture of drug products. When disinfectants are used in a manufacturing environment, care should be taken to prevent the drug product from becoming contaminated with chemical disinfectants as a result of the inherent toxicity of the disinfectants. The requirements for aseptic processing include readily cleanable floors, walls, and ceilings that have smooth and nonporous surfaces; particulate, temperature, and humidity controls; and cleaning and disinfecting procedures to produce and maintain aseptic conditions. The cleaning and sanitization program should achieve specified cleanliness standards, control microbial contamination of products, and be designed to prevent the chemical contamination of pharmaceutical ingredients, product-contact surfaces and/or equipment, packaging materials, and ultimately the drug products. These principles also apply to nonsterile dosage forms where the microbial contamination is controlled by the selection of appropriate pharmaceutical ingredients, utilities, manufacturing environments, sound equipment cleaning procedures, products especially formulated to control water activity, inclusion of suitable preservatives, and product packaging design.

In addition to disinfectants, antiseptics are used to decontaminate human skin and exposed tissue and may be used by personnel prior to entering the manufacturing area. Chemical sterilants may be used to decontaminate surfaces in manufacturing and sterility testing areas. Furthermore, sterilants may be used for the sterilization of Pharmacopeial articles, and UV irradiation may be used as a surface sanitizer.

This general information chapter will discuss the selection of suitable chemical disinfectants and antiseptics; the demonstration of their bactericidal, fungicidal, and sporicidal efficacy; the application of disinfectants in the sterile pharmaceutical manufacturing area; and regulation and safety considerations. Biofilm formation and its relationship to disinfectants are outside the scope of this chapter. Additional information not covered in the chapter may be obtained from standard texts on disinfectants and antiseptics.<sup>1</sup>

#### DEFINITIONS

Antiseptic—An agent that inhibits or destroys microorganisms on living tissue including skin, oral cavities, and open wounds.

Chemical Disinfectant—A chemical agent used on inanimate surfaces and objects to destroy infectious fungi, viruses, and bacteria, but not necessarily their spores. Sporicidal and antiviral agents may be considered a special class of disinfectants. Disinfectants are often categorized as high-level, intermediate-level, and low-level by medically oriented groups based upon their efficacy against various microorganisms.

Cleaning Agent—An agent for the removal from facility and equipment surfaces of product residues that may inactivate sanitizing agents or harbor microorganisms.



Decontamination—The removal of microorganisms by disinfection or sterilization.

Disinfectant—A chemical or physical agent that destroys or removes vegetative forms of harmful microorganisms when applied to a surface.

Sanitizing Agent—An agent for reducing, on inanimate surfaces, the number of all forms of microbial life including fungi, viruses, and bacteria.

Sporicidal Agent—An agent that destroys bacterial and fungal spores when used in sufficient concentration for a specified contact time. It is expected to kill all vegetative microorganisms.

Sterilant—An agent that destroys all forms of microbial life including fungi, viruses, and all forms of bacteria and their spores. Sterilants are liquid or vapor-phase agents.

Microorganisms differ greatly in their resistance to disinfection agents. The order of resistance of clinically significant microorganisms to chemical disinfectants from most to least resistant is listed in [Table 1](#).

Table 1. The Resistance of Some Clinically Important Microorganisms to Chemical Disinfectants (Listed in Order of Decreasing Resistance)

Type of Microorganisms	Examples
Bacterial spores	Bacillus subtilis and Clostridium sporogenes
Mycobacteria	Mycobacterium tuberculosis
Nonlipid-coated viruses	Poliiovirus and rhinovirus
Fungal spores and vegetative molds and yeast	Trichophyton, Cryptococcus, and Candida spp.
Vegetative bacteria	Pseudomonas aeruginosa, Staphylococcus aureus, and Salmonella spp.
Lipid-coated viruses	Herpes simplex virus, hepatitis B virus, and human immunodeficiency virus

#### CLASSIFICATION OF DISINFECTANTS

Chemical disinfectants are classified by their chemical type. This includes aldehydes, alcohols, halogens, peroxides, quaternary ammonium compounds, and phenolic compounds (see [Table 2](#)).

Table 2. General Classification of Antiseptics, Disinfectants, and Sporicidal Agents

Chemical Entity	Classification	Example
Aldehydes	Sporicidal agent	2% Glutaraldehyde
Alcohols	General purpose disinfectant, antiseptic, antiviral agent	70% Isopropyl alcohol, 70% alcohol
Chlorine and sodium hypochlorite	Sporicidal agent	0.5% Sodium hypochlorite
Phenolics	General purpose disinfectant	500 µg per g Chlor cresol, 500 µg per g chloroxylenol
Ozone	Sporicidal agent	8% Gas by weight
Hydrogen peroxide	Vapor phase sterilant, liquid sporicidal agent, antiseptic	4 µg per g H2O2 vapor, 10%–25% solution, 3% solution
Substituted diguanides	Antiseptic agent	0.5% Chlorhexidine gluconate
Peracetic acid	Liquid sterilant, vapor phase sterilant	0.2% Peracetic acid, 1 µg per g peracetic acid
Ethylene oxide	Vapor-phase sterilant	600 µg per g Ethylene oxide
Quaternary ammonium compounds	General purpose disinfectant, antiseptic	200 µg per g Benzalkonium chloride
$\beta$ -Propiolactone	Sporicidal agent	100 µg per g $\beta$ -Propiolactone

The effectiveness of a disinfectant depends on its intrinsic biocidal activity, the concentration of the disinfectant, the contact time, the nature of the surface disinfected, the hardness of water used to dilute the disinfectant, the amount of organic materials present on the surface, and the type and the number of microorganisms present. Under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), the Environmental Protection Agency (EPA) registers chemical disinfectants marketed in the United States and requires manufacturers to supply product information on the use dilution, type of microorganisms killed, and the necessary contact time. Certain liquid chemical sterilizers intended for use on critical or semicritical medical devices are defined and regulated by the U.S. Food and Drug Administration (FDA).

#### SELECTION OF AN ANTISEPTIC FOR HAND AND SURGICAL SITE DISINFECTION

Hands and surgical sites are disinfected in a hospital setting to reduce the resident flora and to remove transient flora (e.g., *Streptococcus pyogenes*) and methicillin-resistant *S. aureus* and *P. aeruginosa* that have been implicated in hospital-associated infection. Use of antiseptics to disinfect hands has been shown to be more effective than soap and water in reducing the counts of bacteria on the skin; repeated antiseptic use further reduces these counts. These principles may be applied to clean-room operators in the pharmaceutical industry.

Common antiseptics include 4% chlorhexidine, 10% povidone-iodine, 3% hexachlorophene, 70% isopropyl alcohol, and 0.5% chlorhexidine in 95% alcohol.

#### SELECTION OF A DISINFECTANT FOR USE IN A PHARMACEUTICAL MANUFACTURING ENVIRONMENT

When selecting a disinfectant for use in a pharmaceutical manufacturing area, the following points should be considered: the number and types of microorganisms to be controlled; the spectrum of activity of commercially available disinfectants; the reputation of the disinfectant supplier; the claims as a sterilant; the disinfectant or sanitizer supported by the EPA registrations; the concentration, application method, and contact time of the disinfectant; the nature of the surface material being disinfected and its compatibility with the disinfectant; the amount of organic compounds on the surface that may inactivate a disinfectant; the possible need to maintain a residual bactericidal activity of the disinfectant on the surface; the corrosiveness of the disinfectant to equipment with repeated application; the safety considerations for operators applying the disinfectant; the compatibility of the disinfectant with cleaning agents and other disinfectants; the planned disinfectant rotation; and the steps that need to be taken to avoid the contamination of pharmaceutical products by a disinfectant.<sup>2</sup>

#### THEORETICAL DISCUSSION OF DISINFECTANT ACTIVITY

Plots of the log of the number of microorganisms per mL surviving in a disinfectant solution indicate that first-order kinetics can be applied as a gross approximation to the reduction in microbial count with respect to time. In practice, the plots show a more sigmoid curve with a slower initial reduction in numbers followed by an increasing rate with respect to time.

The rate constant, K, for the disinfection process can be calculated by the formula:

$$(1 / t)(\log NO / N)$$

in which t is the time, in minutes, for the microbial count to be reduced from NO to N; NO is the initial number of organisms, in cfu per mL; and N is the final number, in cfu per mL, of organisms.

As with a first-order chemical reaction, the same concentration of disinfectant reduces the number of organisms more rapidly at elevated temperatures. This can be expressed as a

temperature, T, coefficient per  $10^{\circ}$  rise in temperature, Q10, calculated by the formula:

$$\text{Time to decontamination at } T^{\circ} / \text{Time to decontamination at } T$$

$$\text{in which } T \text{ is } T^{\circ} - 10.$$

Further evidence that a first-order reaction is an inadequate description of disinfection is that the Q10 values for chemical and enzyme reactions are 2 to 3, while the common disinfectants phenol and alcohol have a Q10 of 4 and 45, respectively.

Critical to the successful employment of disinfectants is an understanding of the effect of disinfectant concentration on microbial reduction. A plot of the log of the time to reduce the microbial population in a standard inoculum to zero against the log of the disinfectant concentration is a straight line with the slope of the line termed the concentration exponent, n. The relationship can be expressed as follows:

$$n = (\log \text{ of the kill time at concentration } C_2) - (\log \text{ of the kill time at concentration } C_1) / (\log C_1 - \log C_2)$$

in which C1 and C2 are the higher and lower disinfectant concentrations, respectively.

The wide differences in concentration exponents, n, have practical consequences in picking the use dilution of different disinfectants and in using dilution to neutralize a disinfectant in disinfectant-effectiveness testing and routine microbial monitoring of the manufacturing environment. For example, mercuric chloride has a concentration exponent of 1, so a 3-fold dilution will reduce the disinfectant activity by 31 (or by one-third), while phenol with a concentration exponent of 6 will have a 36 (or a 729-fold) reduction in disinfectant activity. Disinfectants with a larger concentration exponent or dilution coefficient rapidly lose activity when diluted. The concentration exponents for some disinfectants are listed in [Table 3](#).

Table 3. Concentration Exponents of Common Antiseptics, Disinfectants, and Sterilants

Disinfectant	Concentration Exponents
Hydrogen peroxide	0.5
Sodium hypochlorite	0.5
Mercuric chloride	1
Chlorhexidine	2
Formaldehyde	1
Alcohol	9
Phenol	6
Quaternary ammonium compounds	0.8 to 2.5
Aliphatic alcohols	6.0 to 12.7
Phenolic compounds	4 to 9.9

Another important consideration may be the pH of the disinfectant. Many disinfectants are more active in the ionized form, while others are more active in the nonionized form. The degree of ionization will depend on the pKa of the agent and the pH of the disinfection environment. For example, phenol, with a pKa of 10, will be more effective at a pH below 7 where it is nonionized, while acetic acid will be more effective at a pH below 4 where it is ionized.

#### MECHANISM OF DISINFECTANT ACTIVITY

[Table 4](#) lists the sites and modes of action of some representative disinfectants.

Table 4. Mechanism of Disinfectant Activity Against Microbial Cells

Target	Disinfectant
Cell wall	Formaldehyde, hypochlorite, and mercurials
Cytoplasmic membrane, action on membrane potential	Anilides and hexachlorophene
Membrane enzymes, action on electron-transport chain	Hexachlorophene
Action on ATP	Chlorhexidine and ethylene oxide
Action on enzymes with -SH groups	Ethylene oxide, glutaraldehyde, hydrogen peroxide, hypochlorite, iodine, and mercurials
Action on general membrane permeability	Alcohols, chlorhexidine, and quaternary ammonium compounds
Cell contents, general coagulation	Chlorhexidine, aldehydes, hexachlorophene, and quaternary ammonium compounds
Ribosomes	Hydrogen peroxide and mercurials
Nucleic acids	Hypochlorites
Thiol groups	Ethylene oxide, glutaraldehyde, hydrogen peroxide, hypochlorite, mercurials
Amino groups	Ethylene oxide, glutaraldehyde, and hypochlorite
General oxidation	Ethylene oxide, glutaraldehyde, and hypochlorite

#### MICROBIAL RESISTANCE TO DISINFECTANTS

The development of microbial resistance to antibiotics is a well-described phenomenon. The development of microbial resistance to disinfectants is less likely, as disinfectants are more powerful biocidal agents than antibiotics and are applied in high concentrations against low populations of microorganisms usually not growing actively, so the selective pressure for the development of resistance is less profound. However, the most frequently isolated microorganisms from an environmental monitoring program may be periodically subjected to use dilution testing with the agents used in the disinfection program to confirm their susceptibility.

#### DISINFECTANT CHALLENGE TESTING

Under FIFRA, the EPA requires companies that register public health antimicrobial pesticide products including disinfectants, sanitization agents, sporicidal agents, and sterilants to ensure the safety and effectiveness of their products before they are sold or distributed. Companies registering these products must address the chemical composition of their product, include toxicology data to document that their product is safe if used as directed on the label, include efficacy data to document their claims of effectiveness against specific organisms and to support the directions for use provided in the labeling, and provide labeling that reflects the required elements for safe and effective use. While these directions provide valuable information, they may not be helpful in terms of the products' use as disinfectants in a manufacturing environment.

In the United States, the official disinfectant testing methods are published by AOAC International<sup>3</sup> and include the Phenol-Coefficient Test, Use-Dilution Method Test, Hard Surface Carrier Method, and Sporicidal Carrier Test. A scientific study submitted for EPA review in support of disinfectant registration must be conducted at a laboratory facility that follows the Good Laboratory Practices (GLP) regulations (21 CFR 58). To demonstrate the efficacy of a disinfectant within a pharmaceutical manufacturing environment, it may be deemed necessary to conduct the following tests: (1) use-dilution tests (screening disinfectants for their efficacy at various concentrations and contact times against a wide range of standard test organisms and environmental isolates); (2) surface challenge tests (using standard test microorganisms and microorganisms that are typical environmental isolates, applying

disinfectants to surfaces at the selected use concentration with a specified contact time, and determining the log reduction of the challenge microorganisms); and (3) a statistical comparison of the frequency of isolation and numbers of microorganisms isolated prior to and after the implementation of a new disinfectant. This is considered necessary because critical process steps like disinfection of aseptic processing areas, as required by GMP regulations, need to be validated, and the EPA registration requirements do not address how disinfectants are used in the pharmaceutical, biotechnology, and medical device industries. For the surface challenge tests, the test organisms are enumerated using swabs, surface rinse, or contact plate methods. Neutralizers that inactivate the disinfectants should be included in either the diluent or microbiological media used for microbial enumeration or both

(see [Table 5](#)). Additional information on disinfectant neutralization may be found in [Validation of Microbial Recovery from Pharmacopeial Articles](#) (1227).

Table 5. Neutralizing Agents for Common Disinfectants

Disinfectant	Neutralizing Agent
Alcohols	Dilution or polysorbate 80
Glutaraldehyde	Glycine and sodium bisulfite
Sodium hypochlorite	Sodium thiosulfate
Chlorhexidine	Polysorbate 80 and lecithin
Mercuric chloride and other mercurials	Thioglycolic acid
Quaternary ammonium compounds	Polysorbate 80 and lecithin
Phenolic compounds	Dilution or polysorbate 80 and lecithin

Universal neutralizer broths may be formulated to contain a range of neutralizing agents. For example, Dey/Engley (D/E) broth contains 0.5% polysorbate 80, 0.7% lecithin, 0.1% sodium thioglycolate, 0.6% sodium thiosulfate, 0.25% sodium bisulfite, 0.5% tryptone, 0.25% yeast extract, and 1.0% dextrose; Itephen broth contains 0.5% polysorbate 80, 0.07% lecithin, 1.0% peptamin, 0.5% beef extract, and 0.5% sodium chloride; and Tryptone-Azolectin-Tween (TAT) broth base + tween 20 contains 4.0% (v/v) polysorbate 20, 0.5% lecithin, and 2.0% tryptone.

In practice, sufficient organisms need to be inoculated on a 2-inch × 2-inch square of the surface being decontaminated, i.e., a coupon, to demonstrate at least a 2 (for bacterial spores) to 3 (for vegetative bacteria) log reduction during a predetermined contact time (i.e., 10 minutes or above the recovery observed with a control disinfectant application). The efficacy of the neutralizers and their ability to recover inoculated microorganisms from the material should be demonstrated during the use-dilution or surface-challenge studies. Points to remember are that disinfectants are less effective against the higher numbers of microorganisms used in laboratory challenge tests than they are against the numbers that are found in clean rooms (see [Microbiological Evaluation of Clean Rooms and Other Controlled Environments](#) (1116)); that inocula from the log growth phase that are typically employed in laboratory tests are more resistant, with the exception of spores formed during the static phase, than those from a static or dying culture or stressed organisms in the environment; and that microorganisms may be physically removed during actual disinfectant application in the manufacturing area.

Although not all inclusive, typical challenge organisms that may be employed are listed in [Table 6](#).

Table 6. Typical Challenge Organisms

AOAC Challenge Organisms	Typical Environmental Isolates
Bactericide: Escherichia coli, ATCC 11229; S. aureus, ATCC 6538; P. aeruginosa, ATCC 15442	Bactericide: Micrococcus luteus, S. epidermidis, <i>Corynebacterium jeikeium</i> , <i>P. vesiculans</i>
Fungicide: C. albicans, ATCC 10231 or 2091; Penicillium chrysogenum, ATCC 11709; Aspergillus niger, ATCC 16404	Fungicide: <i>P. chrysogenum</i> , <i>A. niger</i>
Sporicide: <i>B. subtilis</i> , ATCC 19659	Sporicide: <i>B. sphaericus</i> , <i>B. thuringiensis</i>

Because a wide range of different materials of construction are used in clean rooms and other controlled areas, each material needs to be evaluated separately to validate the efficacy of a given disinfectant. [Table 7](#) contains a list of common materials used in clean room construction.

Table 7. Typical Surfaces to be Decontaminated by Disinfectants in a Pharmaceutical Manufacturing Area

Material	Application
Stainless steel 304L and 316L grades	Work surfaces, filling equipment, and tanks
Glass	Windows and vessels
Plastic, vinyl	Curtains
Plastic, polycarbonate	Insulation coating
Lexan® (plexiglass)	Shields
Epoxy-coated gypsum	Walls and ceilings
Fiberglass-reinforced plastic	Wall paneling
Tyvek®	Equipment wraps
Terrazzo tiles	Floors

#### DISINFECTANTS IN A CLEANING AND SANITIZATION PROGRAM

The selection of suitable disinfectants and the verification of their effectiveness in surface challenge testing is critical in the development of a cleaning and sanitization program.

Issues associated with the successful implementation of such a program are the development of written procedures, staff training, decisions on disinfectant rotation, institution of application methods and contact times, environmental monitoring to demonstrate efficacy, and personnel safety.

The cGMP 21 CFR 211.67, Equipment Cleaning and Maintenance, details the requirements for written procedures for cleaning, maintenance, and sanitization of pharmaceutical manufacturing equipment. These procedures should address the assignment of responsibility, establishment of schedules, details of cleaning operations, protection of clean equipment prior to use, inspection for cleanliness immediately prior to use, and maintenance of cleaning and sanitization records.

Staff involved in disinfection require training in microbiology, industry practices for cleaning and sanitization, safe handling of concentrated disinfectants, the preparation and disposal of disinfectants, and appropriate application methods. It should be emphasized that the preparation of the correct dilutions is critical because many disinfectant failures can be attributed to use of disinfectant solutions that are too dilute. Typically disinfectants used in aseptic processing and filling areas are diluted with Sterile Purified Water, and are prepared aseptically. Alternately, the disinfectant may be diluted with Purified Water, and then sterile filtered to eliminate microorganisms that may potentially persist in a disinfectant. Diluted disinfectants must have an assigned expiration date justified by effectiveness studies.

Because it is theoretically possible that the selective pressure of the continuous use of a single disinfectant could result in the presence of disinfectant-resistant microorganisms in a manufacturing area, in some quarters the rotation of disinfectants has been advocated. However, the literature supports the belief that the exposure of low numbers of microorganisms on facility and equipment surfaces within a clean room where they are not actively proliferating will not result in the selective pressure that may be seen with the antibiotics. It is prudent to augment the daily use of a bactericidal disinfectant with weekly (or monthly) use of a sporicidal agent. The daily application of sporicidal agents is not generally favored because of their tendency to corrode equipment and because of the potential safety issues with chronic operator exposure. Other disinfection rotation schemes may be supported on the basis of a review of the historical environmental monitoring data. Disinfectants applied on potential product contact surfaces are typically removed with 70% alcohol wipes. The removal of



residual disinfectants should be monitored for effectiveness as a precaution against the possibility of product contamination.

The greatest safety concerns are in the handling of concentrated disinfectants and the mixing of incompatible disinfectants. For example, concentrated sodium hypochlorite solutions (at a concentration of more than 5%) are strong oxidants and will decompose on heating, on contact with acids, and under the influence of light, producing toxic and corrosive gases including chlorine. In contrast, dilute solutions (at a concentration of less than 0.5%) are not considered as hazardous. Under no circumstances should disinfectants of different concentrations be mixed. Material Safety Data Sheets for all the disinfectants used in a manufacturing area should be available to personnel handling these agents. Appropriate safety equipment such as face shields, safety glasses, gloves, and uniforms must be issued to personnel handling the disinfectant preparation, and personnel must be trained in the proper use of this equipment. Safety showers and eye wash stations must be situated in the work area where disinfectant solutions are prepared.

1 Ascenzi, J.M., Ed. Handbook of Disinfectants and Antiseptics, 5th ed.; Marcel Dekker: New York, 1995; Block, S.S., Ed. Disinfection, Sterilization, and Preservation, 5th ed.; Lippincott Williams & Wilkins Publishers: Philadelphia, 2000. Russell, A.D.; Hugo, W.B.; Ayliffe, G.A.J., Eds. Principles and Practices of Disinfection, Preservation and Sterilization, 3rd ed.; Blackwell Science Inc.: London, 1999.

2 Denny, V.F.; Marsik, F.J. Current Practices in the Use of Disinfectants within the Pharmaceutical Industry. PDA J. of Pharmaceutical Sci. and Tech., 1997, 51, (6), 227-228.

3 AOAC International Official Methods of Analysis, 15, 16, and 17th editions. Arlington, VA.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

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## 1074 EXCIPIENT BIOLOGICAL SAFETY EVALUATION GUIDELINES

### INTRODUCTION

This informational chapter presents a scientifically-based approach for the safety assessment of new pharmaceutical excipients (i.e., those excipients that have not been previously used or permitted for use in a pharmaceutical preparation). The guidelines presented herein provide a protocol for developing an adequate database upon which to establish conditions for the safe use of a new excipient intended for use in products administered by various dosage routes. [note—The final section of this chapter, [Definition of Terms](#), lists some terms referred to in this chapter.]

An excipient may perform a variety of functionality roles in a pharmaceutical product; but, unlike pharmacologically active drug entities, the excipient displays either no pharmacological activity or very limited and directed activity. Because of these differences between excipients and active drug substances in terms of risk and benefit relationships and expected biological activities, the approaches for safety assessments of excipients and active drug substances will differ. Therefore, it is important to note that the guidelines presented in this informational chapter apply only to the safety assessment of excipients, not to the safety assessment of active drug substances.

These testing guidelines are informational in nature and are intended to be used by professionals having a knowledge of toxicology and associated sciences. It is also intended that the applicable safety test method requirements of the receiving regulatory authority would be used in a proposal for market entry. For example, if a proposal is to be submitted to the U.S.

Food and Drug Administration, that agency's safety test requirements would have to be met. These guidelines do not provide specific details regarding test methodology and data interpretation. Test procedures that are generally recognized by experts and by the regulatory agencies should be used. Alternatives to the use of living animals are encouraged wherever these alternative procedures have been validated for the intended purpose and where it is known that the alternative procedure will provide sufficient data upon which to base a safety judgment. It is recommended that the Guiding Principles on the Use of Animals in Toxicology of the Society of Toxicology (1996) and, in other countries, the appropriate legal and professional codes, be adhered to in the conduct of all test procedures. All studies must meet the requirements of the appropriate national good laboratory practice guidelines in effect in the country where the studies are being conducted.

In cases of extensive human experience based upon food use, there may be sufficient information to fulfill the requirements of the guidelines for orally-ingested excipients only. In addition, there may be animal-based data, which was developed for other purposes, that may be used to fulfill the testing guidelines requirements. If the data requirements have been met through prior human use experience and pertinent human data have been collected in a scientifically sound manner, there is no need to provide animal data for those endpoints evaluated by prior clinical experience.

Some dosage routes offer unique toxicological challenges, and the guidelines include provisions for these routes (e.g., inhalation). Also, further explanation is provided regarding numbers of species and other basic information (e.g., two species, one rodent and one nonrodent).

The extent of information required to define a set of baseline data, which constitute a toxicological and chemical database, is dependent upon the intended use of, and duration of, dosing of the candidate excipient material. It is critical that a thorough review of background information be conducted before embarking on a testing regimen. In addition to literature database reviews, information should be obtained regarding the physical and chemical properties of the compound; its manufacturing process (or processes); and product specifications including limits of impurities, potential for pharmacological activity, exposure conditions (i.e., dose, duration, frequency of use, dosage formulation, and route of administration), and potential user population. Also, base toxicity information covering the topics is fundamental. Particular attention should be addressed to the absorption/distribution/metabolism/excretion/pharmacokinetics (ADME/PK) studies because much of the later decision process will be dependent upon these data.

These guidelines provide a mechanism for obtaining sets of baseline data for all candidate excipient materials. The background information and baseline toxicity information alone may support the use of the candidate excipient either in a short half-life product that is not administered in a frequency that results in a residual excipient build-up in body tissue or in a product used only once or twice in a lifetime, such as a diagnostic agent. Additional tests, listed under Step 4 of the Safety Assessment Guidelines, are necessary for candidate excipient material that is to be used in a manner that will result in short- or intermediate-term repeated exposure in humans—that is, a pharmaceutical product that will be administered for less than 10 days or for 30 to 90 consecutive days, respectively. For a candidate excipient material that is intended for use in a pharmaceutical product intended for either intermittent or chronic administration over a long time period, such as a treatment for psoriasis or an insulin preparation, further tests are required. These tests are listed under Step 7 of the guidelines and in the appropriate section under Additional Requirements for Specific Exposure Routes. While providing guidance for consumer safety, some of the required tests are intended to provide information to address occupational safety (e.g., skin and eye irritation).

The guidelines are summarized in [Table 1](#). Tests that are required (R) by the guidelines are distinct from those that are recommended conditionally (C). Whether conditional tests are conducted is dependent upon the conditions of use and available biological data. Consideration must also be given to the requirements of the regulatory authorities when making the decision to test.

Table 1. Summary of Excipient Guidelines

Tests	Routes of Exposure for Humans					
	Oral	Mucosal	Dermal/ Topical/ Trans- dermal	Injectable*	Inhalation/ Intranasal	Ocular
Baseline Toxicity Data						
Acute Oral Toxicity	R	R	R	R	R	R

Acute Dermal Toxicity	R	R	R	R	R	R
Acute Inhalation Toxicity	C	C	C	C	R	C
Eye Irritation	R	R	R	R	R	R
Skin Irritation	R	R	R	R	R	R
Skin Sensitization	R	R	R	R	R	R
Acute Injectable Toxicity	—	—	—	R	—	—
Application Site Evaluation	—	—	R	R	—	—
Pulmonary Sensitization	—	—	—	—	C	—
Phototoxicity/Photoallergy	R	—	R	R	R	—
Genotoxicity Assays	R	R	R	R	R	R
ADME/PK-Intended Route	R	R	R	R	R	R
28-Day Toxicity (2 Species)-Intended Route	R	R	R	R	R	R

**Additional Data: Short- or Intermediate-term Repeated Use**

90-Day Toxicity (Most Appropriate Species)	R	R	R	R	R	R
Embryo-Fetal Toxicol.	R	R	R	R	R	R
Additional Assays	C	C	C	C	C	C
Genotoxicity Assays	R	R	R	R	R	R
Immunosuppression Assays	R	C	C	R	C	C

**Additional Data: Intermittent Long-term or Chronic Use**

Chronic Toxicity (Rodent, Nonrodent)	C	C	C	C	C	C
Reproductive Toxicity	R	R	R	R	R	R
Photocarcinogenicity	C	—	C	C	C	—
Carcinogenicity	C	C	C	C	C	C

R = Required

C = Conditional

\* Intravenous, intramuscular, subcutaneous, intrathecal, etc.

**SAFETY ASSESSMENT GUIDELINES**
**Background Information**

Before proceeding to the steps under Data Requirements and Checkpoints, the following points should be reviewed and defined:

- Review literature information using all appropriate databases
  - Define chemical and physical properties
  - Define manufacturing process
- Define product specifications, including impurities and residual solvents (see applicable ICH guidelines)
  - Estimate exposure conditions (dose, duration, frequency route)
    - Define user population
    - Assess potential for pharmacologic activity.

At this point evaluate what is known, and develop the initial approach to testing.

**Data Requirements and Checkpoints**
**STEP 1: Toxicity Data (see Baseline Toxicity Data)**

The toxicity data should take into account the following information:

- Effects of acute exposure by oral and intended routes
- Effects of repeated exposures by intended routes
  - Effects of in vitro genotoxicity assays
- ADME/PK by oral or appropriate routes; single or multiple doses.

**STEP 2: Depending on results of above, evaluate effects of a single dose in humans.**

STEP 3: Checkpoint: Evaluate results of above and proposed exposure conditions and exposed population. The above data might allow use in a single product with a short half-life (e.g., a diagnostic agent).

**STEP 4: Gather the following additional data:**

- Effects of subchronic exposure in appropriate species and routes
- Embryo-fetal development studies via appropriate route of exposure
  - Additional in vitro and in vivo genotoxicity tests.

STEP 5: Depending on results of above, consideration should be given to testing in humans as part of the clinical trials of an active ingredient or as a stand-alone procedure.

STEP 6: Checkpoint: Evaluate all of above information. Data might allow use in a variety of products intended for short-term, repeated intake (e.g., an antibiotic). If the ADME/PK studies for a noninjectable excipient show no absorption, data may permit using a product for 30 to 90 consecutive days.

**STEP 7: Additional data should be obtained for use in a product taken chronically, either daily or intermittently, over a long time period depending on:**

- Results of subchronic studies and long-term toxicity in appropriate mammalian nonrodents
  - Reproductive toxicity studies
- Other test results and human exposure data and long-term toxicity or carcinogenicity in rodents.

**Baseline Toxicity Data**

The following data should be taken into account:

Appropriate Acute Toxicity by Intended Dose Routes: skin sensitization, approximate lethal dose method, limit test, etc.



Other Appropriate Acute Toxicity Studies: oral toxicity by limit test or approximate lethal dose method, skin irritation, etc.

ADME/PK: single or multiple doses.

Genotoxicity: for example, Ames Test, in vitro chromosome aberration test, mammalian cell mutation assay.

28-Day Repeated Dosing Studies in Two Species by Appropriate Routes (One Rodent, One Mammalian Nonrodent): evaluation of injection site or similar considerations might be necessary depending on route of administration.  
notes—

1. In those cases where intended route restrictions (e.g., volume, concentration) preclude an adequate assessment of the toxicity of the excipient, development of a toxicity profile by other relevant routes may be needed.
2. The comparison of toxicity and ADME/PK data between oral and intended routes is critical at this point because that knowledge may set the direction for future toxicity testing (e.g., reproductive toxicity testing conducted by oral route rather than intended route). In addition, relevant studies using the intended route and anticipated duration of exposure may preclude performance of additional studies.

#### Additional Requirements for Specific Exposure Routes

FOR ORAL EXPOSURE: No additional requirements beyond those presented for Baseline Toxicity Data.

FOR MUCOSAL EXPOSURE: No additional requirements beyond those presented for Baseline Toxicity Data.

FOR DERMAL, TOPICAL, OR TRANSDERMAL EXPOSURE:

##### Baseline Toxicity Data—

- Effects of Acute Exposure by Transdermal Dose Route: dermal sensitization study for repeat applications
  - Effects of Repeated Exposures by Transdermal Route
    1. Photoallergy/phototoxicity study
    2. Studies in two species (one rodent, one mammalian nonrodent) by transdermal route.
- Effects of Subchronic Exposure, Reproductive Toxicity Effects—Initial toxicity studies may be performed by the IV route to adequately profile the toxicity of the excipient. This will provide an assessment of potential target organs if an adequate amount of the compound cannot be delivered via a transdermal dosage form. This is dependent upon the results from the ADME/PK studies.

Reproductive studies may also be conducted via oral or IV route with demonstration of absorption (oral) and pharmacokinetic comparisons of the chosen route versus transdermal.

Photocarcinogenicity studies may be required and should be considered if data and the proposed use indicate when evaluating materials to be placed on the skin for prolonged periods of time and exposure to UV light is a factor (e.g., sun block). This also applies to oral, parenteral, and inhalation products where skin drug concentrations exceed plasma drug concentrations for a substantial period of time, or where the candidate material would appear to have the potential for photo-activity or has demonstrated photo-activity.

FOR INJECTABLE DOSAGE FORMS:

##### Background Information—

1. Define compatibility of the dosage form with blood, if appropriate, based on route of exposure.
2. Define the pH and tonicity of injectable dose form, if appropriate, based on the route of exposure.

##### Baseline Toxicity Data—

- Effects of Acute Exposure by Intended Injectable Dose Routes
  1. Include evaluation of injection site irritation in rabbit or dog
  2. Include evaluation of rate of administration.

for inhalation or intranasal exposure:<sup>1</sup>

##### Baseline Toxicity Data—

- Acute Inhalation Toxicity—A limit test that would, for example, use the highest achievable concentration in a 4-hour exposure to vapor, aerosol, or solid particulate. Pulmonary sensitization may be performed along with other appropriate studies. If exposure is to be to an aerosol or solid particulate, particulates of appropriate mass median diameter should be generated.
  - Single and Repeated Dose ADME/PK by Inhalation or Intranasal and Oral Routes
- 28-Day Repeated Dose Inhalation Study in Two Mammalian Species Using Vapor or Particulates of Appropriate Mass Median Diameter: compare to similar oral toxicity data.

for ophthalmic exposure:

Background Information: define pH and osmolarity of topical ocular dose form.

##### Baseline Toxicity Data—

- Effects of Acute Exposure by Ophthalmic Routes: cytotoxicity tests (e.g., agar overlay)
  - Effects of Repeated Exposures by Ophthalmic Routes
    1. Studies in two species (one rodent, one mammalian nonrodent)
    2. Examination of anterior and posterior segments of the eye
    3. Studies on allergenicity potential.

Other Data— Comparison of pharmacokinetic parameters of the route chosen for reproductive studies and the ophthalmic exposure are essential for extrapolation of potential toxicity via the ophthalmic route.

#### DEFINITION OF TERMS

Acute: exposure to a test agent within a single, 24-hour period. Doses may be single, multiple or continuous during a 24-hour period.

Subacute: repeated dosing of a test agent for up to 29 days. Daily doses may be single, multiple or continuous during a 24-hour period.

Subchronic: repeated dosing of a test agent for 30 days to 10% of the lifespan of the test species (90 days in rodents). Daily doses may be single, multiple or continuous during a 24-hour period.

Chronic: repeated dosing of a test agent for more than 10% of the lifespan of the test species (more than 90 days in rodents). Daily doses may be single, multiple or continuous during a 24-hour period.

<sup>1</sup> When designing studies to evaluate use in products intended for use by the inhalation or intranasal route, consideration should be given to the dosing regimen that will be used by humans. The appropriate study protocol for a product intended for inhalation therapy that will result in prolonged exposures (e.g., several hours per day) may differ from that used to evaluate a product that would result in exposure to several metered doses per day.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

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## 1075 GOOD COMPOUNDING PRACTICES

The purpose of this chapter is to provide compounders with guidance on applying good compounding practices for the preparation of compounded formulations for dispensing and/or administration to humans or animals. This chapter is intended to provide information as a supplement to other relevant chapters. The following discussion is applicable to those engaged in compounding preparations in all pharmacies. It is expected that pharmacists or compounders engaged in the compounding of drugs will compound in conformance with applicable state and federal compounding laws, regulations, or guidelines.

### APPLICABLE DEFINITIONS

Compounding (see [Pharmaceutical Compounding—Nonsterile Preparations](#) (795))—Compounding involves the preparation, mixing, assembling, packaging, and labeling of a drug or device in accordance with a licensed practitioner's prescription of medication order under an initiative based on the practitioner/patient/pharmacist/compounder relationship in the course of professional practice. Compounding includes the following:

- a. Preparation of drugs or devices in anticipation of prescription drug orders based on routine, regularly observed prescribing patterns.
- b. Reconstitution or manipulation of commercial products that may require the addition of one or more ingredients as a result of a licensed practitioner's prescription drug order.
- c. Preparation of drugs or devices for the purposes of, or as an incident to, research, teaching, or chemical analysis.

Categories of Compounding—The categories of compounding are intended to provide an understanding among compounders when different forms of preparations are compounded. It is to be understood that there are levels of training associated with each category. In the categories of compounding described below, certain criteria were used to determine the overall classification.

Category 1 Nonsterile—Simple

Generally, the mixing of two or more commercial products

Category 2 Nonsterile—Complex

Generally, compounding with the bulk drug substances or when calculations are required.

Category 3 Sterile—Risk Level 1

(See Low-Risk Level in USP general chapter (797))

Category 4 Sterile—Risk Level II

(See Medium-Risk Level in USP general chapter (797))

Category 5 Sterile—Risk Level III

(See High-Risk Level in USP general chapter (797))

Category 6 Radiopharmaceuticals

Preparation of radiopharmaceuticals

Category 7 Veterinary

Preparation of veterinary pharmaceuticals

Manufacturing—Manufacturing involves the production, propagation, conversion, or processing of a drug or device, either directly or indirectly, by extraction of the drug from substances of natural origin or by means of chemical or biological synthesis. Manufacturing also includes (1) any packaging or repackaging of the substance(s) or labeling or relabeling of containers for the promotion and marketing of such drugs or devices; (2) any preparation of a drug or device that is given or sold for resale by pharmacies, practitioners, or other persons; (3) the distribution of inordinate amounts of compounded preparations or the copying of commercially available drug products; and (4) the preparation of any quantity of a drug product without a licensed prescriber/patient/licensed pharmacist/compounder relationship.

Component—A component is any ingredient used in the compounding of a drug product, including any that are used in its preparation, but may not appear on the labeling of such a product. (See [Pharmaceutical Compounding—Nonsterile Preparations](#) (795) for additional definitions.)

Compounder—A compounder is a professional authorized by the state to perform compounding pursuant to a prescription order by a licensed prescriber.

### RESPONSIBILITIES OF THE COMPOUNDER

- a. Compounders who are engaged in drug compounding or nutriceutical compounding shall be proficient in compounding and should continually expand their compounding knowledge by participating in seminars and/or studying appropriate literature.
- b. A compounder must be familiar with all of the details of [Pharmaceutical Compounding—Nonsterile Preparations](#) (795), [Pharmaceutical Compounding—Sterile Preparations](#) (797), [Pharmaceutical Calculations in Prescription Compounding](#) (1160), and other applicable state or federal compounding guidelines or laws. In addition, the compounder must be responsible for the following:
  - certifying all prescription orders;
  - approving or rejecting all components, drug product containers, closures, in-process materials, and labeling;
  - preparing and reviewing all compounding records to assure that errors have not occurred in the compounding process;
  - assuring the proper maintenance, cleanliness, and use of all equipment used in a prescription compounding practice;
  - assuring that only authorized personnel shall be in the immediate vicinity of the drug compounding operations;
- c. The compounder must ensure that personnel engaged in compounding wear clean clothing appropriate to the type of compounding performed, e.g., coats, gowns, gloves, masks, shoes, aprons, or other items as needed for protection of personnel from chemical exposures and for prevention of drug contamination.
- d. The compounder must implement procedures to prevent cross-contamination when compounding with drugs (e.g., penicillins) that require special precaution to prevent cross-contamination.

### TRAINING

All personnel involved in the compounding, evaluation, packaging, and dispensing of compounded preparations shall be properly trained for the type of compounding conducted. All training activities will be covered by appropriate standard operating procedures (SOPs) and documentation.

All compounders and all personnel involved in compounding must be well trained and must participate in current, relevant training programs. It is the responsibility of the compounder



Ensure that a training program has been implemented and that it is ongoing. Standards of practice require that all employees be adequately trained in their job functions and that the training is properly documented. Steps in the training procedure will include the following:

- a. All employees involved in pharmaceutical compounding shall read and become familiar with [Pharmaceutical Compounding—Nonsterile Preparations](#) (795), [Pharmaceutical Compounding—Sterile Preparations](#) (797), and [Pharmaceutical Calculations in Prescription Compounding](#) (1160).
- b. All employees shall read and become familiar with each of the procedures related to compounding, including those involving the facility, equipment, personnel, actual compounding, evaluation, packaging, storage, and dispensing.
- c. The compounding shall meet with employees to review their work and answer any questions the employees may have concerning SOPs.
- d. The compounding shall demonstrate the procedures for the employee, and will observe and guide the employee throughout the training process. The employee will then repeat the procedure without any assistance from, but under the direct supervision of, the compounding.
- e. When the employee has demonstrated to the compounding a verbal and functional knowledge of the procedure, then and only then, will the employee be permitted to perform the procedure without direct supervision. However, the compounding should be physically present and should check off the final preparation.
- f. When the compounding is satisfied with the employee's knowledge and proficiency, the compounding will sign off on the documentation records to show that the employee was appropriately trained.
- g. The compounding shall continually monitor the work of the employee and assure that the employee's calculations and work are accurate and adequately performed. The compounding is completely responsible for the finished preparation. The compounding will answer any questions the employee may have concerning the SOPs.

#### PROCEDURES AND DOCUMENTATION

All significant procedures performed in the compounding area will be covered by SOPs and will be documented.

Procedures should be developed for the facility, equipment, personnel, preparation, packaging, and storage of compounded preparations to ensure accountability, accuracy, quality, safety (including access to Material Safety Data Sheets), and uniformity in compounding. Implementing SOPs establishes procedural consistency and also provides a reference for orientation and training of personnel.

Documentation enables a compounding, whenever necessary, to systematically trace, evaluate, and replicate the steps included throughout the preparation process of a compounded preparation.

#### DRUG COMPOUNDING FACILITIES

- a. Compounding facilities shall have an adequate space that is specifically designated for compounding of prescriptions. This area may include a space for the storage of equipment and materials.
- b. Sterile compounded preparations shall be compounded in accordance with the provisions in [Pharmaceutical Compounding—Sterile Preparations](#) (797), and aseptic processes shall be conducted in an area separate and distinct from the area used for the compounding of nonsterile products.
- c. The areas used for compounding shall be maintained in clean, orderly, and sanitary conditions.
- d. The areas for drug compounding shall be maintained in a good state of repair. The plumbing system shall be free of defects that could contribute to contamination of any compounded product. Adequate washing facilities shall be easily accessible to the compounding areas. Such facilities shall include, but not be limited to, hot and cold water, soap or detergent, and an air-drier or single-use towels.
- e. Potable water shall be supplied under continuous positive pressure.
- f. The area for compounding shall have adequate lighting and ventilation.
- g. The area for compounding shall be free of infestation by insects, rodents, and other vermin. Trash shall be held and disposed of in a sanitary and timely manner.
- h. Sewage and other refuse in the area of compounding shall be disposed of in a safe and sanitary manner.
- i. Bulk drugs and other chemicals or materials used in the compounding of drugs must be stored as directed by the manufacturer, or according to USP monograph requirements, in a clean, dry area, under appropriate temperature conditions (controlled room temperature, refrigerator, or freezer). The bulk chemicals shall be stored in a manner such that they are protected from contamination. All containers shall be properly labeled.
- j. If parenteral products are compounded, the compounding shall refer to [Pharmaceutical Compounding—Sterile Preparations](#) (797), and [Injections](#) (1) for compounding technique applications.

#### DRUG COMPOUNDING EQUIPMENT

(See also [Pharmaceutical Compounding—Nonsterile Preparations](#) (795).)

- a. The equipment or utensils used for compounding of a drug preparation shall be of appropriate design and capacity. The equipment should be stored in such a manner as to protect it from contamination, and shall be located in such a place as to facilitate operations for its use, maintenance, and cleaning.
- b. The equipment should be of suitable composition such that the surfaces that contact components are neither reactive, additive, nor absorptive and therefore will not affect or alter the purity of the compounded preparations.
- c. Automated, mechanical, electronic, and other types of equipment used in compounding or testing of compounded preparations should be routinely inspected, calibrated as necessary, and checked to ensure proper performance.
- d. Immediately prior to initiation of compounding operations, the equipment shall be inspected by the compounding to determine its suitability for use.
- e. After use, the equipment should be appropriately cleaned. Extra care should be used when cleaning equipment used in compounding preparations requiring special precaution, e.g., antibiotics, cytotoxins, cancer drugs, and other hazardous materials. If possible, special equipment may be dedicated for such use or if the same equipment is being used for all drug products, appropriate procedures must be in place to allow meticulous cleaning of equipment prior to use with other drugs.

#### COMPONENT SELECTION REQUIREMENTS

- a. The compounding must first attempt to use USP—NF drug substances manufactured in an FDA-registered facility.
- b. The compounding shall also first attempt to use inactive components manufactured in an FDA-registered facility.
- c. If components are not obtainable from an FDA-registered facility or if the FDA and/or the providing company cannot document FDA registration, compounders shall use their professional judgment in first receiving, storing, or using the components that meet official compendial requirements or are provided by another high quality source.
- d. If components of compendial quality are not obtainable, components of high quality such as those that are chemically pure, analytical reagent grade, American Chemical Society-certified, or Food Chemicals Codex grade may be used.
- e. When a component is not obtained from an official compendial source or is not obtainable from the sources mentioned above, the component may be obtained from a source deemed acceptable and reliable in the professional judgment of the compounding.
- f. When a component is derived from ruminant animals (e.g., bovine, caprine, ovine) the supplier shall provide written assurance that these animals were born, raised, and slaughtered in countries where bovine spongiform encephalopathy (BSE) and scrapie are known not to exist.
- g. The compounding shall not use components that are listed by FDA to be withdrawn from the market for public health reasons.
- h. Components shall be stored off the floor, handled and stored to prevent contamination, and rotated so that the oldest stock is used first.

#### PACKAGING AND DRUG PREPARATION CONTAINERS

- a. The compounding shall ensure that the containers and container closures used in packaging the compounded preparations meet the requirements under [Containers—Glass](#) (660), [Containers—Plastics](#) (661) and [Containers—Performance Testing](#) (671). The compounding shall obtain written records from the supplier to show that the containers meet USP requirements.



- b. Containers and container closures intended for compounding of sterile preparations and nonsterile preparations must be handled, sterilized (if appropriate), and stored as described in [Pharmaceutical Compounding—Sterile Preparations](#) (797) and [Pharmaceutical Compounding—Nonsterile Preparations](#) (795). The use of commercially available presterilized containers is encouraged for sterile preparations.
- c. The containers and closures shall be stored off the floor, handled and stored to prevent contamination, and rotated so that the oldest stock is used first.
  - d. The containers and container closures shall be stored in such a way as to permit inspection and cleaning of the work area.
  - e. The containers and container closures shall be made of clean materials that are neither reactive, additive, nor absorptive.
  - f. The containers and closures shall be of suitable material so as not to alter the quality, strength, or purity of the compounded drug.
- g. The compounder shall ensure that the containers and container closures selected to dispense the finished compounded prescription, whether sterile or nonsterile or radiopharmaceutical, meet the criteria in sections (a)–(f) above.

#### COMPOUNDING CONTROLS

- a. The compounder should ensure that there are written procedures for the compounding of drug products to assure that the finished products have the identity, strength, quality, and purity that they purport to have. These procedures should be available in either written form or electronically stored with printable documentation.
- b. The compounder shall establish procedures that include a description of (1) components, their amounts, the order of component additives, and the compounding process; (2) the required equipment and utensils; and (3) the drug product container and closure system.
- c. The written procedures described above shall be followed in execution of the compounding process.
- d. The compounder shall accurately weigh, measure, and subdivide as appropriate.
- e. The compounder shall check and recheck each procedure at each stage of the process to ensure that each weight or measure is correct as stated in the written compounding procedures.
- f. If a component is transferred from the original container to another container (e.g., a powder is taken from the original container, weighed, placed in a container, and stored in that other container), the new container shall be identified with the component name, weight or measure, the lot or control number, the expiration or beyond-use date, and the transfer date.
- g. The compounder should have established written procedures that will describe the tests or examinations to be conducted on the preparation compounded (e.g., the degree of weight variation among capsules) to assure uniformity and integrity of compounded drug preparations.
- h. Appropriate control procedures should be established to monitor the output and to validate the performance of those compounding processes that may be responsible for causing variability in the final compounded preparations. Factors that may cause variability include (1) capsule weight variation; (2) adequacy of mixing to assure uniformity and homogeneity; and (3) clarity, completeness, or pH of solutions.
- i. Appropriate written procedures should be designed to prevent microbiological contamination of compounded drug preparations purporting to be sterile, and these procedures shall be followed. Such procedures shall include validation of sterilization processes (see [Pharmaceutical Compounding—Sterile Preparations](#) (797)).
- j. The compounder should establish appropriate beyond-use dates determined either from available USP–NF monographs, appropriate testing, or from peer-reviewed literature.
- k. The compounder should adopt appropriate storage requirements as provided in Preservation, Packaging, Storage, and Labeling under General Notices and Requirements.

#### LABELING

1. The compounder's preparation label should contain information required by state and federal law and accepted standards of practice. [notes—(a) The compounder shall use the established name or distinct common name (cannot use the trademarked name of a manufactured product). (b) The compounder cannot indicate that the compounded product is therapeutically equivalent to a manufactured product. (c) The label should state that this is a compounded preparation. (d) The compounder shall not use an NDC number assigned to another product.]
  2. The compounder shall label any excess compounded products so as to reference them to the formula used, the assigned control number, and beyond-use date based on the compounder's appropriate testing, published data, or USP–NF standards.
  3. Preparations compounded in anticipation of a prescription prior to receiving a valid prescription should not be prepared in an inordinate amount. A regularly used amount should be prepared on the basis of a history of prescriptions filled by the pharmacy. These preparations should be labeled or documentation referenced with the following:
    - a. A complete list of ingredients or preparation name and reference or established name or distinct common name
    - b. Dosage form
    - c. Strength
    - d. Preparation date
    - e. Name and address of compounder
    - f. Inactive ingredients
    - g. Batch or lot number
    - h. Assigned beyond-use date, based on published data, or appropriate testing, or USP–NF standards.
- Storage conditions for these preparations should be dictated by their composition and sterility, e.g., stored in a clean, dry place under appropriate temperature conditions (controlled room temperature, refrigerator, or freezer.)
4. The compounder should examine the preparation for correct labeling after completion of the compounding process.

#### RECORDS AND REPORTS

- a. The compounder shall maintain records, including but not limited to, the hard copy of the prescription to indicate that the prescription is compounded, and to provide formulation records and compounding records.
- b. The compounder shall keep adequate records of controlled drug substances (scheduled drugs) used in compounding.
- c. All records of all compounded preparations shall be kept for a period of time as set forth in the federal and state laws or regulations. Such records shall be readily available for authorized inspection.
- d. The compounding records shall include the manufacturer and lot number of all ingredients.

#### COMPOUNDING FOR A PRESCRIBER'S OFFICE USE

- a. Compounds may prepare compounded drug preparations for a prescriber's office use only where permitted by federal and state requirements.
- b. An order by the prescriber indicating the formula and quantity ordered may be filed in the compounder's facility.
- c. Where compounding for office use is permitted, the compounder shall compound the preparation for the sole purpose of administration by or for the prescriber.
  - d. A record of the compounding process shall be maintained.
  - e. A label must be generated and a number may be assigned.

#### COMPOUNDING VETERINARIAN PRODUCTS

- a. Compounds shall compound prescriptions for animals on the basis of prescription orders.
- b. These prescriptions shall be handled and filled according to the guideline available for compounding of veterinarian products.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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## BACKGROUND

Many of the principles in this general information chapter are derived from an international guidance on the extent and point of application of appropriate good manufacturing practice principles. It is intended to assist excipient manufacturers in determining whether the methods used in, and the facilities and manufacturing controls used for, production adequately ensure that an excipient possesses the quality, purity, safety, and suitability for use that it purports to possess.

The principles and information in this chapter can be applied to the manufacture of all bulk pharmaceutical excipients (referred to throughout this document as "excipient(s)") intended for use in human drugs, veterinary drugs, and biologics. It covers the quality systems and the extent of good manufacturing practices necessary throughout the chain of production up to and including delivery to customers. As an international guidance document, it does not provide information for all national legal requirements nor cover in detail the particular characteristics of every excipient. The quality system standard used as a framework for this chapter is ISO 9002, which is appropriate to manufacturing. Information specific to excipients has been added. Because of the diversity of excipients, some principles in this information chapter may not be applicable to certain products and processes.

This information chapter combines existing governmental regulatory good manufacturing practices principles and international quality management system requirements as developed by The International Organization for Standardization (ISO). In view of the increasing globalization of the pharmaceutical industry and the harmonization of pharmaceutical registration requirements, deference to both schemes is becoming necessary. Therefore, relevant portions of both manufacturing concepts are employed throughout this chapter.

The General Guidance section provides an overview of the appropriate manufacturing practices criteria applicable to excipient manufacture and the point of application of excipient good manufacturing practices and quality systems. This section also recommends measures to limit contamination of an excipient and provides the relationship of excipients to finished dosage forms. For a list of terms and their definitions used in this information chapter, see [Appendix 1](#). The section Excipient Quality Systems provides information on the requirements necessary for compliance with relevant good manufacturing practice principles and implementation of an excipient quality system. Information for production facility requirements are included under Process Control. No attempt has been made to include details specific to particular excipients. The information under [Appendix 2](#), General Auditing Considerations, sets forth key criteria to aid in the audit of an excipient manufacturing facility.

## GENERAL GUIDANCE

International regulations governing drugs require that components of the drugs be manufactured, processed, packed, and held in accordance with good manufacturing practices. Unlike other pharmaceutical products and components, until now there was no guidance that specifically addressed the manufacture of bulk pharmaceutical excipients.

Excipients are substances, other than the active drug substance or finished dosage form, that have been appropriately evaluated for safety and are included in drug delivery systems 1) to aid in the processing of the drug delivery system during its manufacture; 2) to protect, support, or enhance stability, bioavailability, or patient acceptability; 3) to assist in product identification; or 4) to enhance any other attribute of the overall safety, effectiveness, or delivery of the drug during storage or use.

The application of good manufacturing practices to excipients is relevant when it is determined that a chemical is intended for use as a component of a drug product. Excipient manufacture should be carried out in accordance with the manufacturing practice concepts consistent with the information in this chapter. The objective of excipient good manufacturing practices is to ensure that excipients are manufactured with the appropriate quality characteristics.

Excipients generally are manufactured on a large scale, which means that the use of automated process controls and continuous stream processing are more likely to be utilized. Production equipment and operations will vary depending on the type of excipient being produced, the scale of production, and the type of operation (e.g., lot or batch versus continuous). The use of automated equipment is appropriate when adequate inspection is conducted and calibration and maintenance procedures are followed.

Manufacturing practice requirements increase as the process progresses. At some logical processing step, usually well before the final finishing operation, appropriate manufacturing practices should be imposed and maintained throughout the remainder of the process. To determine the processing step at which these manufacturing practices should be implemented, good judgment and a thorough knowledge of the process are required. A detailed process flow should identify the unit operations, equipment used, stages at which various substances are added, key steps in the process, critical parameters (time, temperature, pressure, etc.), and monitoring points.

ISO 9000 series is a quality system standard of general application that can be applied to cover every aspect of manufacturing to the benefit of both the manufacturer and customer. It has taken several years since its introduction in 1987 for the ISO 9000 series to be utilized worldwide. There is no current regulatory requirement in Europe, Japan, or the United States for third party certification. A manufacturer may apply the standard with or without certification. However, certification has the benefit of providing assurance to customers that conformance to this quality system has been independently confirmed. Incorporation of GMP requirements into the ISO 9000 quality system enhances not only the quality system, but a company's operational procedures as well. Final dosage formulators worldwide increasingly regard compliance with ISO 9002 as an essential qualification for their suppliers.

Obtaining certification is a business decision and is not discussed in this general information chapter.

## Excipient Purity

The processes used for the production of bulk pharmaceutical excipients and those used for the production of bulk pharmaceutical chemicals are similar. Both can be manufactured by chemical synthesis, recombinant DNA technology, fermentation, enzymatic reactions, recovery from natural materials, or any combination of these processes. Impurities, contaminants, carriers, vehicles, inert ingredients, diluents, or unwanted crystalline or molecular forms may be present in the raw materials. Therefore, the starting materials for excipients may not be required to be manufactured in accordance with the manufacturing practices specified in this chapter because often the starting materials (or their derivatives) undergo significant chemical change and physical modification or blending, with the result that many of the impurities present in the starting materials are removed. The ultimate manufacturing objective is purification and physical or chemical alteration, which is accomplished by various chemical, physical, or biological processing steps. The effectiveness of these steps is confirmed by chemical, biological, and physical testing of the excipient. Excipients, once synthesized or isolated, normally undergo additional, extensive purification during manufacture.

Many excipients have applications other than for pharmaceutical uses and are used in food, cosmetics, or industrial products. Thus, environmental conditions, equipment, and operational techniques employed in excipient manufacture often reflect the chemical industry rather than the pharmaceutical industry. Many chemical processes have the potential to produce toxic impurities from side reactions. Therefore, careful process control may be essential. Also, the manufacturing environments may contain deleterious substances. However, chemical processes used to manufacture excipients are either performed in closed systems that afford protection against such contamination—even when the reaction vessels are not enclosed in buildings—or else these processes are in environments that must be controlled.

It is important that manufacturers identify and set appropriate limits for impurities. These limits should be based upon appropriate toxicological data, or limits described in national compendia as requirements, as well as sound manufacturing practice considerations. Manufacturing processes should be adequately controlled so that the impurities do not exceed such established specifications.

## Excipients in Finished Dosage Forms

The formulator of finished dosage forms is highly dependent on the excipient manufacturer to provide bulk pharmaceutical excipients that are uniform in chemical and physical characteristics. This is particularly important in the context of the product approval process where bioequivalency comparisons are made between pivotal clinical biobatch production and commercial scale-up lots or batches. To provide adequate assurance of drug product performance, the excipient used to manufacture commercial lots or batches should not significantly differ from those used in biobatches. Where significant differences do occur, additional testing by the manufacturer of finished dosage forms may be required to establish the bioequivalence of the finished product. It remains equally important to ensure that the bioequivalence of subsequent, post-approval commercial lots or batches of drug product is



not adversely affected over time.

In general, excipients are used as purchased. Consequently, impurities present in the excipient will be present in the finished dosage form. While manufacturers of dosage forms may have limited control over excipient quality through specifications, the excipient manufacturer has greater control over physical characteristics, quality, and the presence of impurities in the excipient.

Excipients are used in different types of dosage forms where physical characteristics, such as particle size, may be important. While it is primarily the responsibility of the manufacturer of finished dosage forms to identify the particular physical characteristics needed, it is the responsibility of the excipient manufacturer to adequately control processes to ensure the excipient's consistent conformance to specifications. The excipient's end use should be identified and considered during inspection of excipient manufacturers' facilities.

Particularly important is whether the excipient is a direct component of a drug dosage form, whether the excipient will be used in the preparation of a sterile dosage form, or whether the excipient is represented as pyrogen free. The excipient manufacturer is responsible for ensuring that excipients are pyrogen free if the manufacturer makes such a representation in specifications, labeling, contractual agreement, or a Drug Master File (DMF).

#### EXCIPIENT QUALITY SYSTEMS

The information described below can be used as the basis for a quality system in the manufacture of excipients. Procedures that are utilized in the manufacture and control of excipients should be written. Conformance to those procedures should be documented. A quality manual is a documented base and is intended to describe the quality policy and the commitment of the supplier to quality. The procedural system should have adequate formal controls related to procedure approval, revision, and distribution. These controls should provide assurance that the proper version of a procedure is being utilized throughout the operation.

##### Management and Employee Responsibility

**Quality Policy**— Management should demonstrate commitment to a quality policy that should be implemented within the operational unit. Management should participate in the development of the company's quality policy and provide the resources necessary for development, maintenance, and review of such policy and quality system at least annually. Management should be committed to this policy and should appoint appropriate company personnel to be responsible for coordination and implementation of the quality system.

**Organization**— There should be a quality unit, independent of production, that has the responsibility and authority to approve or reject all components, in-process materials, packaging materials, and finished excipients. The quality unit should have the authority to review production records to ensure that no errors have occurred or, if errors have occurred, that they have been fully investigated. The quality unit should be responsible for approving or rejecting excipients manufactured, processed, packaged, or held under contract by another company. The quality unit can delegate these responsibilities if proper controls, such as periodic audits and documentation of training, are in place. Adequate laboratory facilities for the testing and approval or rejection of raw materials, packaging materials, in-process materials, and finished excipients should be available to the quality control unit.

It is the responsibility of an independent unit, usually the quality assurance group, which is independent of production, to participate in issuing procedures, authorizing changes to processes, specifications, procedures, and test methods and in investigating failure and complaints.

An organization chart by function should be available showing interdepartmental relationships as well as relationships to the management of the company. As a minimum, all quality assurance, quality control, production maintenance, and engineering functions should have clear job descriptions.

##### Personnel—

**Employee Responsibility**— Employees engaged in the manufacture, processing, packaging, or holding of an excipient should wear clean clothing appropriate for the duties they perform. Protective apparel, such as head, face, hand, and arm coverings, should be worn as necessary to protect excipients from contamination. Only employees authorized by supervisory personnel should enter those areas of the buildings and facilities designated as limited-access areas.

Employees should practice good sanitation and health habits. Any person shown at any time (either by medical examination or supervisory observation) to have an apparent illness or open lesions that may adversely affect the safety or quality of excipients should be excluded from direct contact with components, excipient containers and closures, in-process materials, and finished excipients until the condition is corrected or determined by competent medical personnel not to jeopardize the safety or quality of the excipients. All employees should be instructed to report to supervisory personnel any health conditions that may have an adverse effect on excipients.

**Other Requirements**— There should be an adequate number of qualified personnel to perform and supervise the manufacture, processing, packaging, or holding of each excipient in a manner consistent with the information in this guide. Management should establish adequate and continued good manufacturing practices and personal hygiene training for all employees handling products so that they understand the precautions necessary to prevent the contamination of excipients.

**Consultants**— Consultants advising on the manufacture, processing, packaging, or holding of excipients should have sufficient education, training, and experience, or any combination thereof, to advise on the subject for which they are retained. Records should be maintained listing the name, address, and qualifications of any consultants and the type of service they provide.

**Technical Assistance**— The excipient manufacturer should establish and maintain procedures for providing such technical assistance as may be required.

##### Manufacturer and User Responsibilities

**Contract Review**— The manufacturer and user should mutually agree upon the excipient specifications. The manufacturer must have the facility and process capability to consistently meet the mutually agreed upon specifications of the excipient(s). Subcontracting or significant changes to a supplier's audited process that could affect the physical, chemical, or functionality of the excipient in a final dosage form should be immediately communicated or pre-approved as mutually agreed upon between customer and supplier.

**Document and Data Control**— The excipient manufacturer should have a system to control all documents and data that relate to the requirements of the quality system. When these documents were issued and where they are located should be recorded. To identify the most recent document, each document should include a unique identifier, date of issue, and revision number on each page. The issuing department also should be identified. All changes, where practical, and the reasons for the change should be documented. Documents and subsequent changes to the documents should be reviewed and approved by designated qualified personnel before issuance to the appropriate areas identified in the documents.

**Purchasing**— The purchaser should verify that the supplier of raw materials, components, and services for the manufacture of excipients has the capability to consistently meet the agreed-upon requirements. This may include periodic audits of the vendor's plant, if deemed necessary. Purchasing agreements should contain data clearly describing the product ordered, including where applicable, the following:

- The name, type, class, style, grade, item code number, or other precise identification traceable to the raw material specification.
- Drawings, process requirements, inspection instructions, and other relevant technical data, including requirements for approval or qualification of product, procedures, process equipment, and personnel.

These requirements also apply to selection and control of subcontractors. Subcontractors include toll manufacturers and contract laboratories.

**Control of Customer Supplied Products**— The manufacturer should establish and maintain procedures for verification, storage, and maintenance of customer supplied products intended for incorporation into the customer's excipients. Verification by the manufacturer does not relieve the customer of the responsibility to provide an acceptable product. Any product that is lost, damaged, or is otherwise unsuitable for use should be recorded and reported to the customer. In this case, procedures should be in place for acceptable disposition and replacement of the product.

**Product Identification and Traceability**— All items, from the received raw materials, through the in-process goods, to the finished products, should be clearly identified and traceable through a documented system. The system should allow the traceability of product upstream and downstream. Identification of raw materials used in the production of processed materials should be traceable using a batch numbering system or any other appropriate system. The finished product should be traceable to the customer and retrievable in case of the need for a product recall.

**Labeling**— Labeling requirements for excipient packages are subject to applicable national and international regulatory requirements, which may include transportation and safety measures. Procedures should be employed to protect the quality and purity of the excipient when it is packaged, and to ensure that the correct label is applied to all containers. A good system of labeling should have, at a minimum, the following features: the name of product; the manufacturer and distributor; a lot or batch number from which the complete lot or batch



...story can be determined; a file of master labels [note—A designated individual should review incoming labels or labels printed on demand against the appropriate master labels], storage of labels in separate containers or compartments to prevent mix-ups; formal issuance of labels by requisition or other document; issuance of an exact number of labels, sufficient for the number of containers to be labeled, retention copies, and calculated excesses, if any; reconciliation of the number of labels issued with the number of unit packages and retention labels, together with the destruction of excess labels bearing lot or batch numbers; and avoidance of labeling more than one lot or batch at a time without adequate separation and controls.

In instances where excipients are labeled on the packaging line, packaged in pre-printed bags, or bulk shipped in tank cars, there should be documentation of the system used to satisfy the intent of the above requirements.

If the need for special storage conditions exists (e.g., protection from light, heat, etc.), such restrictions should be placed on the labeling.

**Retained Samples**— Reserve samples of an excipient should be retained for one year after the expiration or re-evaluation date, or for one year after distribution is complete, whichever is longer. Sample size should be twice the amount required to perform specification testing.

#### Process Control

**Buildings and Facilities**— Any building or buildings used in the manufacture, processing, packaging, or holding of an excipient should be of suitable size, construction, and location to facilitate cleaning, maintenance, and proper operations.

**Cross-contamination Prevention**— Cross-contamination should be a consideration in the design of the manufacturing process and facility. The minimization of the degree of cross-contamination should be dependent on the safety and intended use of the excipient.

It is expected that the degree of precautions taken in minimizing cross-contamination be appropriate to the conditions of the manufacturing facility. Where two different grades of the same excipient are manufactured in the same building or the same equipment, a trace carryover from the production of the previously produced grade to the present production may occur. This can be considered acceptable if shown that the extent of commingling does not change the functionality and safety of the excipient.

When the excipient product is initially recovered, it should be in a clean environment and not exposed to airborne contaminants such as dust, other excipients, or industrial chemicals. The primary consideration is that the building and facilities be designed so that operations performed within do not contribute to an actual or potential contamination of the excipient.

**Air Handling**— Excipient plant air handling systems should be designed to prevent cross-contamination. For dedicated areas processing the same excipient, it is permissible to recycle a portion of the exhaust air back into the same area. The adequacy of such a system of operation for multi-use areas, especially if several products are processed simultaneously, should be carefully analyzed.

In multi-use areas where several products are completely confined in closed vessels and piping systems, the extent of filtration of the supply air (combined fresh make-up air and recycled air) is acceptable if the conditions are consistent with other existing regulations (e.g., environmental, safety). However, there should be data to demonstrate adequacy of the air handling system. Where for process reasons an inert gas is needed, these same rules should apply.

**Cleaning and Sanitary Conditions**— Adequate cleanliness is an important consideration in the design of excipient manufacturing facilities. Any building used in the manufacture, processing, packaging, or holding of an excipient should be maintained in an appropriately clean and sanitary condition. There should be written procedures assigning responsibility for sanitation and describing in sufficient detail the cleaning schedules, methods, equipment, and materials used in cleaning the buildings and facilities; such written procedures should be followed and periodically reviewed. Conformance should be documented.

All buildings should be free of infestation by rodents, birds, insects, and other vermin. Waste should be held and disposed of in a timely and appropriate manner. However, many starting materials, particularly botanicals, may have some unavoidable contamination, such as rodent or other animal filth or infestation. The manufacturer should have sufficient control methods to prevent the increase of such contamination or infestation in holding areas or its spread to other areas of the plant.

**Other Facility Concerns**— Any building used in the manufacture, processing, packaging, or holding of an excipient should be maintained in a good state of repair. The following items are of particular concern:

**LIGHTING**— Adequate light should be provided in all areas.

**PLUMBING**— Water that comes in contact with an excipient should be supplied under continuous positive pressure in a plumbing system free of defects that could contribute contamination to the excipient. Drains should be of adequate size and, where connected directly to a sewer, should be provided with an air break, or other mechanical device, to prevent back-siphoning.

**WASHING AND TOILET FACILITIES**— Adequate washing facilities should be provided, including hot and cold water, soap or detergent, air dryers or single service towels, and clean toilet facilities easily accessible to working areas.

**Equipment**— Equipment used in the manufacture, processing, packaging, or holding of an excipient should be of appropriate design, adequate size, and in a suitable location to facilitate its operation, cleaning, and maintenance.

**Outside Equipment**— Some fermentation tanks, reaction vessels, and certain other equipment may not be situated within a building, and a considerable amount of processing may occur out-of-doors. Such processing is acceptable provided it occurs in a closed system.

**Multipurpose Equipment**— Many excipients are produced using multipurpose equipment. With few exceptions, such multiple usage is satisfactory provided the equipment can be adequately cleaned according to validated written procedures. The program should take into consideration the need for different cleaning procedures, depending on the safety considerations of the product or intermediate and what product or intermediate was previously produced. Products that leave residues that cannot be easily removed should be produced in dedicated equipment.

Where multipurpose equipment is in use, it is important to be able to determine previous usage when investigating cross-contamination or the possibility of such contamination. An equipment cleaning and use log, while desirable and perhaps preferable, is not the only method of determining prior use. Any documentation system that clearly identifies the previous lot or batch and shows that the equipment was cleaned is acceptable.

Cleaning and disinfection procedures should be properly established by competent personnel using the model product approach, when applicable. These procedures should be designed to meet or exceed the particular needs of the product and process involved and be set down in a written schedule available for the guidance of employees and management. An effective and regular cleaning program should be put in place to remove product residues and dirt, which may also contain microorganisms and act as a source of contamination.

The supplier should demonstrate the effectiveness and efficiency of the cleaning and disinfection procedures for each piece of equipment, and the cleaning status of equipment should be recorded. Validation data should prove that the cleaning procedure is acceptable. An evaluation should consider the potential impact that traces of contaminant may have on the product supplied to the customer. All equipment that has been in contact with contaminated material should be thoroughly cleaned and disinfected before coming in contact with an excipient.

**Controlled Environment**— A controlled environment may be necessary to avoid microbial contamination or degradation caused by exposure to heat, air, or light. The degree of protection required may vary depending on the stage of the process. Equipment should be designed to minimize the possibility of contamination caused by direct operator contact in such activities as the unloading of centrifuge bags, use of transfer hoses (particularly those used to transfer powders), and the operation of drying equipment and pumps. The sanitary design of transfer and processing equipment should be evaluated. Those with moving parts should be assessed in regard to the integrity of seals and packing materials to avoid their contaminating the product.

Special environments required by some processes should be monitored at all times to ensure product quality (e.g., inert atmosphere, protection from light). Where inert atmosphere is required, the gas should be treated as a raw material. If interruptions in a special environment occur, adequate evidence and appropriate rationales should be documented to show that such interruptions have not compromised the quality of the excipient. Such environmental concerns become increasingly important after purification of the excipient has been completed.

**Construction**— Process equipment should be constructed so that their contact surfaces will not be reactive, additive, or absorptive so as to alter the quality attributes of the excipient. Substances required for operation, such as lubricants or coolants, should not come into contact with components, excipient containers, closures, in-process materials, or finished



excipients. Materials acceptable for food grade use should be used where product exposure or contamination is possible.

Maintenance— Equipment and utensils should be maintained and sanitized (where necessary) at appropriate intervals to prevent malfunctions or contamination that would alter the standards and characteristics of the excipient beyond the official, or otherwise established, requirements. Written procedures should be established and followed for maintenance of critical equipment, including utensils, used in the manufacture, processing, packaging, or holding of the excipient. Records should be kept of preventive maintenance of equipment and utensils, a description of the maintenance performed, and the batch or lot number of the excipient that was present in the equipment before and after the activity.

These records can be in the form of a log, computer database, or other appropriate documentation, provided the system can properly identify who was responsible for performing each function.

Water Systems and Water Quality— Potable water may be used in the production of excipients, provided that established water quality standards are consistent with regulatory requirements for source drinking water. If the manufacturer specifies water of pharmacopeial quality in the specifications or DMF, the water should meet the particular pharmacopeial standards. Data from periodic testing should be available to show compliance with chemical and microbiological standards, including freedom from pathogenic organisms. Data need not be generated by the manufacturer if such data are available from municipal water authorities.

While drinking water is used for many excipient processes, Purified Water is also widely used in the manufacture of excipients. Because of the well-recognized potential for microbial growth in deionizers and ultrafiltration or reverse osmosis systems used to produce Purified Water, such systems should be properly validated and controlled.

Proper control methods include the establishment of water quality specifications and corresponding action levels, remedial action when microbial levels are exceeded, and adequate maintenance procedures such as regeneration and sanitation or sterilization.

Appropriate specifications for chemical and microbial quality should be established and periodic testing conducted. Such specifications will vary depending on the process and the point in the process when the water is used. The water quality standards should reflect the intended use of the excipient. The frequency of microbial and chemical testing of Purified Water is dependent upon a variety of factors, including the test results and the point in the process (e.g., final wash in centrifuge) at which such water is used.

Similar principles to those discussed above for Purified Water apply to Water For Injection used in sterile and pyrogen-free excipient processing. The water for injection (WFI) system should be monitored for microorganisms, and the validation data and reports of monitoring should be reviewed as is required for finished dosage forms.

Most purified and WFI water systems, including ultrafiltration and reverse osmosis systems, have the potential for the development of endotoxins. If the final excipient product purports to be pyrogen free or sterile, or will be used in preparing parenteral products, validation of the system to control endotoxins should be conducted and routine testing of the process water for endotoxins should be performed (preferably by the LAL method).

Aseptic and Sterile Manufacturing— The manufacture of sterile excipients for use in aseptic or sterile processing presents technical challenges. Because humans are the primary source of contamination in an aseptic operation, the process should be designed to eliminate this direct contact. Those aseptic excipient operations that utilize considerable operator involvement should have adequate controls.

The excipient manufacturer should document the sanitizing of critical processing equipment. Processes used for the sterilization of equipment should be validated. The manufacturer also should verify that no chemical interaction with the product occurs.

There are guidelines and compliance programs that provide detailed guidance for the manufacture of sterile products. These documents should be reviewed in association with sterile excipient manufacturing inspections.

Validation of Process and Control Procedures— Excipient manufacturers are expected to adequately determine and document that all significant processing steps are performed consistently. The type of excipient, the breadth of the specification relative to the degree of process control, and other factors determine the extent of the process development and documentation required.

An important factor in the assurance of product quality includes the adequate design and control of the manufacturing process because product testing alone is not sufficient to reveal variations that may have occurred. Each step of the manufacturing process should be controlled, to the extent necessary, to ensure that the excipient meets established specifications. The concept of process validation is a key element in assuring that these quality assurance goals are met. Documentation describing the process reactions, operating parameters, purifications, impurities, and key tests needed for process control should be written, thus providing the basis for validation.

Many manufacturers already possess the data necessary to validate that their processes perform in a consistent manner. For example, limitations of a reaction or purification step are usually identified in the development phase. Known impurities and tests used to determine their levels are also established at this phase. Thus, when the process is scaled up to production of a lot or batch size, a comparison can be made with development lots or batches. Scale-up and development reports, along with purity profiles, would constitute an appropriate validation report.

Stability— While many excipient products are very stable and may not require extensive testing to ensure stability, the stability of excipients is an important contributing factor to the stability of the finished dosage form. The stability of excipients may be affected by undetected changes in raw material specifications or subtle changes in manufacturing procedures. Excipient products also may be shipped in a large variety of different packaging types that can affect their stability (e.g., drums that are metal and plastic, bags and bottles that are plastic or glass, tank cars, etc.).

Some excipients may be available in different grades (i.e., various molecular weights of a polymer or different monomer ratios, etc.) or some may be mixtures or blends of other excipients. These excipients may be very similar to others within a product group. Minor quantitative differences of some of the components may be the only significant variation from one product to another. For these types of excipients, a model product approach may be appropriate to assess the stability of similar excipients. Stability studies would involve the selection of several model products that would be expected to simulate the stability of the product group being assessed. This election should be based on scientifically sound theories.

Data from stability studies of these model products can be used to determine theoretical stability for similar products.

There should be a documented testing program designed to assess the stability characteristics of excipients. The results of such stability testing should be used in determining appropriate storage conditions and their re-evaluation or expiration dates. The testing program should be ongoing and should include the following: the number of lots per year, sample size, and test intervals; the storage conditions for samples retained for testing; those test methods that are necessary to indicate stability; and a simulation of containers and storage time equivalent to those conditions in the marketplace, if possible.

For excipients that have been in the market for a long time, historical data may be used to assign the shelf life and storage conditions. The testing program can also be modified based on available historical data.

Expiration Dating and Re-evaluation— If testing indicates a short shelf life under anticipated storage conditions, the excipient should either be labeled with an expiration date or be re-evaluated at appropriate intervals to determine its continued suitability for use. If the excipient has been in the market for a long time, the expiration date or its re-evaluation must be derived from appropriate stability testing or from historical data. With few exceptions, expiration dates are not presently considered to be a general requirement for all excipients. Thus, the absence of an expiration date is not objectionable.

Process Changes (Change Control)— The excipient manufacturer should establish and maintain written procedures for the identification, documentation, appropriate review, and approval of changes within the production processes. An independent group (such as regulatory affairs, quality assurance, etc.) should have the responsibility and authority for the management and final approval of changes. Significant operational changes should be based on validated excipient studies. The effect of the changes should be communicated to both internal and external customers.

Lot or Batch Production Records— There is increased use of computer systems to initiate, monitor, adjust, and otherwise control manufacturing processes. These operations may be accompanied by recording charts that show key parameters (e.g., temperature) at suitable intervals, or even continuously throughout the process. In other cases, key measurements (e.g., pH) may be momentarily displayed on a monitor screen but not available in hard copy. In both cases, conventional hard-copy lot or batch production records such as those showing the addition of ingredients, actual performance of operations by identifiable individuals, and other information usually seen in conventional records may not be generated.

Therefore, documentation of the excipient manufacturing process should include a written description of the process and production records.

As a practical matter, when computers and other sophisticated equipment are employed, the following considerations are essential: systems and procedures that show the equipment



and software is in fact performing as intended; checking and calibrating the equipment at appropriate intervals; retention of suitable backup systems, such as copies of the program and files; and assurance that changes in the program are documented, validated, and made only by authorized personnel.

In-Process Blending or Mixing— In-process blending or mixing to ensure batch uniformity or to facilitate processing is acceptable provided it is adequately controlled and documented.

In order to ensure batch uniformity, homogeneous mixing of all materials, to the extent feasible, and reproducibility from batch to batch is essential. Blending of batches or lots that individually do not conform to specifications with other lots that do conform (to salvage or hide adulterated material) is not an acceptable practice.

In-process blending or mixing, which is performed to facilitate processing, includes the use of holding tanks, reprocessing, and repeated crystallizations. Incidental carryover is another type of in-process mixing that frequently occurs and is usually acceptable because cleanup between successive lots or batches of the same excipient is not normally required to maintain quality levels during a production operation.

Solvents, Mother Liquors, and Second Crops— Many excipients are extracted from, or purified by, the use of organic solvents. These solvents are normally removed by drying the moist excipient. It is important that excipient specifications include tests and limits for residues of solvents.

Solvents may be recovered and reused in the same process or different processes provided that the recovered solvents are shown to meet appropriate standards prior to reuse or commingling with other approved material. Mother liquors or filtrates containing recoverable amounts of excipients, reactants, or intermediates are frequently reused. Recovery procedures for such excipients are acceptable if the recovered excipient meets its specifications and recovery procedures are indicated in lot or batch production records. Recovery procedures for reactants and intermediates are acceptable if the recovered materials meet suitable specifications.

#### Inspection and Testing

Testing programs should include the establishment of scientifically sound and appropriate specifications, standards, sampling plans, and test procedures designed to ensure that components, containers, closures, in-process materials, labeling, and finished excipients conform to established standards.

It is not appropriate to retest a sample, which previously gave a nonconforming test result, and to release the material based solely on the retest results. This would only be appropriate if it is demonstrated that the original test result was erroneous based on a documented investigation. Otherwise, statistical treatment of all the test data, including both the original and retest, should be used to justify release of the lot. These same principles apply when the representative of the sample is suspect. The excipient manufacturer should establish procedures for identifying adequate statistical techniques that may be required for verifying the acceptability of process capability and product characteristics.

Test Status— There should be a system to identify the inspection status of all materials including raw materials, intermediates, and finished products. While storing materials in properly identified locations is preferred, any means which clearly identifies the test status is satisfactory.

Upon receipt, raw materials should be placed in quarantine status and should not be used prior to acceptance. Effective quarantine can be established with suitable identifying labels or signs, or validated documentation systems. With increasing frequency, quarantine and documentation is widely accomplished with a computer system in lieu of a physical stock control system. This is acceptable provided that system controls are adequate to prevent use of unreleased material.

Raw Material Testing— Raw materials should be tested or otherwise verified prior to use. Verification should include a supplier certificate of analysis and, wherever feasible, at least an identification test. There should be clear guidelines established for the approval of each raw material.

Raw material specifications should be written documents, even if only minimal requirements are specified. Such specifications will vary in depth and sophistication, depending on various factors such as the critical nature of the raw material, its function in the process, the stage of manufacture, and the hazards associated with sampling. The specifications should be organized to separate those tests that are routine from those that are performed infrequently or only for new suppliers.

Raw materials are usually subjected to an identity test and additional testing to determine if they meet appropriate specifications. Laboratory controls should include a comprehensive set of meaningful analytical procedures designed to substantiate that the raw materials meet established specifications. Some raw materials may not be acceptance tested by the manufacturer because of the hazards involved. This is acceptable where there is a reason based on safety or other valid considerations. In such a circumstance, assay certification for each lot from the vendor should be on file. There should always be some evidence of an attempt by the excipient manufacturer to establish identity, even if it is only a visual examination of containers, examination of labels, and recording of the lot or batch numbers from the labels.

In-Process Testing— Excipients are normally subject to various in-process tests to show that a manufacturing process is proceeding satisfactorily. Such tests often are performed by production personnel in production laboratory facilities. Approval to continue with the process is often issued within the production department. The important considerations are that specified tests are performed by trained personnel and recorded, and that results are within specified limits.

In-process inspection and testing should be performed based upon monitoring the process or actual sample analysis at defined locations and times. The results should conform to established process parameters or acceptable tolerances. Work instructions should delineate the procedure to follow and how to utilize the inspection and test data to control the process.

Finished Product Testing and Release— Finished product testing should be performed by the quality unit and should conform to written specification. There should be a procedure that ensures that appropriate manufacturing documentation, in addition to the test data, is evaluated prior to release.

All appropriate records relating to inspection and testing should be available for review. Where the process is continuously monitored, acknowledgment that the process was monitored and the results of the monitoring should be available.

Control of Nonconforming Product— Any raw material, intermediate, or finished excipient found not to meet specifications should be clearly identified and segregated to prevent inadvertent use or release for sale. A record of nonconforming product should be maintained. All incidence of nonconformance should be investigated to identify the root cause. This investigation should be documented and corrections made to prevent recurrence of the problem.

Procedures should exist for the evaluation and fate of nonconforming products. Nonconforming product should be reviewed in accordance with documented procedures to determine its final outcome. The nonconforming product may be reprocessed or reworked to meet the specified requirements and then accepted with agreement by the customer, regraded for alternative applications, or destroyed.

Returned Excipient Products— Returned excipient products should be identified as such and held. If the conditions under which the products have been held, stored, or shipped before and during return, or if the condition of the container casts doubt about its safety, identity, strength, quality, or purity, the product should be destroyed unless examination, testing, or other investigations prove that the excipients meet appropriate standards of safety, quality, or purity.

Records of returned products should include the name and lot number (or control batch number), reason for the return, quantity returned, date of disposition, and ultimate fate of these products. Procedures for the holding, testing, and reprocessing should be written and followed.

Corrective and Preventive Actions— The supplier should establish, document, and maintain procedures for

- investigating the cause of nonconforming product, returns, and complaints along with the corrective action needed to prevent recurrence;
- analyzing all processes, work operations, concessions, quality records, and service reports to detect and eliminate potential causes of nonconforming product;
  - initiating preventive actions to deal with problems to a level corresponding to the risks encountered;
  - applying controls to ensure that corrective actions are taken and that they are effective; and
  - implementing and recording changes in procedures resulting from corrective action.

Reprocessing or Reworking— Reprocessed or reworked product should be re-inspected in accordance with documented procedures. Reprocessing or reworking of an excipient may be acceptable. However, merely relying on final testing of the reprocessed excipient as a means of demonstrating compliance to specification and neglecting the investigation and evaluation of the manufacturing process is unacceptable.

Reprocessed material should also be evaluated and documented to ensure that the lot or batch will conform with all established standards, specifications, and characteristics equivalent to those of the original material. There should be a sufficient investigation, evaluation, and documentation to show that the reprocessed excipient is at least equivalent to other acceptable products and that the nonconformance did not result from an inadequate process. If the need for reprocessing resulted from human error, it may indicate other deficiencies, such as inadequate training or work instructions.



processing or rework that is not a normal part of the validated process should not be performed without the review and approval of an independent group such as regulatory affairs or quality assurance, etc.

#### Inspection, Measuring, and Test Equipment

Calibration of all in-process instruments identified as quality instruments, as well as test equipment used in the laboratory, should be traceable to recognized standards. Laboratory instruments, such as spectrometers, viscosimeters, and other apparatus, as well as reagents, buffer solutions, and standard solutions would be included.

The control program should include the standardization or calibration of reagents, instruments, apparatus, gauges, and recording devices at suitable intervals in accordance with an established written program containing specific directions, schedules, limits for accuracy and precision, and provisions for remedial action in the event accuracy or precision limits are not met. Reagents, instruments, apparatus, gauges, and recording devices not meeting established specifications should not be used.

Computer systems used to verify that the product conforms to specifications should be audited to ensure satisfactory performance.

#### Handling, Storage, Preservation, Packaging, and Delivery

**Handling, Storage, and Preservation**— Excipient products, intermediates, and raw materials should be handled and stored under appropriate conditions of temperature, humidity, and light so that the identity, strength, quality, and purity are not affected. Storage and handling procedures should protect containers and closures from contamination and deterioration and should prevent mix-ups (e.g., between containers that have different specifications but are similar in appearance).

Raw materials, including solvents, are sometimes stored in silos or other large containers, making precise separation of lots or batches difficult. If such materials are used, this should be noted in an inventory or other record, with reasonable accuracy.

Outdoor storage of raw materials (e.g., acids, other corrosive substances, explosive materials) is acceptable provided the containers give suitable protection to their contents, identifying labels remain legible, and containers are adequately cleaned prior to opening and use.

**Packaging System**— An excipient packaging system should include, at a minimum, the following features:

- Written specifications, examination or testing methods, and cleaning procedures where so indicated.
- Tamper evident seals, particularly if the excipient claims to be sterile or nonpyrogenic or if returned material is to be restocked.
- Evaluation of the container closure system, in which it is demonstrated that there is adequate protection from deterioration and contamination and that the excipient is not altered beyond its established specifications.
- All previous labeling removed or defaced if returnable excipient containers are reused. If the containers are repetitively used solely for the same excipient, all previous lot or batch numbers or the entire label should be removed or completely obliterated.

**Delivery**— The manufacturer should arrange for the protection of the quality of product after final inspection and test. Where contractually specified, this protection should be extended to include delivery to the destination.

Distribution records should be kept that document all shipments of finished products. To facilitate its recall, if necessary, these records should identify by excipient batch or lot where and to whom the product was shipped, the amount shipped, the carrier, and the date of shipment.

#### Quality Record Control

The manufacturer should establish and maintain procedures for identification, collection, indexing, filing, storage, maintenance, and disposition of quality records. Quality records should be maintained to demonstrate achievement of the required quality and the effective operation of the quality system. Pertinent subcontractor quality records should be an element of the data.

All quality records should be legible and identify the product involved. Quality records should be kept for at least as long as samples are retained or in accordance with legislative requirements. These records should be stored in facilities that provide a suitable environment to minimize deterioration or damage and to prevent loss and should be maintained in such a way that they are readily retrievable.

Batch production and control records should be prepared for each batch of excipient produced and should include complete information relating to the production and control of each batch. These records should include an accurate reproduction of the appropriate master production or control record, checked for accuracy, dated, and signed; and documentation that each significant step in the manufacture, processing, packing, or holding of the batch was accomplished, including the following:

- Dates
- Identity of individual major equipment and lines used, and specific identification of each batch of component or in-process material used
  - Weights and measures of components used in the course of processing
    - In-process and laboratory control results
  - Inspection of the packaging and labeling area before and after use
- A statement of the actual yield and a statement of the percentage of theoretical yield at appropriate phases of processing
  - Complete labeling control records, including specimens or copies of all labeling used
    - Description of drug product containers and closures
    - Any sampling performed
  - Identification of the persons performing and directly supervising or checking each significant step in the operation
    - Any investigation made for failures and discrepancies
  - Results of examinations made during final product inspection.

#### Internal Quality Audits

The excipient manufacturer should carry out a comprehensive system of planned and documented internal quality audits to verify whether quality activities comply with planned arrangements and to determine the effectiveness of the quality system. Audits should be scheduled on the basis of the status and importance of the activity. The audits and follow-up actions should be carried out in accordance with documented procedures.

The results of the audits should be documented and brought to the attention of the management personnel having responsibility in the area audited. The management personnel responsible for the area should take corrective action on the deficiencies found by the audit.

#### Training

The excipient manufacturer should establish and maintain procedures for identifying and providing the training needs of all personnel performing activities affecting quality. Appropriate records of training should be maintained. Training should directly relate to the employee's function or performance of specific operations and to good manufacturing practices. This training should be conducted by qualified individuals on a continuing basis and with sufficient frequency to ensure that employees remain familiar with any applicable manufacturing practice requirements.

#### APPENDIX 1. DEFINITIONS

**Active Ingredient:** a substance or bulk pharmaceutical chemical that is intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease or to affect the structure or any function of the body of man or other animals.

**Adulterated Substance:** a material that has either been contaminated with a foreign substance or not manufactured using good manufacturing practices. This definition does not pertain to materials that do not meet physical or chemical specifications.

**Aseptic:** an environment free from pathogenic microorganisms.



Batch (Lot): a defined quantity of raw material, intermediate material, packaging components, or final product processed so that it is expected to be homogeneous. In a continuous process, a batch corresponds to a defined portion of the production, based on time or quantity (e.g., vessel volume, one day's production, etc.).

Batch Number (Lot Number): a distinctive combination of numbers or letters from which the complete history of the manufacture, processing, packing, coding, and distribution of a batch can be determined.

Batch Numbering System: a standard operating procedure (SOP) describing the details of assigning batch numbers.

Batch Record: documentation that provides the history of a batch from the raw material stage to completion of the batch or lot.

Blending (Mixing): intermingling different conforming grades into a homogeneous lot.

Certificate of Analysis: a document relating specifically to the results of testing a representative sample drawn from the material to be delivered.

Clean Area: an area with defined environmental control of particulate and microbial contamination, constructed and used in such a way as to reduce the introduction, generation, and retention of contaminants in the area.

Commingling: the blending of trace carryover material from one grade of an excipient with another, usually due to a continuous process.

Contaminant: an impurity not intended to be present in an excipient, which may be introduced by poor cleaning, processing, or lack of appropriate environmental and personnel controls during the manufacturing process.

Continuous Process: a manufacturing process that continually produces an excipient from a continuous supply of raw materials.

Critical Process: a manufacturing process step that may cause variation in quality attributes.

Cross-contamination: contamination during production of a raw material, intermediate, or of a finished excipient with another raw material, intermediate, or product.

DMF: detailed information submitted to the United States Food and Drug Administration concerning a specific facility, process, or product intended for incorporation by reference into a new drug application, supplemental new drug application, abbreviated new drug application, or investigational new drug application.

Excipient: any substances, other than the active drug or product, that have been appropriately evaluated for safety and are included in a drug delivery system to either aid the processing of the drug delivery system during its manufacture, protect, support or enhance stability, bioavailability, or patient acceptability, assist in product identification, or enhance any other attribute of the overall safety and effectiveness of the drug delivery system during storage or use.

Expiration Date: the date beyond which the product may no longer conform to relevant specifications.

Finished Dosage Form (Drug Product): a finished pharmaceutical product, prepared for consumer applications, containing excipients and the active drug substance.

Finished Product: any pharmaceutical product that has undergone all stages of production, including packaging and labeling.

Finished Process Materials: any material that has undergone all stages of production and is released from quality control.

Homogeneous Material: throughout the batch, material of uniform consistency and composition.

Impurity: a substance contained in a product other than the desired substance.

In-Process Testing: monitoring checks performed during production to ensure that the product conforms to its specifications.

In-Process Material: any material that must undergo further manufacture before it becomes a bulk product.

Intermediate Product: any material that must undergo further manufacturing steps before it becomes a bulk product.

Lot: See Batch.

Manufacturer: the company that performs the final production steps and release of the product.

Manufacturing Process: all steps necessary to produce a finished product from raw materials.

Master Formula (Master Formula Record): documentation describing the manufacture of the excipient from raw material to completion of the lot or batch.

Material Review Board: a committee or group selected to evaluate the disposition of potentially nonconforming material.

Model Product: a product that simulates a group of like products.

Mother Liquor: a concentrated solution from which the product is obtained by evaporation, freezing, or crystallization.

Re-evaluation Date: that date beyond which the bulk pharmaceutical excipient should not be used without prior adequate re-examination.

Representative Sample: a sample drawn according to an appropriate sampling plan, which may involve regular or random selection.

Reprocessing: introducing back into the process previously processed material that did not conform to standards or specifications and repeating steps that are already part of the normal manufacturing process.

Nonconforming Material: any material that does not meet manufacturer's specifications or applicable good manufacturing practices.

Packaging: the act of filling and labeling a container with a product.

Packaging Material: the containers, closures, and labels employed in the packaging of a product.

Processing Instructions: the manufacturing procedures set forth in the master formula.

Production: all operations involved in the preparation of an excipient pharmaceutical product, from receipt of raw materials through the completion of a finished product.

Purification: the process of removing impurities from a substance.

Quality: the totality of features and characteristics of a product that bear on its ability to satisfy stated or implied needs.

Quality Assurance: all those planned and systematic actions necessary to provide confidence that a product or a service will satisfy given requirements for quality.

Quality Control: all activities such as measuring, examining, testing, or gauging one or more characteristics of a product (including raw materials) and comparing the findings with specified requirements to determine conformity.

Quality Control Instruments: measurement instruments used to monitor the manufacturing process, in-process controls, and the finished excipient products for final quality control approval.

Quarantine: the status of any material isolated physically or by other effective means while awaiting a decision on its use.

Raw Material: any substance used in the production of a product excluding packaging materials.

Reserve (Retained) Sample: a representative sample of the final excipient batch of sufficient quality and quantity necessary to perform quality control analyses twice.

Returned Products: finished products sent back to the manufacturer.

Reworking: introducing previously processed material that did not conform to standards or specifications to processing steps that are different from the normal process.

Significant Processing Step: processing steps that are required to produce an excipient that meets the established physical and chemical criteria.

Shelf Life: the length of time during which the excipient exhibits stability.

Specifications: the quality parameters that serve as a basis for quality evaluation and to which the products or materials must conform.

Stability: the continued conformance of the excipient to its specifications.

Standard Operating Procedures (SOPs): a written authorized procedure that gives instructions for performing operations.

Validation: documentation that states that any procedure, process, equipment, material, or activity consistently leads to the expected results.

Vendor: an organization contracted to supply a material or perform a service.

## APPENDIX 2. GENERAL AUDITING CONSIDERATIONS

### Evaluation

Prevention of Contamination— In evaluating the adequacy of measures taken to prevent contamination of materials in the process, it is appropriate to consider the following factors:

- Type of system (e.g., open or closed. Closed systems in chemical plants are often not closed when they are being charged or when the final product is being emptied. Also, the same reaction vessels are sometimes used for different reactions)
  - Form of the material (e.g., wet or dry)
- Stage of processing and use of the equipment and/or area (e.g., multi-purpose or dedicated)
  - Continuous versus (discrete) batch production.

Other factors that should be considered in evaluating an excipient plant are the degree of exposure of the material to adverse environmental conditions, the potential for cross-contamination from any source, the relative ease and thoroughness of clean-up, and sterile versus nonsterile operations.

Documentation— An excipient manufacturer should recognize the need for appropriate evaluation and utilization of proper standards and test procedures for raw materials before they are introduced into the process. In addition, as chemical processing proceeds, a chain of documentation should be established that includes the following:

- A written process
- Identification of critical processing steps
  - Appropriate production records
- Records of initial and subsequent lot or batch numbers
  - Records of raw materials used
- Intermediate test results with meaningful standards.

The production of some excipients involves processes in which chemical and biochemical mechanisms have not been fully characterized; therefore, the methods and procedures used in their production will often differ from those applicable to the manufacture of finished dosage forms.

It should be recognized that all intermediates need not require testing. An excipient manufacturer should, however, be able to identify critical or key points in the process where selective intermediate sampling and testing is necessary in order to monitor process performance. The records should become more complete as the end of the process approaches.

The finishing steps and packaging steps should be conducted under appropriate conditions to avoid contamination and mix-ups and be appropriately documented.

### Inspections

Inspection of an excipient operation may depend on the purpose of the audit and intended use of the excipient. Operational limitations and validation of the significant processing steps of a production process should be examined to determine that the manufacturer adequately controls steps to ensure that the process performs consistently. Overall, an inspection should determine the excipient manufacturer's capability to deliver a product that consistently meets the specifications listed in the marketed application or the product specifications needed for research purposes. A team consisting of auditors, engineers, laboratory analysts, purchasing agents, computer experts, or other appropriate personnel should participate in the inspection when resources permit. Confidentiality of the manufacturers' processes must be respected by external auditors.

A good starting point for an excipient plant inspection is a review of the following areas.

- Nonconformance—This could be because of the rejection of a lot or batch that did not meet specifications, customer complaints, return of a product by a customer, or recall of a product. The cause of the nonconformance should have been determined by the manufacturer, a report of the investigation prepared, and subsequent corrective action initiated and documented. Records and documents should be reviewed to ensure that nonconformances are not the result of a poorly developed or inconsistent process.
- Complaint files—Customers may report some aspects of product attributes that are not entirely suitable for their use. These may be caused by impurities or inconsistencies in the excipient manufacturing process.
  - Change control logs
  - Material Review Board documents or equivalent team reports
- Master formula and lot or batch production records—Frequent revisions may reveal problems in the excipient production process.
  - Specifications for the presence of unreacted intermediates and solvent residues in the finished excipient
  - Storage areas for rejected products.

### Significant Processing Steps

Significant processing steps are those steps that are required to produce an excipient that meets the established physical and chemical criteria. These steps should be identified by the excipient manufacturer. Significant processing steps can involve a number of unit operations or unit processes. Unit operations include physical processing steps involving energy transfer where there is no chemical change of the molecule. Unit processes include those processing steps wherein the molecule undergoes a chemical change.

Significant processing steps can include, but are not limited to, the following:

- Phase changes involving either the desired molecule, solvent, inert carrier or vehicle (e.g., dissolution, crystallization, evaporation, drying, sublimation, distillation, or absorption)
  - Phase separation (e.g., filtration or centrifugation)
- Chemical changes involving the desired molecule (e.g., removal or addition of water of hydration, acetylation, formation of a salt)
  - Adjustments of the solution containing the molecule (e.g., adjustment of pH)
- Precision measurement of added excipient components, in-process solutions, recycled materials (e.g., weighing, volumetric measuring)
  - Mixing of multiple components
- Changes that occur in surface area, particle size, or lot or batch uniformity (e.g., milling, agglomeration, blending).

### Documentation and Record Keeping

Documentation required for the early steps in the process should provide a chain of documentation, but need not be as comprehensive as in latter parts of the process. The minimum documentation that should be applied in order to promote uniformity in excipient GMP inspections is:

- the assignment of a unique lot or batch number to the released or certified excipient
  - the preparation of a lot or batch record
- demonstration that the lot or batch has been prepared using GMP guidelines from the processing point at which excipient manufacturing practices have been determined to

- apply
- demonstration that the lot or batch is homogeneous within the manufacturer's specifications (This does not necessitate final blending of continuous process material if process controls can demonstrate compliance to specifications throughout the lot or batch.)
    - demonstration that the lot or batch is not commingled with material from other lots or batches for the purpose of either hiding or diluting an adulterated batch
    - demonstration that the lot or batch has been sampled in accordance with a sampling plan that ensures a representative sample of the lot or batch
  - demonstration that the lot or batch has been analyzed using scientifically established tests and methods designed to ensure that the product meets standards, specifications, and characteristics
  - demonstration that an excipient has stability data to support the intended period of use. (These data can be obtained from actual studies on the specific excipient or from applicable model product studies that can reasonably be expected to simulate the performance of the specific excipient.)

Complete documentation should exist when:

- the excipient can be identified and quantified for those processes where the molecule is produced during the course of the process (In this regard, a theoretical yield should be established with appropriate limits, and there should be an investigation if the actual yield falls outside the limits.)
- a contaminant, impurity, or other substance likely to adversely affect the purity or form of the molecule is identified and subsequent attempts are made to remove it
  - any significant aberration occurs outside of the normal manufacturing process.

Complete documentation should be continued throughout the remainder of the process for all significant processing steps until the excipient is packaged and transported to the end user.

#### Product Lot or Batch Consistency and Audit

Excipient manufacturing plants often produce laboratory or pilot lots or batches. Scale-up to commercial production may involve several stages, and data should be reviewed to demonstrate the adequacy of the scale-up process. Scale-up may introduce significant problems in consistency among lots or batches. Pilot lots or batches should serve as the basis for establishing in-process and finished product purity specifications.

Typically, manufacturers will generate reports that discuss the development and limitation of the manufacturing process. Summaries of such reports should be reviewed to determine if the plant is capable of adequately producing the excipient. The reports, where appropriate, serve as the basis for the validation of the manufacturing and control process, as well as the basic documentation to demonstrate that the process performs consistently.

A review of a process flow chart is helpful in understanding the various processing stages. As part of the review of the processing records, the critical stages and sampling points should be identified. The normal limits from in-process testing should be determined, along with the action to be taken by the manufacturer should these specifications not be met. For example, an in-process test result may show the presence of some unreacted material which may indicate that the process time should be extended.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Robert H. Lafaver, B.A.</a> Scientist 1-301-816-8335	(EGC05) Excipient General Chapters

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#### 1079 GOOD STORAGE AND SHIPPING PRACTICES

This general information chapter is intended to provide general guidance concerning storing, distributing, and shipping of Pharmacopeial preparations. It describes procedures to maintain proper storage environments for individual articles and to ensure a preparation's integrity, including its appearance, until it reaches the user. There is no change to any applicable requirements under Current Good Manufacturing Practices, approved labeling, state laws governing pharmacies, the USP General Notices and Requirements, or monographs. The section Preservation, Packaging, Storage, and Labeling under General Notices and Requirements provides definitions for storage conditions. All equipment used for recording, monitoring, and maintaining temperatures and humidity conditions should be calibrated on a regular basis. This calibration should be based on NIST or international standards (see [Monitoring Devices—Time, Temperature, and Humidity](#) 1118). A Pharmacopeial preparation may follow several potential routes from the original manufacturer to the patient. [Figure 1](#) documents present-day routes and the associated risks. These risks include exposure to temperature excursions, humidity, light, and oxygen. For a discussion of climates, stability, and mean kinetic temperature, see [Pharmaceutical Stability](#) 1150. Temperature- or humidity-sensitive articles are to be handled in accordance with General Notices.

#### PACKAGING AND STORAGE STATEMENT IN MONOGRAPHS

Most articles have storage conditions identified by their labeling. Otherwise, it is expected that the conditions for storing the article are specified in the monograph according to definitions provided by the General Notices and Requirements in the section Storage Temperature, and Humidity under Preservation, Packaging, Storage, and Labeling. In cases where additional information on packaging and storage is desired, a specific statement can be provided in the Packaging and storage or the Labeling section of the individual monograph.

#### STORAGE IN WAREHOUSES, PHARMACIES, TRUCKS, SHIPPING DOCKS, AND OTHER LOCATIONS

Pharmacopeial articles are to be stored in locations that adhere to conditions established by the manufacturer. Where the desired conditions are not established, use storage conditions described in the General Notices and Requirements or in the applicable monograph.

##### Warehouses

Observation of the temperature variations in a warehouse should be made over a period of time to establish a meaningful temperature profile, including the temperature variations and conditions in the different parts of the warehouse. Such observations provide data and information as to where various products should and should not be stored.

##### establishing temperature profiles

Temperature profiles can be compiled by using a suitable number of thermometers or other temperature recording instruments. They should be placed throughout the warehouse in divided sections and should record the maximum and minimum temperatures during a 24-hour period for a total of three consecutive 24-hour periods. The following factors, some of which may give rise to extreme temperatures, should be considered during the process of temperature profiling: the size of the space, location of space heaters, sun-facing walls, low ceilings or roofs, and geographic location of the warehouse. Temperature profiling for warehouses already in use should be done at known times of external temperature extremes, e.g., for a period of not less than 3 hours when air temperatures are higher than 25° or less than 15°. Profiling should be conducted in both summer and winter. A mean kinetic temperature (MKT) should be obtained for any separate areas within the warehouse (see [Pharmaceutical Calculations in Prescription Compounding](#) 1160 for samples of MKT calculations). The temperature profile report should provide recommendations for the use of each area and identification of any areas that are found unsuitable for storage of Pharmacopeial articles.

##### controlled room temperature

The General Notices provide a definition for Controlled Room Temperature. A temperature profiling study should demonstrate suitability for storing Pharmacopeial articles in areas determined to be at room or controlled room temperature. A suitable number of temperature and humidity recording instruments should be installed to record temperatures and to provide temperature and humidity profiles. Temperature recording should be conducted to meet the recommendations for establishing mean kinetic temperature and to comply with the

warehouse's written procedures. These written procedures should have a reporting mechanism in place whereby a management tree is informed in the event that predefined high or low temperatures or humidity limits have been exceeded. Records can be reviewed as determined by the management system in accordance with established guidelines. Suitable training should be provided to persons who record temperatures, and proper quality accountability and tracking systems should be maintained.

storage at "cool," "cold," "refrigerator," and "freezing" conditions

The General Notices provide definitions for cool, cold, refrigerator, and freezer temperatures. A temperature profiling study can be used to establish suitable areas for storing Pharmacopeial articles designated to be stored under these conditions. Equipment used for storing Pharmacopeial articles at these low temperatures should be qualified according to written procedures provided by the management system. Recording devices can be installed within the equipment and used to enable both air and product temperatures to be recorded at regular intervals. The number and location of monitoring devices should be determined based on the result of the temperature profile. Temperature records should be examined at least once every 24 hours or as provided in the equipment protocol. Cool or cold conditions are moisture-condensing conditions. Humidity-monitoring devices should be used in cases where the repackaged Pharmacopeial article is humidity-sensitive or labeled to avoid moisture. Additionally, there can be installed temperature-monitoring, and where necessary, humidity-monitoring alarm devices that have the capability of alerting personnel in the event that control is compromised. There should be protocols in place to address procedures for responding to failed temperature and humidity ranges both for normal working hours and outside normal working hours. Temperature and humidity should be reviewed

at the times designated by the established protocol. The calibration and functioning of all temperature and humidity monitoring devices, including alarms and other associated equipment, should be checked on an annual or semiannual basis. Regular maintenance protocols should be in place for refrigeration equipment. There should be written agreements in place for all maintenance and evaluation procedures, and this may include an emergency situation protocol.

#### personnel training

Suitable training should be provided for personnel who handle Pharmacopeial articles with special storage temperature requirements. Personnel should know how to monitor temperatures and how to react to situations where adverse temperatures are identified. There should be written procedures in place such that the adverse temperatures are recorded and a report provided to the parties designated in the protocol.

#### qualification of "cold" equipment or stores

Only climate control equipment for which a contractor has provided documentation to assure its suitability for temperature and humidity requirements should be considered for use in cold storage. Qualification procedures on a regular basis should be independently conducted on equipment in cold stores to guarantee suitability and proper functioning. The procedure should demonstrate the temperature profile for both air and product temperatures when empty as well as when loaded. The procedure should also demonstrate the time taken for temperatures to exceed the maximum temperature in the event of a power failure. Qualification should consider thermal fluctuations that occur during stock replenishment and order removal. The results of the qualification should demonstrate the ability of the equipment to maintain the required temperature range in all areas, defining any zones which should not be used for storage such as those areas in close proximity to cooling coils, cold air streams from equipment ventilation, or doors. The variability of the system can be characterized by using the relative standard deviation. Thermal monitoring should establish that the system is rugged in that its temperature profile is consistent and reliable.

### DISTRIBUTION AND SHIPMENT OF PHARMACOPEIAL ARTICLES

As indicated in [Figure 1](#), a drug can take a variety of paths from the manufacturer to the patient. In the simplest form of the distribution system, the manufacturer ships directly to the customer, such as a doctor's office, clinic, or hospital. However, more often, the article leaves the manufacturer's chain of control and enters a complex system of handoffs that involve the distribution chain to the patient.

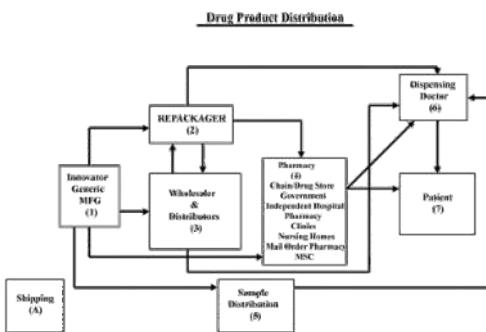


Figure 1. Drug product distribution.

Shippers and distributors are to follow the proper storage and shipping requirements as indicated by the manufacturer. For particular cases, such as shipment of vaccines or other special care products, manufacturers may require special shipping and storage conditions generally referred to as "cold-chain management". For example, manufacturers may attach temperature-monitoring devices and/or ship under specified controlled conditions to ensure that the desired temperature is maintained during distribution (see [Monitoring Devices—Time, Temperature, and Humidity](#) [1118](#)). Validated, available temperature- and/or humidity-monitoring technologies can be used to monitor the overall environmental effect on compendial articles during shipment and distribution. In these cases, the shipping conditions of the package are recorded. In general, extreme temperature conditions (i.e., excessive heat, freezing) should be avoided. Distribution systems chosen to deliver pharmaceutical products from the manufacturer to the consumer should take into account basic operational parameters, including timeliness and accountability. The manufacturer's FDA-approved storage conditions, printed in the labeling of the product, should be observed carefully at each destination of the distribution chain (see [Figure 1](#)), unless specifically instructed otherwise in the immediate label of a shipping container. This may be the case for certain pallet-sized shipping containers where the amount of refrigerant contained (e.g., dry ice, gel packs) is based on an anticipated exterior condition approximating controlled room temperature. In such cases, placing the shipping container in a refrigerator could lead to the product inside freezing, potentially affecting its quality. Items requiring special handling conditions will have those conditions clearly indicated in the labeling for the product. The Prescription Drug Marketing Act of 1987 and the ensuing regulations in 21 CFR Part 203, Prescription Drug Marketing, and Part 205, Guidelines for State Licensing of Wholesale Prescription Drug Distributors, provide the necessary regulations and guidance for several legs of the distribution chain for the prescription drug. The manufacturers and distributors should work together to establish proper distribution and product-handling requirements for the purpose of ensuring appropriate product maintenance in transit. Pharmacists and physicians should educate patients regarding proper storage of products to ensure product integrity at the patient level. Information that may be considered in determining the ability of pharmaceutical articles to maintain their Pharmacopeial requirements of identity, strength, quality, and purity through the distribution channel may include, but is not limited to the following: ICH stability studies, temperature cycling studies, stability shipping studies, ongoing regulatory stability commitment studies, market experience portfolio (i.e., product complaint files, historical product performance data, product development data), and product labeling commitments.

#### Qualification Protocol

Operational and performance testing should be parts of a formal qualification protocol that may use controlled environments or actual field testing based on the projected transportation channel. These should reflect actual load configurations, conditions, and expected environmental extremes. Temperature and humidity monitors should be placed into the product or a representative thereof. Testing consists of consecutive replicate field transportation tests using typical loads, according to an established protocol.

#### Physical Challenges

Most products are sufficiently robust to withstand distribution with minimal protection from routine, well-understood physical and environmental hazards. Several standard test methods are available for evaluating package performance factors under well-documented shock, vibration, and other transit elements. The American Society for Testing and Materials document, "Standard Practice for Performance Testing of Shipping Containers and Systems" (ASTM D4169-98), and the International Safe Transit Association's (ISTA) specifications have similar methods for evaluation of shipping performance for various types of transit modes such as less-than-truckload (LTL), small package, rail car, air freight, etc. From the



Manufacturer's perspective, these tests are very useful in evaluating the product and package durability and fragility. The tests are usually performed on shipping carton quantities or specific stock keeping unit (SKU) as an unbroken whole. Fragility problems can be corrected with package modifications, which could include placing cotton or rayon colers in bottles or placing top and bottom pads in the shipping case to reduce package breakage. Not all protective packaging elements follow the SKU through the system.

Basic packaging principles are observed when separating the contents of the manufacturer's shipping container or pallet load into smaller quantities or when shipping mixed product loads. For example, glass containers are wrapped in a bubble wrap or other shock-absorbent material, and the void spaces are filled with dunnage (e.g., foam "peanuts," shredded or tightly crumpled paper, bubble wrap) to protect the contents from shifting and drop impact. Large-volume liquid containers may be bagged in plastic and kept isolated to prevent leakage to, or damage of, adjacent packages. "Skin packaging," a term describing a heat-shrink film that anchors the load to fiberboard and prevents load shift, can be an excellent method of protecting some products, but it may be inappropriate for heat-sensitive products. The shipping carton should have correct Edge Crush Test (ECT) characteristics for freight being shipped according to Item 222 of the National Motor Freight Classification and Rule 41 of the Uniform Freight Classification.

#### Temperature Challenges

Shipping of temperature-sensitive articles requiring thermally controlled packaging presents a special challenge. Unlike shock, vibration, and other physical hazards, thermal hazards tend to be unique to a given system. Except for temperature-controlled trucks, the distribution environment is widely variable and depends upon a range of factors, including points of origin and destination, article and container sensitivities to cold, accidental freezing or heat, transit mode (e.g., air, truck, combination), time, weather or season, and carrier type (e.g., small package carrier or integrator, freight forwarder, U.S. Postal Service). The shippers should know and understand the systems they use and should design the protective package accordingly. Storage temperature ranges may not be indicative of the allowable tolerances during shipping. Articles labeled for special storage conditions (between 2° and 8°) vary widely in their tolerance of short-term exposure to heat and cold. Some, such as soft gelatin capsules and suppositories, carry specific upper limits on both shipping containers and SKUs. A temperature cycling study intended to identify those articles affected by multiple, short-term excursions beyond the storage temperature limits should be performed. These data provide wholesalers and distributors with clearer identification of those drug products that may require special handling during particular climate conditions.

#### Materials

Two commonly used types of refrigerant are dry ice (frozen carbon dioxide gas) and wet ice (frozen water), which appears as crushed ice or in various refrigerant packs containing water mixtures with specific freezing points. Phase-change materials are also available for specialized needs. Refrigerant packs should have the correct freezing point and be cooled to the proper surface temperature prior to use. Articles harmed by accidental freezing may require a barrier between the refrigerant and the product or some other special packaging. Insulating materials commonly available include foil laminates, bubble pack, corrugated, fabricated, and molded expanded polystyrene (EPS) cartons, and fabricated or molded urethane foam cartons, with or without additional interior components. Recognized standard test methods for evaluating insulated containers are currently limited to ASTM D3103-92, Standard Test Method for Thermal Insulation Quality of Packages and a method under development by ISTA. Neither one fully addresses all of the issues involved, but both include useful information on testing procedures. The tests should be modified based on the specific system adopted by the shipper. The manufacturer may be able to supply helpful data on specific articles and their requirements.

#### SPECIAL HANDLING

Certain classes of Pharmacopeial articles may require special handling. Such articles include products classified as dangerous goods under the Department of Transportation (DOT), state, local, or carrier rules; or products classified as controlled substances by the Drug Enforcement Administration (DEA) or by individual states.

#### Receipt of Pharmacopeial Articles

Upon arrival of Pharmacopeial articles to warehouse loading docks, premises, and other arrival areas, the Pharmacopeial articles are to be transferred to their manufacturer-designated storage environment within 2 hours of receipt. Limitation of the time spent in the uncontrolled environments of the loading dock is important to ensure that the integrity of the preparation is maintained. This is particularly important for temperature-sensitive items. The delivery document should be reviewed at receiving sites to ensure that the Pharmacopeial articles have not been subjected to any delays during shipment that could result in exposure of the article to extremes of temperature, or to any other extreme or undesirable conditions. In addition, to the extent possible, the receiving personnel should ensure that the ruggedness requirements in shipment have been met. For Pharmacopeial articles requiring extreme caution, special handling, or refrigerator temperature storage conditions, those who supply the articles (e.g., wholesalers and manufacturers) and delivery contractors should provide documented evidence to show that the required temperature range has been maintained during transportation. In the event that a deviation from the required temperature range has been observed during shipment of an article requiring such a shipping condition, the supplier or delivery contractors should document the temperature and the length of time the compendial article was not within the designated storage temperature. The pharmaceutical manufacturer may be contacted to determine the significance of unusual variances.

#### Distribution or Shipping Vehicles

Vehicles used for shipping or distribution of Pharmacopeial articles designated for storage at controlled room temperature should be suitably equipped to ensure that the temperature excursions encountered are within those allowed under the definition of controlled room temperature. Steps should be taken so that extremes of temperature, whether above or below the specified temperatures, should not be encountered during delivery procedures.

#### Vehicle Qualification

Where practical, suitable monitoring devices, as determined by the manufacturer and vehicle supplier, should be placed in different areas of the truck to establish a temperature profile of the truck over a 24-hour period during a hot summer day, average high, and a cold winter day, average low, and during a normal or typical day. The derived temperature of the different parts of the truck may be used to determine the location on the truck where Pharmacopeial articles can be stored appropriately during shipping (see [Monitoring Devices—Time, Temperature, and Humidity \(1118\)](#)).

#### Pharmaceutical Delivery Staff

As part of the contractual agreement between the delivery contractors and the manufacturers, the delivery staff should receive appropriate training to ensure that they are aware of the correct procedures to follow in maintaining products at the correct temperature. There may be written procedures that should be documented. In addition, the transportation personnel should have proper knowledge of the temperature profile of the vehicle to ensure proper placement of the Pharmacopeial articles in the vehicle. Pharmacopeial articles requiring special handling (e.g., refrigeration) or environmentally sensitive preparations should be transported in a suitably equipped vehicle to ensure that the articles are maintained at the correct temperature during distribution, shipping, and delivery and up to the point of receipt. Special arrangements should be made to inform receiving personnel, pharmacists, or other appropriate customers that the package includes articles with special storage and handling specifications and are to be transferred immediately to the appropriate storage location. The manufacturer, shipper, or delivery agency should provide appropriate evidence to show that the required temperature has been maintained throughout shipment and distribution.

#### SHIPMENT FROM MANUFACTURER TO WHOLESALER

##### Wholesaler

The wholesaler receiving the pharmaceutical articles should ensure that on arrival, the pharmaceutical articles are transferred to the correct environment without delay, as directed by the manufacturer, ideally within 2 hours of receipt. The wholesaler should examine the delivery documentation to ensure that the products have not been subjected to any delays during shipping and distribution that could result in products being exposed to extreme temperatures (see also the previous section, Pharmaceutical Delivery Staff, for staff expectations). The vehicles used for shipping of Pharmacopeial articles to the wholesaler, especially products requiring storage at low temperatures, should be suitably equipped to ensure that products are maintained at the correct temperature during shipping and distribution and up to the point of receipt. The receiving wholesaler staff should be informed that the articles are transferred to appropriate storage locations without delays. The vehicles used for shipping of Pharmacopeial articles requiring storage at room or controlled room temperatures should be suitably equipped to ensure that extremes of temperature, either above or below the specified temperature, do not occur during delivery procedures. Warehouse staff may receive appropriate training to ensure that the correct procedures are followed to maintain required temperature conditions (see Pharmaceutical Delivery Staff). Where necessary, a monitoring device for temperature and/or humidity should be used during shipping and distribution.



#### Compromised Temperature Conditions

A procedure should be in place in the warehouse to define the action that should be taken in the event of deviation from required storage conditions. Suitable records should be maintained to explain the reason for deviation and the resulting action that is taken. The product in question should then be placed in a quarantine status. Advice on the suitability of the product for use should be sought from the manufacturer or supplier of the product. The manufacturer's response should be documented prior to issuing the product to the customer, if that product is to be issued to the customer.

#### SHIPMENT FROM MANUFACTURER OR WHOLESALER TO PHARMACY

##### Pharmacy

The pharmacy receiving the pharmaceutical articles should ensure that on arrival, the pharmaceutical articles are transferred to the correct environment without delay, as directed by the manufacturer, ideally within 2 hours of receipt. The pharmacy personnel should examine the delivery documentation to ensure that the products have not been subjected to any delays during shipping and distribution, which could result in the products being exposed to extreme temperatures (see also the section, Pharmaceutical Delivery Staff, for staff expectations). The vehicles used for shipping of Pharmacopeial articles to the pharmacy, especially products requiring storage at low temperatures, should be suitably equipped to ensure that products are maintained at the correct temperature during shipping and distribution and up to the point of receipt. Receiving pharmacy staff should be informed that the articles are to be transferred to appropriate storage without delays. The vehicles used for shipping of Pharmacopeial articles requiring storage at room or controlled room temperatures should be suitably equipped to ensure that extremes of temperature, either above or below the specified temperature, do not occur during delivery procedures. Pharmacy staff may receive appropriate training to ensure that the correct procedures are followed to maintain required temperature conditions (see Pharmaceutical Delivery Staff). Where necessary, a monitoring device for temperature and/or humidity may be used during shipping and distribution.

#### Compromised Temperature Conditions

The pharmacy should maintain appropriate procedures to define action that should be taken in the event of deviation from the required storage conditions. Suitable records should be maintained to explain the reason for deviation and the resulting action taken (including whether the product is issued to the patient or customer). Advice on the suitability of the product for use as an acceptable drug article should be sought from the manufacturer or supplier of the product.

#### SHIPMENT FROM PHARMACY TO PATIENT OR CUSTOMER

The pharmacy should provide an appropriate label on the package sent through air or surface routes so that the deliverer does not place the package in a mailbox exposed to extremes in temperature. In the event that no one is available to receive the package, the deliverer should return the package to the post office or service office, and store it in a cool or air-conditioned area until the patient can receive the medication. In the event that the package has not been delivered for more than 2 days, the package may be returned to the pharmacy. For temperature-sensitive articles, it is important that proper arrangements be made to protect the drug from exposure to high temperatures, or in some cases, from freezing conditions. Such arrangements may include the following: insulating the packaging, or packaging with coolant included; overnight shipping; and pre-arranged pick-up. In such cases, the pharmacy should provide on the external package a statement of an acceptable period of delay for delivery. The patient or customer should examine the delivery documentation to ensure that the package has not been subjected to any unacceptable delays during shipping and distribution. The patient or customer receiving the pharmaceutical articles, either by mail, delivery vehicle from the pharmacy, or directly from the physician or pharmacy, should be advised that upon receipt the articles are to be transferred to appropriate storage conditions without delay, as directed by the pharmacy, ideally within 2 hours of receipt. The vehicle used for air or surface shipping and distribution of pharmaceutical packages to the patient or customer, especially those requiring low temperatures, should contain the article suitably packaged in containers that maintain the desired storage conditions until the article reaches the patient or customer. The vehicles used for shipping and distribution of pharmaceutical articles to patient or customer, especially those requiring storage at room or controlled room temperatures, should be suitably equipped during extreme temperature conditions such that the packages are not exposed to extremes of temperature either in winter or summer months. In the event that the vehicle is not adequately equipped with air conditioning or heating to protect the product, the time that the article is exposed to ambient conditions should be strictly limited, ideally not more than 2 hours. Where appropriate, a monitoring device may be used to ensure that required temperatures are maintained until the package reaches the patient or customer. If stability studies for the Pharmacopeial preparation indicate that it is particularly sensitive to environmental insults or if appropriate shipping safeguards described in this section are not feasible, then the preparation should be shipped by a different method whereby environmental control can be maintained.

#### Compromised Temperature Conditions

There should be appropriate procedures in the pharmacy that ships the article to the patient or customer defining the action that should be taken in the event that a patient reports that there has been a deviation from required storage conditions for an article, including any environmentally sensitive preparations, prior to the point of receipt. Advice on the suitability of the product for use should be provided to the patient or customer after the manufacturer or supplier's advice has been sought by the pharmacy. If the patient is advised to use the article, such advice should be documented and noted appropriately by the pharmacy. Otherwise, appropriate arrangements should be made to promptly replace the suspect article. For mail order items, replacement from local pharmacies may be an option to ensure an uninterrupted supply of medication.

#### RETURNS OF PHARMACEUTICAL ARTICLES FROM PATIENTS OR CUSTOMERS

The wholesaler, manufacturer, and pharmacy personnel should evaluate the validity of the request for return, and maintain an auditable account of the return receipt. For products in unopened manufacturer's containers that have been at variance during shipment, arrangement may be made to return the products to the manufacturer, wholesaler, or pharmacy preferably within 3 working days of receipt. The supplier may request records or written confirmation by the patient to show that the product was stored properly while in possession of the customer.

#### STORAGE OF PHYSICIAN SAMPLES HANDLED BY SALES REPRESENTATIVES IN AUTOMOBILES

Storage of physician samples by sales representatives is regulated under 21CFR 203.34(b)(4); each manufacturer or distributor is to have appropriate policies in place to ensure that proper storage is maintained. The following suggestions may be considered in response to this need and are of interest to practitioners who may observe actual practices. Automobile trunks or passenger cabins used for the storage and distribution of physician samples should be monitored to determine the temperature profile of the trunk or passenger cabin. Suitable monitoring devices as determined by the sales representative may be placed in different areas of the trunk or passenger cabin on a hot summer and a cold winter day. Measurements should also be made during typical 24-hour periods, and the derived temperature should be used for calculation of the mean kinetic temperature at which the sample is stored (see [Pharmaceutical Calculations in Prescription Compounding](#) (1160) for examples of MKT calculations). If the Pharmacopeial article designated for storage requires storage at controlled room temperature, then suitable measures should be taken to maintain the sample within the allowable limits of the storage parameters. Environmentally-sensitive preparations should not be stored in automobile trunks or passenger cabins. Medications stored in automobile trunks or passenger cabins should be removed at the end of 3 days. Sales representatives should consider parking automobiles in shaded areas to avoid extreme heat during the summer and in garages to avoid freezing temperatures during the winter. The use of vouchers from the manufacturer that patients could use to obtain medication samples from participating pharmacies is an alternative way of providing drug samples.

#### STABILITY, STORAGE, AND LABELING

The design of stability studies of Pharmacopeial articles is based on knowledge of the behavior, properties, and stability of the drug substance and experience gained from clinical formulation studies.<sup>3</sup> The length of the studies and the storage conditions for a Pharmacopeial article should be sufficient to cover storage, shipment, distribution, and subsequent use of a Pharmacopeial article. The data gathered from ICH accelerated testing or from testing at an ICH intermediate condition may be used to evaluate the effect of short-term excursions outside the label storage conditions such as those that might occur during shipping. See [Pharmaceutical Stability](#) (1150).

#### STATEMENTS/LABELING OF THE IMMEDIATE CONTAINERS OR PACKAGE INSERT

Storage statements should be based on the stability evaluations of the Pharmacopeial drug substances and in accordance with national and international requirements.



Temperature Storage Statements— For products with a storage statement reading, "Store at controlled room temperature," the labeling should read as follows on the package insert: "Store at 20°C to 25°C (68°F to 77°F), excursions permitted between 15°C and 30°C (between 59°F and 86°F). Brief exposure to temperatures up to 40°C (104°F) may be tolerated provided the mean kinetic temperature does not exceed 25°C (77°F); however, such exposure should be minimized."

On the immediate container label, the following may read for controlled room temperature (CRT): "Store at 20°C to 25°C (68°F to 77°F), excursions permitted between 15°C and 30°C (between 59°F and 86°F)."

Cool Storage Statement— The storage statement for labeling may be as follows: "Store in a cool place, 8°C to 15°C (46°F to 59°F)."

Refrigerator Storage Statement— The storage statement for labeling may be as follows: "Store in a refrigerator, 2°C to 8°C (36°F to 46°F)."

Freezer Storage Statement— The storage statement for labeling may be as follows: "Store in a freezer, -25°C to -10°C (-13°F to 14°F)."

See the General Notices for all other applicable storage conditions, such as Storage Under Nonspecific Conditions and store in a Dry Place. Additional cautionary statements to protect the Pharmacopeial drug product from extreme temperature and humidity conditions may be included on the container label and package insert, as the manufacturer desires.

1. See International Conference on Harmonization EWG Q1 A&B; see also FDA Guidance for Industry: Stability Testing of Drug Substances and Drug Products ([www.fda.gov](http://www.fda.gov)).

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Desmond G. Hunt, Ph.D.</a> Scientist 1-301-816-8341	(PS05) Packaging and Storage 05

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## 1080 BULK PHARMACEUTICAL EXCIPIENTS—CERTIFICATE OF ANALYSIS

### BACKGROUND

This general information chapter is derived from the Certificate of Analysis Guide for Bulk Pharmaceutical Excipients, prepared by The International Pharmaceutical Excipients Council of the Americas (IPEC-Americas), an international guidance document on the preparation and appropriate use of a Certificate of Analysis (COA) for these excipients, referenced throughout the chapter as "excipient(s)". The chapter defines the suggested elements of a Certificate of Analysis, provides a template for organizing required and optional data in a logical manner, and assists in establishing a uniform understanding of the roles and responsibilities of excipient manufacturers, distributors, and users.

The principles and information in this chapter can be applied to the manufacture of all bulk pharmaceutical excipients intended for use in human drugs, veterinary drugs, and biologics. As an international guidance document, it cannot specify all national legal requirements nor cover in detail the particular characteristics of every excipient. When considering how to use this chapter, each manufacturer, distributor, or user should consider how it may apply to that specific manufacturer's product and processes. The diversity of excipients means that some principles of the chapter may not be applicable to certain products and processes.

The chapter is divided into several parts. The first part provides background discussion necessary for the design and suggested elements of a COA. A template is provided to show the format and placement of information in the COA. This is followed by a detailed discussion to ensure that the purpose and meaning of the specific information contained in the COA is understood. For a list of terms used in this information chapter and their definitions, see Appendix 1.

### GENERAL GUIDANCE

International regulations governing drugs require that components of the drugs be manufactured, processed, packed, and held in accordance with good manufacturing practices (GMPs). For a thorough discussion of GMPs that apply to excipient manufacture, see [Good Manufacturing Practices for Bulk Pharmaceutical Excipients](#). The excipient is often a natural substance, mixture, or polymer whose chemical and physical properties are difficult to quantify and that is often used with a broad range of active pharmaceutical ingredients and in a diverse range of finished dosage forms. Until now, there were no guidance documents that specifically focused on the content or format of COAs for excipients and that addressed the diversity of both the excipients and their usage.

**Preparation and Appropriate Use of a Certificate of Analysis—** The Certificate of Analysis for excipients should be prepared and issued by the supplier of the material, following the general guidelines discussed below. Primary responsibility for the preparation of the COA belongs to the excipient manufacturer. It is most important that a complete and accurate COA be provided to the excipient user for specific lots or batches intended for use in the pharmaceutical industry. Additional considerations should be made for the preparation and issuance of a COA by a distributor of excipients.

The user of a bulk pharmaceutical excipient should always receive a COA for material to be used in the manufacture of a drug product. At a minimum, the user should perform adequate identification tests on each lot of excipient received before releasing it for use in the drug product. Specific identity tests should be used whenever possible. It is a regulatory requirement that excipients be assessed for conformity with all appropriate specifications. However, testing of all specification parameters may not be required for lot release if adequate compliance assurances are provided on the supplier's COA. Before using an excipient in a pharmaceutical product based on COA data, the user also should have an understanding of the supplier's control systems and compliance with GMPs, through appropriate auditing or qualification of the supplier.

Nevertheless, it is the responsibility of the user of the excipient to verify any of the analytical data contained in the COA if knowledge of such information is deemed essential to the use of that excipient. Such testing may go beyond the scope of the compendial methods described in the NF, or beyond those used to develop the information in the COA.

To use test results from a COA, the user must also establish the reliability of the supplier's COA test results by periodically performing all required tests and comparing the results obtained to the supplier's test results. Occasionally, it may not be possible to perform all the required tests because of special equipment requirements, etc., that may not be available to the user. Performing fewer than all these tests may be acceptable provided that the reliability of the supplier has been adequately determined using other appropriate supplier qualification techniques.

It is important to understand that these results may not always specifically correlate, especially when an excipient is produced as a continuous lot. However, the user's test results should demonstrate compliance with the specification requirement.

**Use of Contract Facilities—** Contract facilities are frequently used in the manufacture, testing, and distribution of excipients. When such facilities are used, the supplier of the excipient has the obligation to ensure that the facilities operate under appropriate quality standards (i.e., cGMP, GLP, etc.).

### DESIGN AND SUGGESTED ELEMENTS OF A CERTIFICATE OF ANALYSIS

The suggested elements of a COA are listed below and are included in the following Certificate of Analysis Template section of this chapter. Excipient suppliers may organize the suggested elements presented in the COA template at their discretion; however, the parts of the template were designed to present the suggested and optional information in a logical manner. For a detailed description of each element and examples of statements, see the appropriate section below in this chapter.

The origin and the identity of the excipient are typically established in a Header section. The manufacturer and manufacturing site should be identified if different from the supplier and supplier location, enabling the user to make certain that the excipient comes from a qualified source. Although the manufacturer should be made known to the user, the use of codes for manufacturers and manufacturing sites on the COA to protect confidentiality is acceptable. The identity of the excipient must be definitively established by stating the compendial



and trade name, the grade of the material, and applicable compendial designations.

A lot/batch number or other means of uniquely identifying the quantity of material covered by the COA and information relating specifically to it are typically included in a Body section. The lot number or other unique identification of the material, its date of manufacture, and product code or number should be stated and traceable to a specified lot. If applicable, the expiration date, recommended re-evaluation date, or other relevant statement regarding the stability of the excipient is typically included in this section. Any information required by the customer would also be included here.

The actual test results applicable to the quantity of material covered by the COA are included in an Analysis section. The test name, the result, the acceptance criteria or specifications, and a reference to the test method used should be included for each characteristic listed. Reporting of actual data and observations is recommended rather than nonspecific "passes" or "conforms" statements. If the reported results are derived from a skip-lot or reduced frequency testing program, or an average or in-process test result, this should be noted on the COA.

The Certification and Compliance Statement section is used to list various types of statements that may be required depending on the excipient and specific user needs. These statements are usually negotiated between supplier and user based on specific application requirements. Any declaration of the supplier that includes compliance of additional compendial or other regulatory requirements is typically included in this section.

Many excipients have applications other than pharmaceuticals, such as food, cosmetics, or industrial products. Any product listed as being in compliance with specific regulations should meet the specifications and requirements of that regulation and must be manufactured under appropriate GMPs.

The identity of the individual approving the content of the COA should appear on the COA. The page number and total number of pages should also appear on the COA. This information is usually included in a Footer section.

#### CERTIFICATE OF ANALYSIS TEMPLATE

Listed below is a template for the content and format of a COA.

##### Header

- Titled "Certificate of Analysis"
- Company Name, Address, Phone Number, and Identity of Manufacturer and Manufacturing Site
  - Name (compendial/trade) of Excipient
  - Grade of Excipient
  - Compendial Designation

##### Body

- Lot/Batch Number
- Date of Manufacture
- Product Code or Number
- Expiration Date (if required)
- Recommended Re-Evaluation Date (if required)
  - Stability Statement (if required)
  - Customer Required Information

##### Analysis

- Test Name
- Test Results
- Acceptance Criteria (i.e., specifications)
  - Reference to the Test Method
- Reference to Skip-Lot Testing (if appropriate)
- Reference to Average or In-Process Test Results (if appropriate)
  - Date Retested (if appropriate)
  - Summary of Noncompendial Testing (if any)

##### Certification and Compliance Statements

- GMP Compliance
- Additional Regulatory References
- Potential to Meet Additional Compendial Standards
- Content Listing and Grade of Ingredients (if a mixture)
- Other Specific Compliance Statements [e.g., organic volatile impurities (OVI), residual solvents, transmissible spongiform encephalopathy (TSE), etc.]

##### Footer

- Identity of Authorized Individual for Approval
  - Date of Approval
  - Page Number (i.e., 1 of \_\_)

#### COMPENDIAL DESIGNATION

For a supplier to claim a compendial grade on the COA for an excipient, two requirements should be met. The first requirement is that the excipient be manufactured according to recognized principles of GMPs (see General Notices and Requirements). Adequate conformance to GMPs should also be demonstrated for subsequent steps in the distribution of the excipient. The second requirement is that the excipient meet all the specifications contained in the appropriate compendial monograph, unless its difference is stated on its label, as defined under General Notices and Requirements. When an excipient is listed as compendial grade, it is understood that the above requirements have been met for the material, and the user would be able to confirm this through an appropriate audit of the supplier.

Compendial standards define what is considered an acceptable article and also give test procedures that demonstrate that the article is in compliance. These standards apply at any time in the life of the article from production to consumption. The supplier's release specifications and compliance with GMPs are developed and followed to ensure that the article, when stored according to recommended conditions, will comply with compendial standards until its expiration or recommended re-evaluation date.

Every compendial article shall be so constituted that when examined in accordance with these assay and test procedures, it meets all the requirements in the monograph defining it, as well as meeting any provisions under General Notices and Requirements and in the general chapters, as applicable. However, it is not to be inferred that application of every analytical procedure in the monograph to samples from every production batch is necessarily a prerequisite for ensuring compliance with compendial standards before the batch is released for distribution.

Data derived from manufacturing process validation studies and from in-process controls may provide greater assurance that a batch meets a particular monograph requirement than analytical data derived from examination of finished units drawn from the batch. On the basis of such assurances, the analytical procedures in the monograph may be omitted by the supplier when judging compliance of the batch with the compendial standards.

#### DATES ON A CERTIFICATE OF ANALYSIS



Part of the overall goal to standardize COA for excipients includes a provision for the consistent reporting of appropriate, meaningful, and well-defined dates. The discussion below indicates specific dates that are expected on the COA, along with definitions of the dates, in order to provide suppliers and users of excipients with a mutual understanding of their meaning. Use of the recommended terminology will be helpful in reducing the number of questions on dating information reported for excipients. Use of terminology other than that discussed below is discouraged, because the terms may be ill-defined and have different meanings for the excipient supplier and user. Examples of such terms that should not be used include "shelf life", "use-by date", "warranty date", and "expiration period".

In reporting dates on COA for excipients, it is important that a clear and unambiguous format be used to prevent possible misinterpretation. To accomplish this, it is recommended that an alpha designation be used for the month (may be abbreviated), rather than a numerical representation. It is also recommended that the year include all 4 digits (e.g., Jan. 1, 2005, or 1 Jan. 2005).

**Date of Manufacture**— The date of manufacture should be included on the COA for each excipient lot and should be assigned by the suppliers on the basis of their established policies and procedures. It is recognized that excipients may be manufactured using a variety of processes (e.g., continuous or batch) that may require a period of several days or more to complete. In addition, some excipients may be mixtures or blends of other excipients, and excipient production may include reprocessing steps. Because of this diversity, the date of manufacture should be clearly defined by the supplier and consistently applied for the particular excipient and process. In reporting the date of manufacture, the excipient supplier should indicate the date of completion of the final manufacturing process (as defined by the supplier).

It is important to note that repackaging alone is not considered a processing step to be used in determining the date of manufacture. To provide traceability for a specific excipient lot, other dates may be required in addition to the date of manufacture in order to reflect additional steps such as repackaging.

**Expiration Date and Recommended Re-Evaluation Date**— The stability of excipients may be an important factor in the stability of the finished pharmaceutical dosage forms that contain them. Many excipients are very stable and may not require extensive testing to demonstrate continued conformance to appropriate specifications. Other excipients may undergo chemical, physical, and microbiological changes over time that cause the material to fall outside established specifications.

Appropriate expiration and/or recommended re-evaluation dates for excipients should be established from the results of a documented stability-testing program or from historical data. The testing program should include defined and controlled storage conditions (e.g., temperature and humidity), a consideration of different packaging types that may be used as market containers, and meaningful, specific test methods to adequately assess the stability characteristics of the excipient. Stability testing should determine whether possible degradation, moisture gain or loss, viscosity changes, or other possible changes occur to make the excipient unacceptable for use (e.g., unstable or hygroscopic materials). For additional information on excipient stability, see [Good Manufacturing Practices for Bulk Pharmaceutical Excipients](#) (1078).

The expiration date for an excipient is defined as the date after which the supplier recommends that the material should not be used. Prior to the assigned expiration date, the excipient is expected to remain within established specifications, if stored according to the supplier's recommended conditions.

The recommended re-evaluation date for an excipient is the date suggested by the supplier after which the material should be re-evaluated to ensure continued compliance with specifications. Re-evaluation of the excipient may include physical inspection and appropriate chemical, physical, and microbiological testing. Prior to the re-evaluation date, the excipient is expected to remain within established specifications, provided it has been stored according to the supplier's recommended conditions. But beyond the recommended re-evaluation date, the excipient should not be used without adequate evaluation at appropriate intervals, to determine whether the material continues to be acceptable for use. The recommended re-evaluation date differs from the expiration date in that the excipient may be re-evaluated to extend the length of time the material may be used, if supported by the results of the evaluation and appropriate stability data.

In reporting the expiration and recommended re-evaluation dates, the excipient supplier is providing important information to the user about the stability of the material. As discussed previously, the assignment of an expiration date and a recommended re-evaluation date should be based on appropriate evaluation of potential changes that may occur in the material's properties. It is acceptable to report both an expiration date and a recommended re-evaluation date on the COA for excipients, if applicable, but both dates may not always be required. Expiration and recommended re-evaluation dates should not be reported by a supplier without sufficient stability data or product history to support the assigned dates.

For excipients determined to be very stable (greater than 2 years), either the specific expiration date and/or the recommended re-evaluation date should be reported on the COA for the material, or a general stability statement may be included (e.g., stability greater than 2 years). If available data indicate that an excipient has limited stability (2 years or less) under anticipated storage conditions, a specific expiration date and/or recommended re-evaluation date should be reported on the COA for the material.

If data from formalized stability studies are not available for an excipient, an appropriate statement should be included on the COA to indicate what is known about the stability of the material and whether stability studies are in progress.

**Date Retested**— If retesting is performed by an excipient supplier and the results are used to extend the length of time that the material may be used, the date retested should also be reported on the COA. The specific tests that were subject to retesting should be clearly identified, and the results obtained upon retesting should be reported. After retesting, a new recommended re-evaluation date should be reported on the COA.

**Additional Dates**— Other dates may appear on a COA, if desired by the excipient supplier or requested by the user. Examples include the release date, shipping date, date of testing, and date the COA was printed or approved. Any additional dates that appear on a COA for excipients should include a clear indication of what the date represents or means.

#### TESTING FREQUENCY

For the excipients listed in the USP–NF, the product specifications are set by the supplier to include all parameters listed in the monograph. It is not required that analysis of all specification parameters be made on each lot (see General Notices and Requirements). However, sufficient analysis and process validation data should exist to ensure that the lot meets all specifications before it is released. This is an established practice that has been successfully used in industry for many years. Periodic testing of all parameters should be performed to revalidate the control system. The frequency of these periodic tests should be determined by the suppliers on the basis of their understanding of the manufacturing control system. At a minimum, the parameters should be checked once a year.

For excipients that are not included in USP–NF, specifications should be set by the supplier to ensure that the quality of the material is maintained on a continuing basis and reflects both the excipient manufacturing process and inherent properties. The analytical methods used to evaluate the characteristics of noncompendial excipients may be the same as those contained in the compendia, or may be unique to the supplier or the material. The methods should be demonstrated to provide accurate, reproducible, and consistent results for the characteristic being tested. It may be appropriate for noncompendial excipients to have some tests performed at reduced frequency.

The excipient user should evaluate the supplier's specifications and methods to ensure that they are appropriate and acceptable for the quality control needed for the manufacturing process of their drug product. The user should determine which of the supplier's specifications and methods are required for release of the excipient for use in their process. If additional tests or alternative methods are required by the user, appropriate specifications and methods, along with responsibility for performing the testing, must be agreed upon by the excipient supplier and user.

#### Reduced Frequency Testing

When analysis of some parameters is carried out at a reduced frequency (for example, every 10th lot), this should be clearly stated on the COA. Each specific test subject to reduced frequency testing should be indicated. Reduced frequency testing should be used only for excipients manufactured using a stable process. There should be a sound technical basis and sufficient documentation to support testing any parameter at a reduced frequency. This would normally include the following points:

- Appropriate validation of the manufacturing process
- Process control—attribute charting (when appropriate)
- GMP controls

As part of the justification for reduced testing, it is important that there be assurances in place showing that the manufacturer's process complies with appropriate excipient GMP requirements.

Some tests, because of their significance, should always be performed on each lot, whereas others may be candidates for reduced frequency testing. Attribute testing results in



qualitative data that provide pass/fail results or results expressed as less than or greater than a specified value. The result merely establishes compliance with a specification parameter. There are no data to indicate how well the material complies, as would be obtained from variable or quantitative test results.

Reduced frequency testing of an attribute requires that the manufacturer show that the qualitative parameter is in a state of statistical control. This necessitates tabulating the test results for consecutive lots produced.

**Skip-Lot Testing**— Skip-lot testing may be applied to an excipient that is made by either a batch or a continuous process. Various commonly accepted statistical sampling plans may be used to demonstrate appropriate process control. Examples of each are listed below.

**example 1:** For an average outgoing quality level (AOQL) of 1% and a test frequency of 1 in 10, the supplier should find 100 consecutive lots in conformance. At a 2% AOQL and a test frequency of 1 in 10, the supplier would test 50 consecutive lots. For a 1% AOQL and a 1 in 5 test frequency, the supplier would test 70 consecutive lots. Nomographs are available to determine the test requirements.

**example 2:** When the excipient is manufactured by a continuous process, no discrete lot is produced. The sampling plan again is based upon the risk of approving a lot that was nonconforming. By testing 140 consecutive lots before going to a test frequency of 1 in 10, the plan establishes a low risk of approving a lot that is noncompliant.

Once the requirement is met, the supplier can monitor conformance to the specification parameter by testing 1 in 10 lots. Should any lot fail the analysis, the supplier should return to 100% testing until the results once again meet the specification above.

Because excipients vary greatly in chemical and physical properties, the supplier of the excipient should determine which tests should be routinely performed and which tests may be appropriate for reduced frequency testing. This determination must be justified and documented on the basis of the adequacy of the supplier's control system. Documentation should be kept detailing the assumptions and the data supporting the skip-lot testing plan.

**Type A and Type B Tests**— Only certain types of tests are appropriate for reduced frequency testing. Type A is defined as tests that may not be easily controlled through standard process control techniques or that may change with time. These tests should normally be performed on each lot. Type B is defined as tests that normally can be controlled using standard process control techniques and that are not expected to change with time. These tests are candidates for reduced frequency testing. Examples of both types of tests are listed below.

**TYPE A: EXAMPLES OF TESTS THAT TYPICALLY NEED TO BE PERFORMED ON EVERY LOT**

- Identification—Required by GMPs for users (candidate for reduced frequency testing by suppliers)
  - Assay—Critical quality parameter (if specified)
  - Viscosity—Usually indicates grade
- Loss on drying (or moisture determination)—Indication of stability and appropriate process controls
  - Color—Indication of stability and appropriate process controls
  - pH—Indication of stability and appropriate process controls

**TYPE B: EXAMPLES OF TESTS THAT MAY BE CANDIDATES FOR REDUCED FREQUENCY TESTING**

- Manufacturing impurities—Based on starting materials and processes (e.g., Chloride, Sulfate, Nitrate, Glyoxal)
  - Heavy metals
    - Lead
    - Arsenic
  - Residue on ignition
  - Residual solvents

This is not meant to be an exhaustive list of tests. It simply provides some direction on how a supplier can assess the importance of each test to the overall control of the process. Tests listed as possible candidates for reduced frequency testing (Type B) may need to be routinely tested (Type A), depending on the raw materials and process. Determinations can also be made for some Type A tests to become Type B tests. In a dedicated facility, identification testing by the supplier may not be necessary.

**Documentation**— The supplier of an excipient should develop and maintain documentation that outlines the process control systems and validation data to justify the use of reduced frequency testing. This documentation should also include procedures for handling the impact of significant changes on the reduced frequency testing program.

The minimum number of lots to be fully tested for all specification parameters after a change has been made depends on the process and the significance of the change and should be based on sound statistical considerations.

Additionally, the documentation should contain procedures for re-evaluating the reduced frequency testing program when a testing failure occurs. Decisions regarding the continuance of reduced frequency testing should be justified on the basis of the reasons for the failure and the supplier's ability to provide assurances that the reduced frequency testing program or other in-process parameters would identify these types of failures in the future.

**Justifications for Reduced Frequency Testing**— The following are examples of situations where a sound technical basis can be demonstrated and where reduced frequency testing might therefore be justified. [note—There may be other such examples.]

- An impurity, by-product, or unreacted raw material could not be present in the product because the raw materials and chemical reactions used could not contain or generate such substances above the specified limits.
- The process capability index ( $C_p$ ) on the relevant parameter is high and based on a stable process. Statistical analysis of the reduced frequency data should show that the property remains stable and within specifications. A process is considered stable when the output of the process, regardless of the nature of the processing (batch or continuous), can be demonstrated by appropriate means to show a level of variability that consistently meets all aspects of the stated specification (both Pharmacopeia-specific and customer-specific) and is thus acceptable for its intended use. For continuous processing, it is also important to demonstrate that the material has been produced under conditions in which the process has achieved a form of "steady state", i.e., in which there is minimal operator intervention and in which the in-process parameters have been stabilized (see Appendix 2 for further definition of this concept and for determining levels of control).
- For a continuous process, the in-process analyses show that the property that is determined at reduced frequency is stable and within specification. Repeating the test on each lot would be redundant.
- An analysis that is determined on every lot has been shown to strongly correlate with an analysis that is run at a reduced frequency. The correlation shows that if a lot is within specification on the first analysis, it will be within specification on the second analysis.

#### USE OF ELECTRONIC SIGNATURES

Because of the growing dependence on computers and the need to accommodate paperless record systems, an electronic alternative to handwritten records and signatures is suggested. Excipient suppliers have added computer information systems to enhance productivity.

The primary issue with transfer of a COA without a handwritten signature is the validation of data. There are several considerations that should be met before an electronic signature or name attachment to a COA is considered acceptable.

- Computer systems access must be limited to authorized individuals: access is gained only after inputting a user name and a password. The system should require frequent changes of each individual password.
  - A confirmation of the integrity and accuracy of the information stored in the system should be completed.
- The operation of the system must be checked routinely to ensure that the correct information is transferred from the database to the printed record.
  - Data entered into a database from which information is extracted for a COA should be accompanied by time- and date-stamped audit trails.

When these criteria are met, the issuance of COAs with electronic signatures or the responsible person's name attached to the document, in lieu of a handwritten signature, is acceptable. [note—Computer systems are currently regulated by 21 CFR 11 of the FDA. Users should monitor the FDA's approach to compliance in this area.]



## DISTRIBUTOR INFORMATION

The presentation of a COA issued by a distributor presents some challenges. Because COAs are important documents characterizing the excipients and the state of their quality, the source of that information becomes very important to the end user(s). Because distributors take on different roles in fulfilling the services for which they are contracted, it is necessary to ensure that procedures and methods are appropriate for the functions performed.

Distributors may function in a number of different capacities relating to the movement of excipients and to services associated with their production. Some are simply pass-through locations in which nothing is done to the excipient with the exception of storage and handling. Others serve as extensions of the manufacturer's process by taking bulk quantities and repackaging them for the manufacturer. Still others purchase excipients and repackage them under a different label for sale and distribution. These scenarios should be understood and properly documented with programs that will protect the integrity and safety of the excipients as they move through the distribution process.

**Original Manufacturer and Manufacturing Site**— The identity of the original manufacturer and the manufacturing site should be included on the COA for excipients. This information is important because it provides traceability for specific excipient lots and assures the excipient users that they are consistently obtaining material from the same manufacturer and site.

Reporting the identity and location of the manufacturer does not represent an issue when the original manufacturer is also the direct supplier of the excipient to the pharmaceutical customers. However, it is recognized that this information may be considered proprietary by an excipient distributor. To adequately address this issue, excipient distributors should either list the specific information identifying the original manufacturer and location or provide the information by reporting an appropriate code, which is assigned in order to unambiguously identify the original manufacturer and manufacturing site. To protect the secrecy of this information, the meaning of the code does not have to be revealed to intermediary distributors.

**Certificate of Analysis Data**— When a distributor is primarily used as a pass-through of the excipient without any changes to the excipient and packaging, the COA that accompanies the excipient from the manufacturer can be passed on in the original form. If the data are extracted, translated, or rewritten on other letterhead, a system should be in place to check the rewritten information, and justification should be demonstrated upon request. Alternatively, the source of the data should be indicated on the document.

For a distributor that takes bulk quantities of an excipient from a manufacturer and introduces the bulk quantities into a process (e.g., conveyance and storage system), analysis of the packaged excipient should be performed to demonstrate the same quality as the lot (batch) introduced. Appropriate analytical data should be included on the COA to verify the quality.

The distributor should use equivalent methodology and equipment for the analytical evaluation. Some data may be used from the original manufacturer's COA with appropriate justification.

In all scenarios, it is expected that the distributor will have the appropriate level of GMP in place.

## APPENDIX 1

## DEFINITIONS

**Acceptance Criteria**— The specifications and acceptance or rejection limits—such as acceptable quality level or unacceptable quality level with an associated sampling plan—that are necessary for making a decision to accept or reject a lot or batch of raw material, intermediate, packaging material, or excipient.

**Batch (or Lot)**— A defined quantity of excipient processed so that it could be expected to be homogeneous. In a continuous process, a batch corresponds to a defined portion of the production, based on time or quantity (e.g., vessel's volume, 1 day's production, etc.).

**Batch Number (or Lot Number)**— A unique and distinctive combination of numbers and/or letters from which the complete history of the manufacture, processing, packaging, coding, and distribution of a batch can be determined.

**Batch Process**— A manufacturing process that produces the excipient from a discrete supply of raw materials that is present before the completion of the reaction.

**Certificate of Analysis (COA)**— A document relating specifically to the results of testing a representative sample drawn from the batch of material to be delivered.

**Chemical Property**— A quality parameter that is measured by chemical or physicochemical test methods.

**Continuous Process**— A manufacturing process that continually produces the excipient from a continuous supply of raw material.

**Contract Facility**— An internal or external facility that provides services to the manufacturer or distributor of an excipient. These can include, but are not limited to, the following: manufacturing facilities, laboratories, repackaging facilities (including labeling), and warehouses.

**Date of Manufacture**— A date indicating the completion of the final manufacturing process (as defined by the supplier for the particular excipient and process).

**Date Retested**— The date when retesting is performed by an excipient supplier to extend the length of time that the material may be used.

**Distributor**— A party other than the manufacturer who sells the excipient.

**Excipient**— Any substance, other than the active pharmaceutical ingredient or drug product, that has been appropriately evaluated for safety and is included in a drug delivery system to aid the processing of the drug delivery system during manufacture; to protect, support, or enhance stability, bioavailability, or patient acceptability; to assist in product identification; or to enhance any other attribute of the overall safety and effectiveness of the drug delivery system during storage or use.

**Expiration Date**— The date after which the supplier recommends that the material should not be used.

**Impurity**— Any component of an excipient that is not the intended chemical entity but is present as a consequence of either the raw materials used or the manufacturing process.

**Lot**— See Batch.

**Lot Number**— See Batch Number.

**Manufacturer**— A party who performs the final processing step.

**Packaging**— The container and its components that hold the excipient for storage and transport to the customer.

**Periodic Testing Program**— See Skip-Lot Testing Program.

**Physical Property**— A quality parameter that can be measured solely with mechanical equipment.

**Process**— The set of operating instructions describing how the excipient is to be synthesized, isolated, purified, etc.

**Process Capability Index (Cp)**— A statistical measurement that can be used to assess whether the process is adequate to meet specifications. A state of statistical control can be said to exist if the random variation in test results for a process parameter is such that the calculated process capability is greater than 1.33 (see Appendix 2 for further definition).

**Process Step**— An instruction to the excipient manufacturing personnel directing that an operation be performed.

**Recommended Re-Evaluation Date**— The date suggested by the supplier when the material should be re-evaluated to ensure continued compliance with specifications. Differs from the Expiration Date in that the excipient may be re-evaluated to extend the length of time the material may be used, if supported by the results of the evaluation and appropriate stability data.

**Reduced Frequency Testing Program**— See Skip-Lot Testing.

**Repackaging**— Transfer of an excipient from one container to another.

**Reprocessing**— Introducing previously processed material that did not conform to standards or specifications back into the process and repeating steps that are already part of the normal manufacturing process.



Significant Change— Any change that alters an excipient's physical or chemical property from the norm or that is likely to alter the excipient's performance in the dosage form.

Site— A location where the excipient is manufactured. This may be within the facility but in a different operational area, or at a remote facility, including a contract manufacturer.

Skip-Lot Testing Program— Periodic or intermittent testing performed for a particular test parameter that is justified by historical data demonstrating a state of statistical process control.

Specification— The quality parameters to which the excipient, component, or intermediate must conform and that serve as a basis for quality evaluation.

Stable Process— A process whose output, regardless of the nature of the processing (batch or continuous), can be demonstrated by appropriate means to show a level of variability that consistently meets all aspects of the stated specification (both USP-specific and customer-specific) and is thus acceptable for its intended use.

Supplier— A manufacturer or distributor who directly provides the excipient to the user.

User— A party who uses an excipient in the manufacture of a drug product or another excipient.

## APPENDIX 2

### STATE OF STATISTICAL CONTROL: PROCESS CAPABILITY PARAMETERS FOR DETERMINING LEVELS OF CONTROL

A process is considered to be in a state of statistical control if variations among the observed sampling results from the process can be attributed to a constant system of chance causes. Process capability index ( $C_p$ ) or capability index adjusted for the process average ( $C_{pk}$ ) or performance index ( $P_p$ ) or performance index adjusted for the process average

( $P_{pk}$ ) can be used to assess whether the process is adequate to meet specifications. Values of these parameters exceeding 1.33 show that the process is adequate to meet specifications. Values between 1.00 and 1.33 indicate that the process, although adequate to meet specifications, will require close control. Values below 1.00 indicate that the process is not adequate to meet specifications and that the process and/or specifications should be changed.  $P_p/P_{pk}$  will always be less than or equal to  $C_p/C_{pk}$ , respectively. The essential

difference between the capability and the performance indices is the data used. Capability indices require the calculation of  $\sigma$ , the population standard deviation, whereas the performance indices require the calculation of  $s$ , the sample standard deviation. Thus for pharmaceutical excipients a state of statistical control can be said to exist if the random variation in test results for a process parameter is such that the calculated process capability index or performance index is greater than 1.33.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Robert H. Lafaver, B.A.</a> Scientist 1-301-816-8335	(EGC05) Excipient General Chapters

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### 1081 GEL STRENGTH OF GELATIN

Pipet 105 mL of water at  $10^{\circ}$  to  $15^{\circ}$  into a standard Bloom bottle, add 7.5 g of Gelatin, and stir. Allow to stand for 1 hour, then bring to a temperature of  $62^{\circ}$  in 15 minutes by placing in a water bath regulated at  $65^{\circ}$  (the substance may be swirled several times to aid solution). Finally mix by inversion, allow to stand for 15 minutes, and place in a water bath at  $10 \pm 0.1^{\circ}$ . Chill, without disturbance, for 17 hours. Determine the gel strength in a Bloom Gelometer (a device developed to make this determination under standardized conditions) adjusted for 4-mm depression and to deliver  $200 \pm 5$  g of shot per 5 seconds, using the 12.7-mm diameter (nonbeveled) plunger.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Hong Wang, Ph.D.</a> Scientist 1-301-816-8351	(EGC05) Excipient General Chapters

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### 1086 IMPURITIES IN OFFICIAL ARTICLES

#### INTRODUCTION

This general information chapter covers impurities or degradation products in drug substances and degradation products in drug products. Impurities or degradation products in drug substances can arise during the manufacturing process or during storage of the drug substance. The degradation products in drug products can arise from drug substances or reaction products of the drug substance with an excipient or an immediate container–closure system. Biological, biotechnological, and radiopharmaceutical products are not covered in this chapter.

Concepts about purity change with time and are inseparable from developments in analytical chemistry. If a material previously considered to be pure can be resolved into more than one component, that material can be redefined into new terms of purity and impurity. Inorganic, organic, biochemical, isomeric, or polymeric components can all be considered impurities. Microbiological species or strains are sometimes described in similar terms of resolving into more than one component.

Communications about impurities or degradation products in compendial articles may be improved by including in this Pharmacopeia the definitions of terms and the contexts in which these terms are used. (See Definitions below.) There has been much activity and discussion in recent years about term definition. Certain industry-wide concerns about terminology and context deserve widespread publication and ready retrievability and are included here. See Foreign Substances and Impurities in the section Tests and Assays under General Notices and Requirements, as well as the general chapter [Ordinary Impurities](#) (466). Some other general chapters added over the years have also addressed topics of purity or impurity as these have come into focus or as analytical methodology has become available. Analytical aspects are enlarged upon in the chapter [Validation of Compendial Procedures](#) (1225).

Monographs for drug substances usually cite one of three types of purity tests: (1) a chromatographic purity test coupled with a nonspecific assay; (2) a chromatographic purity-indicating method that serves as the assay; or (3) a specific test and limit for a known impurity, an approach that usually requires a Reference Standard for that impurity. Modern separation methods clearly play a dominant role in scientific research today because these methods simultaneously separate and measure components and fulfill the analytical ideal of making measurements only on purified specimens. Nevertheless, the more classical methods based on titrimetry, colorimetry, spectrophotometry, single or multiple partitions, or changes in physical constants (or any other tests or assays) lose none of their previous validities. The purity profile of a specimen that is constructed from the results of experiments using a number of analytical methods is the ultimate goal.

Purity or impurity measurements for drug products present a challenge to Pharmacopeial standards-setting. Where degradation of a drug product over time is at issue, the same analytical methods that are stability-indicating are also purity-indicating. Resolution of the active ingredient(s) from the excipients necessary to the preparation presents the same qualitative problem. Thus, many monographs for Pharmacopeial preparations feature chromatographic assays. Where more significant impurities are known, some monographs set forth specific limit tests. In general, however, this Pharmacopeia does not repeat impurity tests in subsequent preparations where these appear in the monographs of drug substances and where these impurities are not expected to increase. It is presumed that adequate retention specimens are in storage for the exact batch of drug substances used in any specific lot of a drug product. Whenever analysis of an official article raises a question of the official attributes of any of the drug substances used, subsequent analysis of retention specimens is in order.



Pharmaceutical manufacturers interact with regulatory agencies in developing new drug substances and new drug products, and cooperate with the compendia in writing official monographs for the compendial articles the manufacturers produce. Establishment of impurity limits in drug substances and drug products should proceed on a rational basis so that everyone involved in the development and approval phases can carry on their work in a predictable manner. Although drug development in the United States is the primary focus of this section of the chapter, the subject also has broad applicability across national boundaries.

Manufacturers share with regulatory agencies and with the compendia the goal of making available to the public high-quality products that are both safe and efficacious. This goal continues to be achieved through rational approaches to the complex process of drug development. Tests used at all stages of drug development and marketing should not be interpreted individually but as a whole. Controls on raw materials and on manufacturing as well as those on drug substances, along with toxicological and clinical studies performed, ensure the safety and efficacy of drug products. It is more rational to identify impurities or degradation products and to set limits based on the factors detailed here, relying on the scientific judgments of manufacturers, the compendia, and regulators to arrive at sets of acceptable limits for identified and unidentified impurities or degradation products.

Limits are set for impurity levels or degradation products as one of the steps in ensuring the identity, strength, quality, and chemical purity of drug substances or drug products. The ultimate goal is to produce a final drug product of high quality that is safe and efficacious and remains so throughout its shelf life. The setting of limits for impurities or degradation products in drug substances is a complex process that considers a number of factors:

1. the toxicology of a drug substance containing typical levels of impurities and/or the toxicology of impurities relative to a drug substance;
2. the route of administration, e.g., oral, topical, parenteral, or intrathecal;
3. the daily dose, i.e., frequency and amount (micrograms or grams) administered of a drug substance;
4. the target population (age and disease state), e.g., neonates, children, or senior citizens;
5. the pharmacology of an impurity, when appropriate;
6. the source of a drug substance, e.g., synthetic, natural product, or biotechnology;
7. the duration of therapy, i.e., administration over a long period (treatment of chronic conditions) versus administration intended for a short duration (treatment of acute conditions); and
8. the capability of a manufacturer to produce consistently high-quality material.

Concepts for setting impurity or degradation product limits in drug substances are based on chemistry and safety concerns. As such, limits for organic and inorganic impurities and residual solvents should be established for drug substances. The basic tenet for setting limits is that levels of impurities or degradation products in a drug substance must be controlled to ensure its safety and quality throughout its development to use in a drug product.

For drug products the concept for setting degradation product limits is based on sound scientific judgment as applied to available data on the safety and stability of the drug product, which may include the degradation pathways of the drug substance, the manufacturing process, known excipient interactions, any safety assessment studies, stability studies conducted under the recommended storage conditions, and ancillary studies that may provide additional information on the stability profile of the drug product. A rationale should be provided for exclusion of those impurities that are not degradation products (e.g., process impurities from drug substance and impurities arising from excipients).

For drug substances and drug products, limits are appropriately set no higher than the level that can be justified (e.g., safety data, literature references, etc.) and no lower than the level achievable by the manufacturing process and analytical capability. Where there is no safety concern, limits should be based on (a) data generated on actual batches of the drug substance or drug product, allowing sufficient latitude to deal with normal process and analytical variation, and (b) stability characteristics.

Documented evidence that the analytical procedure used to evaluate impurities or degradation products is validated and suitable for the detection and quantification of impurities or degradation products should be established.

The setting of limits on impurities or degradation products is an evolutionary process, beginning in the United States before an investigational new drug (IND) is filed and continuing until well after the approval of a new drug application (NDA). Therefore, it is appropriate to address different stages in drug development as separate issues. There are three points in the drug development process where the setting of limits may be significantly different: (1) at the initial IND application, (2) at the filing of the NDA, and (3) after NDA approval. The filing of an abbreviated new drug application (ANDA) is another activity in which limits are set on impurities or degradation products. Since the approach may vary from that of filing an NDA, it is addressed as a separate issue. The underlying assumption is that the analytical methods used to evaluate impurities or degradation products are suitable for their intended purpose at each stage in the development.

#### INITIAL IND FILING

**Drug Substances**— At the initial IND filing, the chemical nature of a drug substance has generally been defined. The manufacturing process normally is in an early stage of development, and materials may be produced on a laboratory scale. Usually few batches have been made and, therefore, little historical data is available. The reference materials of a drug substance may be relatively impure. Limits for the purity of a drug substance are set to indicate drug quality. The setting of limits on related substances and process contaminants can be characterized as follows.

1. Limits are set on total impurities, and an upper limit may be set on any single impurity. The limit for total impurities should maintain, if possible, a nominal composition material balance.
2. Impurity profiles are documented. These are profiles of the lots of drug substances used in clinical studies and in toxicological studies that establish the safety of drug substances. The lots used in these studies should be typical products of the manufacturing process in use at that time.
3. Limits for residual solvents are based on the known toxicology of the solvents and on the manufacturing capabilities and dosing regimens.
4. General inorganic impurities are monitored by appropriate tests such as a heavy metals limit test and/or a test for residue on ignition. Traditional compendial limits are applied unless otherwise indicated. Specific residual metals that appear during manufacturing should be monitored by appropriate analytical techniques, and limits should be set based on the toxicological properties of these metals.
5. Appropriate limits are set for impurities known to be toxic.
6. If appropriate, enantiomeric purity is controlled.

Although water is not classified as an impurity, limits for water content may be needed to ensure the stability or ease of processing a drug substance.

**Drug Product**— At the initial IND filing, the dosage form of a drug product has been identified, which is appropriate for early clinical studies (and may or may not be representative of the drug product that will eventually be marketed). Usually, few batches have been made and, therefore, little historical data is available.

1. Qualitative and quantitative limits on degradation products may not be established at this point due to the limited data available. Typically, degradation products will be monitored as part of the stability evaluation of the drug product.
2. Dating for use of a drug product in clinical studies will be based on data from ongoing stability studies. If the data indicate the presence of degradation products, dating and storage conditions are controlled to ensure any degradation products are controlled within industry accepted limits or within limits established through safety assessment studies.
3. Limits for residual solvents, if appropriate, are based on the known toxicology of the solvents and on the manufacturing capabilities and dosing regimens. When amendments to the IND are filed, limits for impurities in drug substance and degradation products in drug substance or drug product may be updated based on additional data as they become available.

#### NDA FILING

**Drug Substances**— During the IND phases of drug development, the manufacturing process for a drug substance may undergo a number of revisions. Generally, the scale will have changed from laboratory size and will approach or reach full production batch size. A number of batches will normally have been produced, and a historical data base of the results of testing for impurities will exist. When significant changes in a manufacturing process are made, the impurity profile should be reviewed to determine if the toxicological studies are still supportive.

At the NDA stage a reference standard of defined purity is available, analytical methods have been validated, impurity and degradation profiles are known, and enantiomeric purity has



been evaluated. The setting of limits on related substances and process contaminants can be characterized as follows.

1. Consistency of the impurity profile of a drug substance has been established.
2. IND limits for total and individual impurities (identified and unidentified) are reviewed and adjusted based on manufacturing experience and toxicological data.
3. Impurities present in significant amounts are identified and individual limits are set. However, it is not always possible to identify or prepare authentic substances for impurities. The labile nature of some impurities precludes this possibility. Limits may be set on these substances based on comparison of lots produced and used in toxicological and clinical studies.
4. The impurity profiles of the lots designated for marketing should not be significantly higher than those of the lot(s) used for toxicological and clinical studies.
5. The composition material balance should be used, if possible, to evaluate the adequacy of the controls.
6. Limits for residual solvents are based on the known toxicology of the solvents and on the manufacturing capabilities and dosing regimens.
7. Limits are set for inorganic impurities by appropriate tests such as a heavy metals limit test and/or by a test for residue on ignition. Traditional compendial limits are applied unless otherwise indicated. Based on toxicological properties, limits may be set for specific residual metals that appear during manufacturing.
8. Additional guidance for setting limits can be found in various ICH and FDA guidance documents.

Drug Product— Similarly, for the drug product, the dosage form may change, the number or scale of batches may increase, and more stability data will have become available. Methods will have been validated. The setting of limits can be characterized as follows:

1. IND limits for total and individual degradation products (identified and unidentified) are reviewed and adjusted based on manufacturing experience, stability data, and toxicological data.
2. Degradation products present in significant amounts are identified and individual limits are set. However, it is not always possible to identify or prepare authentic substances for degradation products.
3. The degradation product profiles of the lots designated for marketing should not be significantly higher than those of the lot(s) used for toxicological and clinical studies.
4. The mass balance should be used, if possible, to evaluate the adequacy of the controls.
5. Limits for residual solvents, if appropriate, are based on the known toxicology of the solvents and on the manufacturing capabilities and dosing regimens.
6. Additional guidance for setting limits can be found in various ICH and FDA guidance documents.

#### POST NDA APPROVAL

After approval and marketing of a pharmaceutical product, significant changes may be made in manufacturing the drug substance. There may be technological, ecological, economic, or safety reasons for these changes. If they occur, the Pharmacopeial and NDA impurity and degradation product limits and rationale should be reviewed; the limits should be revised when indicated to ensure similar or improved quality of the drug substance or drug product.

#### ANDA FILING

The drug substance for a pharmaceutical product eligible for ANDA status is frequently an official article and should be well characterized analytically. Drug substances are typically available from multiple sources, and each source may have a different manufacturing process. Therefore, it is essential that the dosage form manufacturer evaluate each supplier's drug substance impurity or degradation profiles. Limits can then be set based on the more detailed concepts described for NDA filing, including review of compendial monographs for appropriateness.

#### DEFINITIONS

**Concomitant Components**— Concomitant components are characteristic of many drug substances and are not considered to be impurities in the Pharmacopeial sense. Limits on contents, or specified ranges, or defined mixtures are set forth for concomitant components in this Pharmacopeia. Examples of concomitant components are geometric and optical isomers (or racemates) and antibiotics that are mixtures. Any component that can be considered a toxic impurity because of significant undesirable biological effect is not considered to be a concomitant component.

**Foreign Substances (Extraneous Contaminants)**— Foreign substances (extraneous contaminants), which are introduced by contamination or adulteration, are not consequences of the synthesis or preparation of compendial articles and thus cannot be anticipated when monograph tests and assays are selected. The presence of objectionable foreign substances not revealed by monograph tests and assays constitutes a variance from the official standard. Examples of foreign substances include ephedrine in Ipecac or a pesticide in an oral liquid analgesic. Allowance is made in this Pharmacopeia for the detection of foreign substances by unofficial methods. (See Foreign Substances and Impurities in the section Tests and Assays under General Notices and Requirements.)

**Identified Impurities and Identified Degradation Products**— Impurities or degradation products for which structural characterizations have been achieved.

**Inorganic Impurities**— Inorganic impurities can result from the manufacturing process (e.g., residual metals, inorganic salts, filter aids, etc.). Inorganic impurities are typically controlled by tests such as [Heavy Metals](#) (231) and [Residue on Ignition](#) (281). Information found in [Plasma Spectrochemistry](#) (730) and [Ion Chromatography](#) (1065) may also be of value.

**Ordinary Impurities**— Some monographs make reference to ordinary impurities. For more details see [Ordinary Impurities](#) (466).

**Process Contaminants**— Process contaminants are identified or unidentified substances (excluding related substances and water), including reagents, catalysts, other inorganic impurities (e.g., heavy metals, chloride, or sulfate); and may also include foreign substances (extraneous contaminants). These contaminants may be introduced during manufacturing or handling procedures.

**Related Substances**— Related substances are structurally related to a drug substance. These substances may be (a) identified or unidentified impurities arising from synthesis manufacturing process such as intermediates or by-products and do not increase on storage or (b) identified or unidentified degradation products that result from drug substance or drug product manufacturing processes or arise during storage of a material.

**Residual Solvents**— Refer to [Residual Solvents](#) (467).

**Specified Impurities and Specified Degradation Products**— Previously referred to as Signal Impurities, specified impurities or specified degradation products are impurities or degradation products that are individually listed and limited with specific acceptance criteria in individual monographs as applicable. Specified impurities or specified degradation products can be identified or unidentified.

**Toxic Impurities**— Toxic impurities have significant undesirable biological activity, even as minor components, and require individual identification and quantification by specific tests. These impurities may arise out of the synthesis, preparation, or degradation of compendial articles. Based on validation data, individualized tests and specifications are selected. These feature comparison to a Reference Standard of the impurity, if available. It is incumbent on the manufacturer to provide data that would support the classification of such impurities as toxic impurities.

**Unidentified Impurities and Unidentified Degradation Products**— Impurities or degradation products for which structural characterizations have not been achieved and that are identified solely by qualitative analytical properties (e.g., chromatographic retention times).

**Unspecified Impurities and Unspecified Degradation Products**— Impurities or degradation products that are limited by general acceptance criteria but not individually listed with their own specific acceptance criteria in individual monographs.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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**1087 APPARENT INTRINSIC DISSOLUTION—DISSOLUTION TESTING PROCEDURES FOR ROTATING DISK AND STATIONARY DISK**

This general information chapter [Apparent Intrinsic Dissolution—Dissolution Testing Procedures for Rotating Disk and Stationary Disk](#) **1087** discusses the determination of dissolution rates from nondisintegrating compacts exposing a fixed surface area to a given solvent medium. Compact, as used here, is a nondisintegrating mass resulting from compression of the material under test using appropriate pressure conditions. A single surface having specified physical dimensions is presented for dissolution. Determination of the rate of dissolution can be important during the course of the development of new chemical entities because it sometimes permits prediction of potential bioavailability problems and may also be useful to characterize compendial articles such as excipients or drug substances. Intrinsic dissolution studies are characterization studies and are not referenced in individual monographs. Information provided in this general information chapter is intended to be adapted via a specific protocol appropriate to a specified material.

Dissolution rate generally is expressed as the mass of solute appearing in the dissolution medium per unit time (e.g., mass sec<sup>-1</sup>), but dissolution flux is expressed as the rate per unit area (e.g., mass cm<sup>-2</sup> sec<sup>-1</sup>). Reporting dissolution flux is preferred because it is normalized for surface area, and for a pure drug substance is commonly called intrinsic dissolution rate. Dissolution rate is influenced by intrinsic solid-state properties such as crystalline state, including polymorphs and solvates, as well as degree of noncrystallinity. Numerous procedures are available for modifying the physicochemical properties of chemical entities so that their solubility and dissolution properties are enhanced. Among these are coprecipitates and the use of racemates and enantiomeric mixtures. The effect of impurities associated with a material can also significantly alter its dissolution properties. Dissolution properties are also influenced by extrinsic factors such as surface area, hydrodynamics, and dissolution medium properties, including solvent (typically water), presence of surfactants, temperature, fluid viscosity, pH, buffer type, and buffer strength.

Rotating disk and stationary disk dissolution procedures are sufficiently versatile to allow the study of characteristics of compounds of pharmaceutical interest under a variety of test conditions. Characteristics common to both apparatuses include the following:

1. They are adaptable to use with standard dissolution testing stations, and both use a tablet die to hold the nondisintegrating compact during the dissolution test.
2. They rely on compression of the test compound into a compact that does not flake or fall free during the dissolution test.
3. A single surface of known geometry and physical dimension is presented for dissolution.
4. The die is located at a fixed position in the vessel, which decreases the variation of hydrodynamic conditions.

A difference between the two procedures is the source of fluid flow over the dissolving surface. In the case of the rotating disk procedure, fluid flow is generated by the rotation of the die in a quiescent fluid, but fluid flow is generated by a paddle or other stirring device for the stationary disk procedure.

#### EXPERIMENTAL PROCEDURE

The procedure for carrying out dissolution studies with the two types of apparatus consists of preparing a nondisintegrating compact of material using a suitable compaction device, placing the compact and surrounding die assembly in a suitable dissolution medium, subjecting the compact to the desired hydrodynamics near the compact surface, and measuring the amount of dissolved solute as a function of time.

Compacts are typically prepared using an apparatus that consists of a die, an upper punch, and a lower surface plate fabricated out of hardened steel or other material that allows the compression of material into a nondisintegrating compact. An alternative compaction apparatus consists of a die and two punches. Other configurations that achieve a nondisintegrating compact of constant surface area also may be used. The nondisintegrating compact typically has a diameter of 0.2 cm to 1.5 cm.

##### Compact Preparation

Attach the smooth lower surface plate to the underside of the die, or alternatively, insert the lower punch using an appropriate clamping system. Accurately weigh a quantity of material necessary to achieve an acceptable compact and transfer to the die cavity. Place the upper punch into the die cavity, and compress the powder on a hydraulic press at a compression pressure required to form a nondisintegrating compact that will remain in the die assembly for the length of the test. Compression for 1 minute at 15 MPa usually is sufficient for many organic crystalline compounds, but alternative compression conditions that avoid the formation of capillaries should be evaluated. For a given substance, the compact preparation, once optimized is standardized to facilitate comparison of different samples of the substance.

Changes in crystalline form may occur during compression; therefore, confirmation of solid state form should be performed by powder X-ray diffraction or other similar technique. Remove the surface plate or lower punch. Remove loose powder from the surface of the compact and die by blowing compressed air or nitrogen over the surface.

##### Dissolution Medium

The choice of dissolution medium is an important consideration. Whenever possible, testing should be performed under sink conditions to avoid artificially retarding the dissolution rate due to approach of solute saturation of the medium. Dissolution measurements are typically made in aqueous media. To approximate in vivo conditions, measurements may be run in the physiological pH range at 37°. The procedure when possible is carried out under the same conditions that are used to determine the intrinsic solubility of the solid state form being tested. Dissolution media should be deaerated immediately prior to use to avoid air bubbles forming on the compact or die surface.<sup>1</sup>

The medium temperature and pH must be controlled, especially when dealing with ionizable compounds and salts. In the latter cases, the dissolution rate may depend strongly on the pH, buffer species, and buffer concentration. A simplifying assumption in constant surface area dissolution testing is that the pH at the surface of the dissolving compact is the same as the pH of the bulk dissolution medium. For nonionizable compounds, this is relatively simple because no significant pH dependence on dissolution rate is expected. For acids and bases, the solute can alter the pH at and near the surface of the compact as it dissolves. Under these conditions, the pH at the surface of the compact may be quite different from the bulk pH due to the self-buffering capacity of the solute. To assess intrinsic solubility, experimental conditions should be chosen to eliminate the effect of solute buffering, alteration of solution pH, and precipitation of other solid state forms at the surface of the compact. For weak acids, the pH of the dissolution medium should be one to two pH units below the pKa of the dissolving species. For weak bases, the pH of the dissolution medium should be one to two pH units above the pKa of the dissolving species.

##### Apparatus

Rotating Disk—A typical apparatus ([Figure 1](#)) consists of a punch and die fabricated out of hardened steel. The base of the die has three threaded holes for the attachment of a surface plate made of polished steel, providing a mirror-smooth base for the compacted pellet. The die has a cavity into which is placed a measured amount of the material whose intrinsic dissolution rate is to be determined. The punch is then inserted in the die cavity and the test material is compressed with a hydraulic press. [note—A hole through the head of the punch allows insertion of a metal rod to facilitate removal from the die after the test.] A compacted pellet of the material is formed in the cavity with a single face of defined area exposed on the bottom of the die.

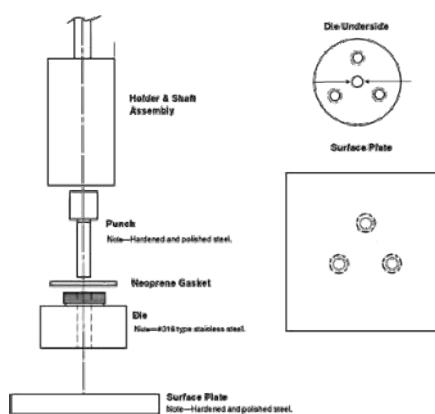


Figure 1

The die assembly is then attached to a shaft constructed of an appropriate material (typically steel). The shaft holding the die assembly is positioned so that when the die assembly is lowered into the dissolution medium (Figure 2) the exposed surface of the compact will be not less than 1.0 cm from the bottom of the vessel and nominally in a horizontal position. The die assembly should be aligned to minimize wobble, and air bubbles should not be allowed to form on the compact or die surface.

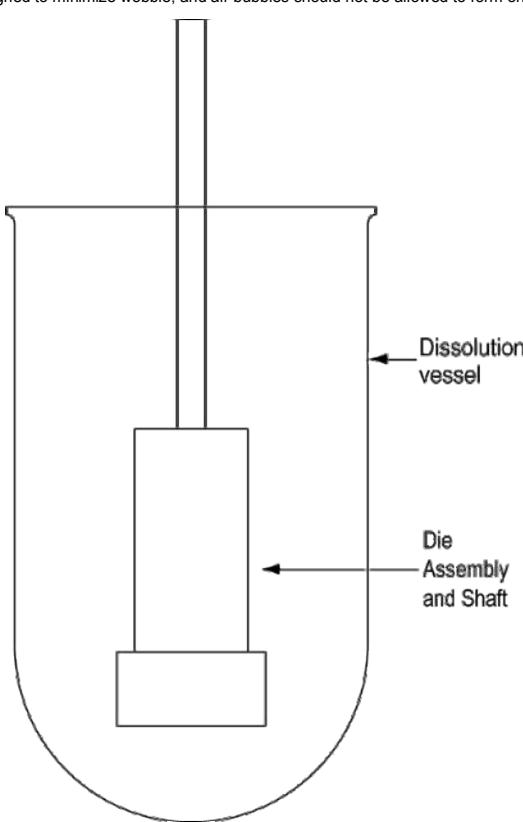


Figure 2

A rotating disk speed of 300 rpm is recommended. Typical rotation speeds may range from 60 rpm to 500 rpm. The dissolution rate depends on the rotation speed used. This parameter should be selected in order to admit at least five sample points during the test, but excessive stirring speeds may create shear patterns on the surface of the dissolving material that could cause aberrant results (i.e., nonlinearity). Typically, the concentration of the test specimen is measured as a function of time, and the amount dissolved is then calculated. The sampling interval will be determined by the speed of the dissolution process. If samples are removed from the dissolution medium, the cumulative amount dissolved at each time point should be corrected for losses due to sampling.

**Stationary Disk**— The apparatus (Figure 3) consists of a steel punch, die, and a base plate. The die base has three holes for the attachment of the base plate. The three fixed screws on the base plate are inserted through the three holes on the die and then fastened with three washers and nuts. The test material is placed into the die cavity. The punch is then inserted into the cavity and compressed, with the aid of a bench top press. The base plate is then disconnected from the die to expose a smooth compact pellet surface. A gasket is placed around the threaded shoulder of the die and a polypropylene cap is then screwed onto the threaded shoulder of the die.

The die assembly is then positioned at the bottom of a specially designed dissolution vessel with a flat bottom (Figure 4). The stirring unit (e.g., paddle) is positioned at an appropriate distance (typically 2.54 cm) from the compact surface. The die assembly and stirring unit should be aligned to ensure consistent hydrodynamics, and air bubbles should not be present on the compact surface during testing. Alternative configurations may be utilized if adequate characterization and control of the hydrodynamics can be established.

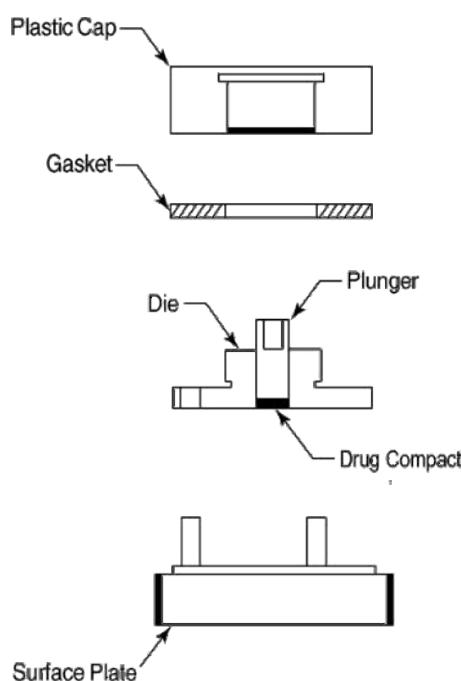


Figure 3

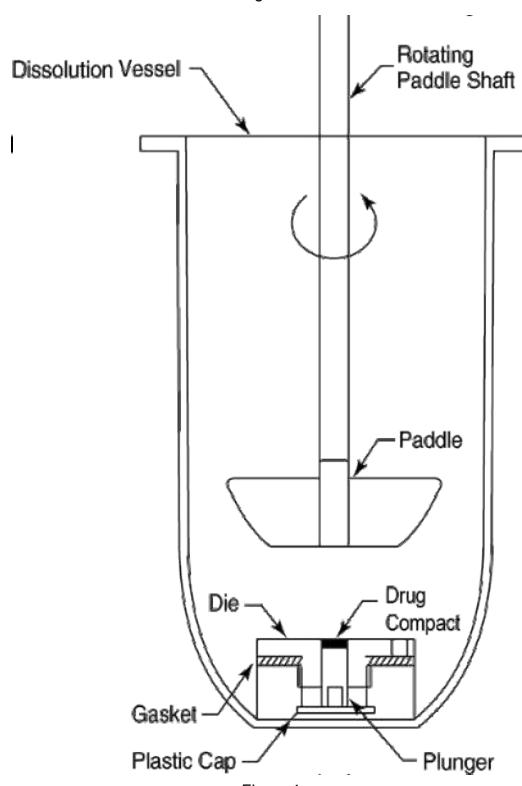


Figure 4

The dissolution rate depends on the rotation speed and precise hydrodynamics that exist. Typically, the concentration of the test specimen is measured as a function of time, and the amount dissolved is then calculated. The sampling interval will be determined by the speed of the dissolution process (see Rotating Disk). If samples are removed from the dissolution medium, the cumulative amount dissolved at each time point should be corrected for losses due to sampling.

#### DATA ANALYSIS AND INTERPRETATION

The dissolution rate is determined by plotting the cumulative amount of solute dissolved against time. Linear regression analysis is performed on data points in the initial linear region of the dissolution curve. The slope corresponds to the dissolution rate (mass sec<sup>-1</sup>). (More precise estimates of slope can be obtained using a generalized linear model that takes into account correlations among the measurements of the cumulative amounts dissolved at the various sampling times.)

The amount versus time profiles may show curvature. When this occurs, only the initial linear portion of the profile is used to determine the dissolution rate. Upward curvature (positive second derivative) of the concentration versus time data is typically indicative of a systematic experimental problem. Possible problems include physical degradation of the compact by cracking, delaminating, or disintegration. Downward (negative second derivative) curvature of the dissolution profile is often indicative of a transformation of the solid form of the compact at the surface or when saturation of the dissolution medium is inadvertently being approached. This often occurs when a less thermodynamically stable crystalline form converts to a more stable form. Examples include conversion from an amorphous form to a crystalline form or from an anhydrous form to a hydrate form, or the formation of a salt or a salt converting to the corresponding free acid or free base. If such curvature is observed, the crystalline form of the compact may be assessed by removing it from the medium and examining it by powder X-ray diffraction or another similar technique to determine if the exposed surface area is changing.

The constant surface area dissolution rate is reported in units of mass sec<sup>-1</sup>, and the dissolution flux is reported in units of mass cm<sup>-2</sup> sec<sup>-1</sup>. The dissolution flux is calculated by



...ding the dissolution rate by the surface area of the compact. Test conditions, typically a description of the apparatus, rotation speed, temperature, buffer species and strength, pH, and ionic strength should also be reported with the analyses.

- 1 One method of deaeration is as follows: Heat the medium, while stirring gently, to about 41°, immediately filter under vacuum using a filter having a porosity of 0.45 µm or less, with vigorous stirring, and continue stirring under vacuum for about 5 minutes. Other deaeration techniques for removal of dissolved gases may be used.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">William E. Brown</a> Senior Scientist 1-301-816-8380	(BPC05) Biopharmaceutics05

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#### 1088 IN VITRO AND IN VIVO EVALUATION OF DOSAGE FORMS

The Pharmacopeia provides for dissolution and drug release testing in the majority of monographs for solid oral and transdermal dosage forms. In recognition of the sensitivity of dissolution tests, where a valid bioavailability-bioequivalence (BA-BE) study is in hand, the policy of this Pharmacopeia has been to give this information dominant consideration in setting dissolution standards. Early practice was to develop dissolution requirements based on the in vitro performance of clinically successful formulations. Similarity in dissolution behavior has long been sought from the perspectives of both bioavailability and quality control considerations.

It is the goal of the pharmaceutical scientist to find a relationship between an in vitro characteristic of a dosage form and its in vivo performance. The earliest achievable in vitro characteristic thought to portend an acceptable in vivo performance was tablet and capsule disintegration. A test for disintegration was adopted in USP XIV (1950). At that time, no quantitative work was done in attempting to demonstrate such a relationship, especially in regard to in vivo product performance. However, advances in instrumental methods of analysis ultimately opened up prospects for this work. The disintegration test was recognized as being insufficiently sensitive by the USP-NF Joint Panel on Physiologic Availability, and in 1968 the Panel directed the identification of candidate articles for the first twelve official dissolution tests that used Apparatus 1.

The state of science is such that conduct of in vivo testing is necessary in the development and evaluation of dosage forms. Also, no product, including suspensions and chewable tablets, should be developed without dissolution or drug release characterization where a solid phase exists. This chapter sets forth, for products intended for human use, guidelines for characterizing a drug that include: (1) developing in vitro test methods for immediate-release and modified-release dosage forms, (2) designing in vivo protocols, and (3) demonstrating and assessing in vitro-in vivo correlations for modified-release dosage forms.

#### IN VITRO EVALUATION

##### Dissolution and Drug Release Testing—Method Development for Immediate-Release Dosage Forms

Dissolution testing is required for all solid oral Pharmacopeial dosage forms in which absorption of the drug is necessary for the product to exert the desired therapeutic effect. Exceptions are for tablets meeting a requirement for completeness of solution or for rapid (10 to 15 minutes) disintegration for soluble or radiolabeled drugs. The apparatus and procedure conform to the requirements and specifications given in the general chapter [Dissolution](#) (711). Generally, experiments are conducted at 37°.

The dissolution medium preferably is deaerated water or, if substantiated by the solubility characteristics of the drug or the formulation, a buffered aqueous solution (typically pH 4 to 8) or a dilute acid (0.001 N to 0.1 N hydrochloric acid) may be used. The usual volume of the medium is 500 to 1000 mL, with the use of greater volumes (up to 2000 mL) allowed for drugs having limited solubility. The quantity of medium used should be not less than 3 times that required to form a saturated solution of the drug substance. The significance of deaeration of the medium should be determined. Addition of solutes (i.e., surfactants) and electrolytes to aid in solubilization of the drug must be balanced against the loss of the discriminatory power of the test. The use of hydroalcoholic media is generally not favored. The use of such media should be supported by a documented in vitro-in vivo correlation. Conversely, it should be recognized that this discriminatory power could in some circumstances be excessive in that it may result in detection of differences in dissolution that are not clinically significant.

The choice of apparatus should be based on knowledge of the formulation design and actual dosage form performance in the in vitro test system. Since dissolution apparatuses tend to become less discriminating when operated at faster speeds, lower stirring speeds should be evaluated and an appropriate speed chosen in accordance with the test data. The most common operating speeds are 100 rpm for Apparatus 1 (basket) and 50 rpm for Apparatus 2 (paddle) for solid-oral dosage forms and 25 rpm for suspensions. A 40-mesh screen is used in almost all baskets, but other mesh sizes may be used when the need is documented by supporting data.

Apparatus 2 is generally preferred for tablets. Apparatus 1 is generally preferred for capsules and for dosage forms that tend to float or that disintegrate slowly. A sinker, such as a few turns of platinum wire, may be used to prevent a capsule from floating. Other types of sinker devices that achieve minimal coverage of dosage form surface are commercially available.

Where the use of a sinker device is employed, it is incumbent on the analyst to assure that the device used does not alter the dissolution characteristics of the dosage form.

Dissolution testing should be conducted on equipment that conforms to the requirements in the chapter [Dissolution](#) (711) and that has been calibrated with both the USP Salicylic Acid and Prednisone Calibrator Tablets. The method of analysis should be validated in accordance with the procedures given in the chapter [Validation of Compendial Procedures](#) (1225).

The test time is generally 30 to 60 minutes, with a single time point specification for pharmacopeial purposes. To allow for typical disintegration times, test times of less than 30 minutes should be based on demonstrated need. Industrial and regulatory concepts of product comparability and performance may require additional time points, and this may also be a feature required for product registration or approval. For registration purposes, a plot of percentage of drug dissolved versus time should be determined. Enough time points should be selected to characterize adequately the ascending and plateau phases of the dissolution curve.

Dissolution test times and specifications usually are established on the basis of an evaluation of dissolution profile data. Typical specifications for the amount of active ingredient dissolved, expressed as a percentage of the labeled content (Q), are in the range of 70% to 80% Q dissolved. A Q value in excess of 80% is not generally used, as allowance needs to be made for assay and content uniformity ranges.

For products containing more than a single active ingredient, dissolution normally should be determined for each active ingredient. Where a dissolution test is added to an existing monograph, the disintegration test is deleted. However, in the case of sublingual preparations, a short disintegration time may be retained as a monograph specification in addition to a dissolution requirement.

##### Dissolution and Drug Release Testing—Method Development for Modified-Release Dosage Forms

Drug release testing is required for all modified-release dosage forms in which absorption of the drug is necessary for the product to exert the desired therapeutic effect. The apparatus and procedure conform to the requirements and specifications given in the general chapter [Drug Release](#) (724).

The dissolution medium preferably is deaerated water or, if substantiated by the solubility characteristics of the drug or the formulation, buffered aqueous solutions (typically pH 4 to 8) or dilute acid (0.001 N to 0.1 N hydrochloric acid) may be used. (See above under Dissolution and Drug Release Testing—Method Development for Immediate-release Dosage Forms.) For modified-release dosage forms, the pH- and surfactant-dependence of the dosage form should be evaluated by in vitro testing in media of various compositions. The volume of medium will vary depending on the apparatus used and the formulation under test.

The choice of apparatus should be based on knowledge of the formulation design and actual dosage form performance in the in vitro test system. Apparatus 1 (basket) or Apparatus 2 (paddle) may be more useful at higher rotation frequencies (e.g., the paddle at 100 rpm). Apparatus 3 (reciprocating cylinder) has been found to be especially useful for bead-type modified-release dosage forms. Apparatus 4 (flow cell) may offer advantages for modified-release dosage forms that contain active ingredients having very limited solubility. Apparatus



(reciprocating disk) has been shown to have application to nondisintegrating oral modified-release dosage forms, as well as to transdermal dosage forms. Apparatus 5 (paddle over disk) and Apparatus 6 (cylinder) have been shown to be useful for evaluating and testing transdermal dosage forms.

At least three test times are chosen to characterize the in vitro drug release profile for Pharmacopeial purposes. Additional sampling times may be required for drug approval purposes. An early time point, usually 1 to 2 hours, is chosen to show that potential dose dumping is not probable. An intermediate time point is chosen to define the in vitro release profile of the dosage form, and a final time point is chosen to show essentially complete release of the drug. Test times and specifications are usually established on the basis of an evaluation of drug release profile data. For products containing more than a single active ingredient, drug release should be determined for each active ingredient.

Where a single set of specifications cannot be established to cover multisource monograph articles, application of a Case Three standard is appropriate. In Case Three, multiple drug release tests are included under the same monograph heading, and labeling requirements are included to indicate with which drug release test a specific product complies and, in some cases, the biological performance to be expected.

Drug release testing should be conducted on equipment that conforms to the requirements in the chapter [Drug Release](#) (724) and that has been calibrated with the appropriate USP calibrators. The method of analysis should be validated in accordance with the procedures given in the chapter [Validation of Compendial Procedures](#) (1225).

#### IN VIVO EVALUATION OF MODIFIED-RELEASE DOSAGE FORMS

In evaluating a modified-release product, a fundamental issue is the types of studies that should be performed to give reasonable assurance of safety and efficacy. While providing important information concerning the release characteristics of the drug from the dosage form, at present in vitro studies are most useful for such purposes as monitoring drug product stability and manufacturing process control. The assessment of safety and efficacy of a modified-release dosage form is best achieved through observing in vivo pharmacodynamics or pharmacokinetics. Moreover, where there is a well-defined, predictive relationship between the plasma concentrations of the drug or active metabolites and the clinical response (therapeutic and adverse), it may be possible to use plasma drug concentration data alone as a basis for the approval of a modified-release preparation that is designed to replace an immediate-release preparation.

The following guidelines are intended to provide guidance in drug substance evaluation and the design, conduct, and evaluation of studies involving modified-release dosage forms. While these guidelines will focus on oral drug delivery systems, the principles may be applicable to other routes of drug administration (e.g., transdermal, subcutaneous, intramuscular, etc.).

##### Characterization of Drug Substance

###### physicochemical properties

Physicochemical information necessary to characterize the drug substance in a modified-release dosage form should generally be no less than for the drug substance in an immediate-release dosage form. Additional physicochemical information may be needed on polymorphism, particle size distribution, solubility, dissolution rate, stability, and other release-controlling variables of the active drug entity under conditions that may react to the extremes of the physiologic environment experienced by the dosage form. For purposes of this chapter, active drug entity is taken to be the official drug substance.

###### pharmacokinetic properties

It is recommended to characterize thoroughly the input absorption profile of the active drug entity from a rapidly available preparation (an intravenous solution or oral solution or a well-characterized FDA-approved immediate-release drug product), which serves in turn as a reference to evaluate the input profile of the modified-release dosage form. This information together with the biological disposition characteristics for the active drug entity can characterize and predict changes in the bioavailability of the drug when input is modified as in the case of the modified-release dosage form. For example, if the active drug entity exhibits saturable first-pass hepatic metabolism, a reduction in systemic availability could result after oral administration if the input rate is decreased.

In designing an oral modified-release dosage form, it may be useful to determine the absorption of the active drug entity in various segments of the gastrointestinal tract (particularly in the colon in the case of dosage forms that may release drug in this region). The effects of food also may be important, and should be investigated.

###### disposition properties

The information required to characterize the processes of disposition of the active drug entity from a modified-release dosage form should include those generally determined for the same drug in an immediate-release dosage form. This may include the following:

1. Disposition parameters—clearance, volume of distribution, half-life, mean residence time, or model-dependent or noncompartmental parameters.
2. Linearity or characterization of nonlinearity over the dose or concentration range which could possibly be encountered.
3. Accumulation.
4. Metabolic profile and excretory organ dependence, with special attention to the active metabolites and active enantiomers of racemic mixtures.
5. Enter hepatic circulation.
6. Protein binding parameters and dialyzability.
7. The effects of age, gender, race, and relevant disease states.
8. Plasma/blood ratios.
9. A narrow therapeutic index or a clinical response that varies significantly as a function of the time of day.

###### pharmacodynamic properties

Prior to developing a modified-release dosage form, information described below should be gathered.

Concentration-response relationships should be available over a dose range sufficiently wide to encompass important therapeutic and adverse responses. In addition, the equilibration time<sup>1</sup> characteristics between plasma concentration and effect should be evaluated. These concentration-response relationships should be sufficiently characterized so that a reasonable prediction of the safety margin can be made if dose-dumping from the modified-release dosage form should occur. If there is a well-defined relationship between the plasma concentration of the active drug entity or active metabolites and the clinical response (therapeutic and adverse), the clinical performance of a new modified-release dosage form could be characterized by plasma concentration-time data. If such data are not available, clinical trials of the modified-release dosage form should be carried out with concurrent pharmacokinetic-pharmacodynamic measurements.

##### Characterization of the Dosage Form

###### physicochemical properties

The variables employed to characterize the physicochemical properties of the active drug entity as it exists or is discernible in the dosage form should be the same as those employed to characterize the drug substance. Solubility and dissolution profiles over a relevant pH range, usually from pH 1 to pH 7.4, should be obtained, with particular attention given to the effect of the formulation (as compared to the active drug entity). Characterization of formulations that are insoluble in aqueous systems may require the addition of sodium lauryl sulfate or other surfactant.

###### pharmacokinetic properties

The types of pharmacokinetic studies that should be conducted are a function of how much is known about the active drug entity, its clinical pharmacokinetic and biopharmaceutic properties, and whether pharmacokinetic studies are intended to be the sole basis for product approval. As a minimum, (1) a single-dose crossover study for each strength of a modified-release dosage form and (2) a multiple-dose, steady-state study using the highest strength of a modified-release dosage form are required to characterize the product. Some appropriate single-dose crossover and multiple-dose steady-state studies are described below.

In some modified-release capsule dosage forms, the strengths differ from each other only in the amount of identical beaded material contained in each capsule. In this case, a single-dose and a multiple-dose steady-state study at the highest dosage strength are sufficient. Other strengths may be characterized on the basis of comparative in vitro dissolution data.



The following pharmacokinetic studies would be needed for most modified-release dosage forms. Such studies may, in this instance, constitute the basis for characterization of the dosage form. If approval is to be sought without conducting clinical trials, it is recommended that there be preconsultation with the regulatory authorities to ensure that an adequate database exists for such approval.

The types of studies generally conducted can be categorized as follows.

Case A—

Case A applies to the original modified-release oral dosage form of an active drug entity already marketed in immediate-release form and for which extensive pharmacodynamic/pharmacokinetic data exist.

Single-dose Crossover Study—

A single-dose crossover study should include the following treatments: the modified-release dosage form administered under fasting conditions; a dosage form that is rapidly available administered under fasting conditions; and the modified-release dosage form administered at the same time as a high-fat meal (or another type of meal that has potential for causing maximum perturbation).

The study of food effects should include provision for control of the fluid intake (e.g., 6 to 8 oz.) and temperature (e.g., ambient) at the time of drug administration. The dosage form should be administered within 5 minutes after completion of the meal.

If there are no significant differences in the rate or extent of bioavailability (AUC, Cmax, and Tmax) as a function of the meal, then additional food effect studies are not necessary.

If significant differences in bioavailability are found, it is necessary to define how food affects the modified-release dosage form, as well as how the food-drug effect relates to time.

The purpose of these studies is twofold: first, to determine whether there is a need for labeling instructions describing special conditions for administration with respect to meals and second, to provide information concerning the pattern of absorption of the modified-release dosage form compared to that of the immediate-release dosage form. The drug input function should be defined for modified-release dosage forms.<sup>2</sup> This will aid in the development of an appropriate in vitro dissolution test. For dosage forms that exhibit high variability, replicate studies are recommended.

Use the following guidelines in evaluating food effect.

1. If no well-controlled studies have previously defined the effects of a concurrent high-fat meal on an immediate-release dosage form, studies should be performed to determine whether a food effect is a result of problems with the dosage form, i.e., food-related changes in release, or food effects that are unrelated to the dosage form, such as changes in the drug's absorption from the gastrointestinal tract or changes in the drug's disposition (i.e., distribution or elimination) that are independent of absorption. The cause of the food effect should be determined by conducting a single-dose crossover study comparing the solution (or immediate-release dosage form) under fed and fasting conditions. If there is no effect of food, then one would conclude that there are problems with the dosage form. If there is an effect of food, then one would conclude that these are unrelated to the dosage form.
2. The effect of timing on the food-drug effect should be tested by performing a four-way crossover study with the modified-release dosage form administered under the following treatment conditions: fasting, taken with a high-fat meal, 1 hour before a high-fat meal, and 2 hours after a high-fat meal.
3. If the food effect on an immediate-release dosage form is determined to result from changes in the dissolved drug's absorption from the gastrointestinal tract or from changes in drug disposition, studies should be designed to define the appropriate relationship between drug dosing and meals.
4. Alternative appropriate studies could be conducted if the applicant were to label the drug for administration with a meal that is not fat-loaded. In this case, an alternative meal composition should be considered.
5. The entire single-dose, modified-release absorption profile should be monitored. Where appropriate (e.g., in a multiple-dose study) for specific drugs and drug delivery systems, blood samples should be taken following breakfast on the second day, before the second dose is administered. This sampling schedule is particularly important for once-a-day products.
6. For delayed-release (enteric-coated) dosage forms, bioavailability studies to characterize adequately the food effects and to support the dosing claims stated in the labeling should be performed.

Multiple-dose, Steady-state Studies—

STUDY I— When data demonstrating linear pharmacokinetics exist for an immediate-release dosage form, a steady-state study should be conducted with the modified-release dosage form at one dose rate (preferably at the high end of the usual dose-rate range) using an immediate-release dosage form as a control. At least three trough-plasma drug concentration (Cmin) determinations should be made to ascertain that steady-state conditions have been achieved. Plasma-drug concentration determinations, over at least one dosing interval of the modified-release dosage form, should be made in each phase of the crossover study. It may be preferable (as in the case of rhythmic variation in absorption or disposition of the drug) to measure concentrations over an entire day in each phase. The presence or absence of circadian variation should be verified. The modified-release dosage form should produce an AUC that is equivalent to the immediate-release dosage form. The degree of fluctuation for the modified-release product should be the same as, or less than, that for the immediate-release dosage form given by the approved regimen. Appropriate concentration measurements should include unchanged drug and major active metabolites. For racemic drug entities, consideration should be given to the measurement of the active enantiomers [enantiomer/diastereomer distinction].

STUDY II— Where comparisons of the pharmacokinetic properties of an immediate-release dosage form at different doses are not available, or where the data show nonlinearity, steady-state crossover studies comparing effects of the modified-release dosage form with those of the immediate-release dosage form should be conducted at two different dose rates: one at the low end of the recommended dosing range and the second at the high end of the dosing range. In each case, the modified-release dosage form must meet the criteria described in Study I with respect to AUC and fluctuations in plasma drug concentrations. If there are significant differences between the modified-release dosage form and the immediate-release dosage form at either the low or the high dosing rate, these data alone are not adequate to characterize the product.

Data can be misleading when obtained from subjects with atypical drug disposition or physiologic characteristics, relative to the target population. Therefore, subject selection should be randomized or from an appropriate target population. If the modified-release dosage form is for use in a specific subpopulation (e.g., for children), it should be tested in that population. Regardless of whether a drug exhibits linear or nonlinear pharmacokinetics, the basis for characterization is equivalence of AUC and of the relative degree of fluctuation of concentrations of the modified-release and immediate-release dosage forms.

Steady-state studies in selected patient population groups or drug interaction studies may also be necessary, depending upon the therapeutic use of the drug and the types of individuals for whom the modified-release dosage form will be recommended. For drugs having narrow therapeutic indices, it may be necessary to perform more extensive plasma concentration measurements to determine the potential for unusual drug-release patterns in certain subpopulations. In such studies, it is advisable to perform more than one AUC measurement per patient to assess variability with both the modified-release and the immediate-release dosage forms.

Case B—

Case B applies to a non-oral, modified-release dosage form of an already marketed active drug entity for which extensive pharmacodynamic/pharmacokinetic data exist.

Case A studies (omitting the food effect studies) would be appropriate for the evaluation of a modified-release dosage form designed for a non-oral route of administration if the pattern of biotransformation to active metabolites is identical for the two routes. If the biotransformation patterns are different, then clinical efficacy studies should be performed with the modified-release dosage form. In addition, special studies may be necessary to assess specific risk factors related to the dosage form (e.g., irritation and/or sensitization at the site of application).

Case C—

Case C applies to a generic equivalent of an approved modified-release dosage form.

A generic equivalent of an approved modified-release dosage form should be bioequivalent to the standard modified-release dosage form in its rate and extent of availability (i.e., AUC, Cmax, Cmin, and degree of fluctuation) in crossover single-dose and steady-state studies. For an oral modified-release dosage form, the food studies described under Case A should



also be performed.

#### Statistical Analysis

An appropriate statistical method should be selected. (See [In Vivo Bioequivalence Guidances \(1090\)](#)).

The currently accepted criteria in the United States for equivalence for most dosage forms require that the mean pharmacokinetic parameters of the test dosage form should be within 80% to 125% of the reference dosage form using the 90% confidence interval (or, equivalently, the two-sided test procedure,  $P = 0.05$ ), and the upper and lower bounds must be within the 90% confidence interval.

### IN VITRO-IN VIVO CORRELATIONS

The term in vitro-in vivo correlation first appeared in pharmaceutical literature as a result of the awareness of the concepts of bioavailability and of in vitro dissolution rate determinations. The term in vitro-in vivo correlation refers to the establishment of a rational relationship between a biological property, or a parameter derived from a biological property produced by a dosage form, and a physicochemical property or characteristic of the same dosage form. The biological properties most commonly used are one or more pharmacokinetic parameters, such as  $C_{max}$  or AUC, obtained following the administration of the dosage form. The physicochemical property most commonly used is a dosage form's in vitro dissolution behavior (e.g., percent of drug released under a given set of conditions). The relationship between the two properties, biological and physicochemical, is then expressed quantitatively.

With the proliferation of modified-release products, it becomes necessary to examine the concept of in vitro-in vivo correlation in greater depth. Unlike immediate-release dosage forms, modified-release products cannot be characterized using a single-time point dissolution test. Furthermore, with a modified-release product a patient is to experience a specific plasma level curve covering a finite time period, usually 12 to 24 hours. There must be some in vitro means of assuring that each batch of the same product will perform identically in vivo. An in vitro-in vivo correlation would satisfy this need. Initially it was thought that developing a meaningful correlation for immediate-release dosage forms would be an easier task than for modified-release products. However, because of the nature of the principles upon which each type is based, it is believed that an in vitro-in vivo correlation is more readily defined for modified-release dosage forms.

#### Modified-Release Dosage Forms general considerations

Initial attempts at developing in vitro-in vivo correlations of modified-release products utilized the same concepts as those employed with immediate-release dosage forms. Thus, numerous attempts have been made to correlate one or more pharmacokinetic parameters, determined from in vivo studies of a product, with the amount released in a given time in an in vitro dissolution test. These were essentially single-point correlations. Such relationships might indicate that increasing or decreasing the in vitro dissolution rate of the modified-release dosage form would result in a corresponding directional change in the product's performance. However, they revealed little about the overall plasma level curve, which is a major factor for drug performance in the patient.

The recognition and utilization of deconvolution techniques as well as statistical moment calculations represented a major advance over the single-point approach in that these two methodologies utilize all of the dissolution and plasma level data available to develop the correlations. Therefore, there are at least three correlation techniques (i.e., deconvolution, statistical moment, and single point), available to the pharmaceutical scientist. There are marked differences in the quality of the correlation obtained with each procedure. Thus, these methods have been categorized and are discussed in terms of the advantages of each along with the resulting potential utility as a predictive tool for the pharmaceutical scientist.

### CORRELATION LEVELS

Three correlation levels have been defined and categorized in descending order of usefulness. The concept of correlation level is based upon the ability of the correlation to reflect the entire plasma drug concentration-time curve that will result from administration of the given dosage form. It is the relationship of the entire in vitro dissolution curve to the entire plasma level curve that defines the correlation.

Level A— This level is the highest category of correlation. It represents a point-to-point relationship between in vitro dissolution and the in vivo input rate of the drug from the dosage form. This latter factor is sometimes referred to as in vivo dissolution. In such a correlation, the in vitro dissolution and in vivo input rate curves are either directly superimposable or may be made to be superimposable by the use of a constant offset value. The mathematical description for both curves is the same. Such a procedure is most applicable to modified-release systems that demonstrate an in vitro release rate that is essentially independent of the typical dissolution media usually employed in pharmaceuticals. However, this is not a requirement for a Level A correlation. With this correlative procedure, a product's in vitro dissolution curve is compared to its in vivo input curve (i.e., the curve produced by deconvolution of the plasma level data). This may be done by use of mass balance model-dependent techniques, such as the Wagner-Nelson procedure or the Loo-Riegelman method, or by model-independent, mathematical deconvolution.

The advantages of a Level A correlation are as follows:

- A point-to-point correlation is developed. This is not found with any other correlation level. It is developed using every plasma level and dissolution point that has been generated. Thus, it reflects the complete plasma level curve. As a result, in the case of a Level A correlation, an in vitro dissolution curve can serve as a surrogate for in vivo performance. Therefore, a change in manufacturing site, method of manufacture, raw material supplies, minor formulation modification, and even product strength using the same formulation can be justified without the need for additional human studies.
- A truly meaningful (in vivo indicating) quality control procedure, which is predictive of a dosage form's performance, is defined for the dosage form.
- The extremes of the in vitro quality control standards can be justified by a convolution or deconvolution procedure.

Level B— Utilizes the principles of statistical moment analysis. The mean in vitro dissolution time is compared to either the mean residence time or the mean in vivo dissolution time. Like correlation Level A, Level B utilizes all of the in vitro and in vivo data but is not considered to be a point-to-point correlation because it does not reflect the actual in vivo plasma level curve, since there are a number of different in vivo curves that will produce similar mean residence time values. For this reason, unlike the case of a Level A correlation, one cannot rely upon a Level B correlation alone to justify formulation modification, manufacturing site change, excipient source change, etc. In addition, in vitro data from such a correlation could not be used to justify the extremes of quality control standards.

Level C— This category relates one dissolution time point ( $t_{50\%}$ ,  $t_{90\%}$ , etc.) to one pharmacokinetic parameter such as AUC,  $C_{max}$ , or  $T_{max}$ . It represents a single point correlation. It does not reflect the complete shape of the plasma level, which is the critical factor that defines the performance of modified-release products. Since this type of correlation is not predictive of actual in vivo product performance, it is generally only useful as a guide in formulation development or as a production quality control procedure. Because of its obvious limitations, a Level C correlation has limited usefulness in predicting in vivo drug performance and is subject to the same caveats as a Level B correlation in its ability to support product and site changes as well as justification of quality control standard extremes.

developing a correlation

This chapter does not define the only procedures for developing an in vitro-in vivo correlation, as any well-designed and scientifically valid approach would be acceptable. To assist the pharmaceutical scientist, one possible procedure for developing a Level A correlation is described below.

- The plasma level or urinary excretion data obtained in the definitive bioavailability study of the modified-release dosage form are treated by a deconvolution procedure. The resulting data may represent the drug input rate of the dosage form. It is also considered to represent in vivo dissolution when the rate-controlling step of the dosage form is its dissolution rate (i.e., drug absorption, after it has dissolved, is considered to be instantaneous). Any deconvolution procedure (i.e., mass balance or mathematical deconvolution) will produce acceptable results.
- The biobatch<sup>3</sup> is subjected to in vitro dissolution evaluation, and the effect of varying the dissolution conditions investigated. Some of the variables that can be studied are the apparatus (it is preferable to use official apparatus), mixing intensity, and dissolution medium (pH, enzymes, surfactants, osmotic pressure, ionic strength, etc.). It is not always necessary to study the dosage form's dissolution behavior under all of the conditions indicated. The number of conditions investigated will depend largely on whether a correlation can be found with the in vitro results obtained under the more commonly investigated conditions such as apparatus, agitation intensity, or dissolution medium and



pH value. Each formulation and every drug represents an individual challenge. The in vitro evaluation of the dosage form should be performed regardless of the correlation level being developed.

- The in vitro dissolution curve is then compared to the drug input rate curve. This can be performed by various methods. Simply positioning one curve on the other can often indicate the existence of a correlation. This may then be quantified by defining the equation for each curve and comparing the corresponding constants. The simplest way to demonstrate a correlation is to plot the fraction absorbed in vivo versus the fraction released in vitro. With a Level A correlation, this relationship is often linear with a slope of 1. The intercept may or may not be 0 depending upon whether there is a lag time before the system begins to release drug in vivo, or the absorption rate is not instantaneous resulting in the presence of some finite quantity of dissolved but unabsorbed drug. In either case, it is a point-to-point or a Level A correlation when the relationship is linear with a slope of 1. This indicates that the curves are essentially superimposable.
- If from the studies indicated in the in vitro dissolution evaluation above, the modified-release dosage form exhibits dissolution behavior that is independent of the variables studied, and a Level A correlation is demonstrated when the in vitro dissolution curve is compared to the drug input rate curve, it is likely that the correlation is general and can be extrapolated within a reasonable range for that formulation of the active drug entity. If, however, the dosage form exhibits dissolution behavior that varies with the in vitro conditions, it must be determined which set of dissolution conditions best correlates with in vivo performance. One can then establish whether the correlation is real or an artifact. This is achieved by preparing at least two formulations having significantly different in vitro behavior. One should demonstrate a more rapid release and the other a slower release than the biobatch. A pilot BA-BE study should be performed with these formulations, and the previously established correlation demonstrated for both. The formulation modifications of these batches should be based upon formulation factors that would be expected to influence the product's modified-release mechanism, and modification of these formulation factors are expected to influence the dosage form's release rate.
- Once a Level A correlation is established, it is possible that in vitro testing may be utilized for establishing the effects of manufacturing modifications such as minor formulation changes, manufacturing site and equipment change, alternative excipient suppliers, and a change in dosage form strength in the same formulation. It is questionable whether such an extrapolation with Level B and C correlations is possible.

#### Establishment of Dissolution Specification Ranges

It is relatively easy to establish a multipoint dissolution specification for a modified-release dosage form. The dissolution behavior of the biobatch may be used to define the amount to be released at each time point. The difficulty arises in the variation to be allowed around each time point. In the case of a Level A correlation, this may be done in two ways, both of which utilize the in vitro-in vivo correlation: convolution and deconvolution.

##### convolution

Reasonable upper and lower dissolution values are selected for each time point established from the biobatch. Historically, dissolution specifications have been selected by using the average dissolution of the development batches, with a range of  $\pm 2.5$  to 3 standard deviations. It is now expected that the average dissolution values are approximately the same as those of the biobatch. The dissolution curves defined by the upper and lower extremes are convoluted to project the anticipated plasma level curves that would result from administration of these formulations to the same panel to which the biobatch was administered. If the resulting plasma level data fall within the 95% confidence intervals obtained in the definitive BA-BE study, these ranges can be considered to be acceptable. An alternative acceptance approach that has been suggested is that when the therapeutic window for a drug has been defined, one may establish an upper and lower limit if the convolution results fall within the therapeutic window, even if they fall outside the confidence interval. If they fall outside the intervals, a more limited range must be established. This should be continued until the predicted values meet the desired ranges.

##### deconvolution

An acceptable set of plasma-level data is established both for a batch of material demonstrating a more rapid release and for one demonstrating a slower release than that of the biobatch. These may be selected by using the extremes of the 95% confidence intervals or  $\pm 1$  standard deviation of the mean plasma level. These curves are then deconvoluted, and the resulting input rate curve is used to establish the upper and lower dissolution specifications at each time point.

In the case of Level B and C correlations, batches of product must be made at the proposed upper and lower limits of the dissolution range, and it must be demonstrated that these batches are acceptable by performing a BA-BE study.

#### Immediate-Release Dosage Forms

##### general considerations

Since the mechanisms for release of drug from modified-release dosage forms are more complex and variable than those associated with immediate-release dosage forms, it would be anticipated that an in vitro-in vivo correlation would be easier to develop with the later formulations. Unfortunately, most of the correlation efforts to date with immediate-release dosage forms have been based on the correlation Level C approach, although there also have been efforts employing statistical moment theory (Level B). Although it is conceivable that the same Level A correlation approach may be utilized with immediate-release dosage forms, until data have been gathered to support this concept, Level B and Level C are the best approaches that can be recommended with these dosage forms.

1 Equilibration time is a measure of the time-dependent discontinuity between measured plasma concentrations and measured effects. The discontinuity is more often characterized by the degree of hysteresis observed when the effect-concentration plot for increasing concentrations is compared with that for decreasing concentrations. Where the equilibration time is very short (i.e., rapid equilibration with no active metabolites generated), there will be little or no hysteresis. That is, the same effect will be observed for a given concentration independent of the interval between the time of dosing and the time that measurements are made.

2 Wagner-Nelson, Loo-Riegelman, and other deconvolution methods are found in textbooks on biopharmaceutics.

3 The batch that was used in the pivotal bioavailability study.

#### Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">William E. Brown</a> Senior Scientist 1-301-816-8380	(BPC05) Biopharmaceutics05

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#### 1090 IN VIVO BIOEQUIVALENCE GUIDANCES<sup>1</sup>

This chapter is divided into two sections that provide guidances related to the general conduct of bioequivalence tests and to bioavailability protocols for specific drugs. These statements were prepared by the Food and Drug Administration, Office of Generic Drugs (FDA, OGD), Division of Bioequivalence, and presented herein. For general background information on related issues, refer to [In Vitro and In Vivo Evaluation of Dosage Forms](#) 1088.

#### DRUG PROTOCOLS

##### Alprazolam Tablets—In Vivo Bioequivalence and In Vitro Dissolution Testing<sup>4</sup>

Clinical Usage/Pharmacology—Alprazolam is widely used in the management of anxiety disorders, panic disorder, social phobia, and depression. It is a triazolo analog of the 1,4-benzodiazepine class of compounds acting on the central nervous system (CNS). The exact mechanism of action of alprazolam is unknown; however, published literature indicates that this class of drugs exerts effects by binding to stereospecific receptors at several sites within the central nervous system. The drug effect is dose-related. The daily dose of alprazolam depends on the individual patient, and averages 1.5 to 4 mg. Although a major metabolite,  $\alpha$ -hydroxyalprazolam, shows some pharmacological activity, no alprazolam metabolites possess any clinically important role, and they do not accumulate to any significant degree.



Pharmacokinetics— Alprazolam is readily absorbed (more than 90%) after oral administration and is distributed widely with a volume of distribution of approximately 1 L per kg in healthy men and women. Peak concentrations in the plasma occur 1 to 2 hours after administration. With doses ranging from 0.5 to 3.0 mg given on an empty stomach, plasma peak levels of 8.0 to 37 ng per mL were observed. The mean plasma elimination half-life of alprazolam has been reported to be about 11.2 hours (range: 6.3 to 26.9 hours) in healthy adults.

The drug absorption is slower when alprazolam is taken after a meal than on an empty stomach, but total absorption is unchanged.

in vivo bioequivalence studies<sup>5</sup>

Product Information—

FDA Designated Reference Product: Xanax® (Upjohn) 1-mg tablets.

Batch Size— The test batch or lot must be manufactured under production conditions and must be of a size that is at least 10% of the largest lot planned for full production or a minimum of 100,000 units, whichever is larger.

Potency— The assayed potency of the reference product should not differ from that of the test product by more than 5%.

Type of Study Required— A single-dose, randomized, fasting, two-period, two-treatment, two-sequence crossover study comparing equal doses of the test and reference products.

Recommended Protocol for Conducting a Single-Dose, Fasted Bioequivalence Study—

Objective— The objective is to compare the rate and extent of absorption of a generic formulation with that of a reference formulation when given as equal labeled doses.

Design— The study design is a single-dose, two-treatment, two-period, two-sequence crossover with a 1-week washout period between Phase I and Phase II dosing. An equal number of subjects should be randomly assigned to the two possible dosing sequences. Before the study begins, the proposed protocol should be approved by an institutional review board.

Facilities— The clinical facilities and analytical laboratory used for the study should be identified along with the names, titles, and curriculum vitae of the medical and scientific or analytical directors.

Selection of Subjects— The sponsors should enroll a number of subjects sufficient to ensure statistical validity of the study. It is recommended that a minimum of 24 subjects be used in this study. Subjects should be healthy, preferably nonsmoking, volunteers aged 18 to 50 years and within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). Subjects should be selected on the basis of acceptable medical history, physical examination, and clinical laboratory test results. Female subjects must be given a pregnancy test prior to beginning the study. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. If smokers are included, they should be identified as such. Written, informed consent must be obtained from all study participants before they are accepted into the study.

Procedure— Following an overnight fast of at least 10 hours, subjects should be administered a single dose of the test or reference product with 240 mL of water.

Restrictions— Study volunteers should observe the following restrictions:

- a. Water may be taken except for 1 hour before and after drug administration, when no liquid is allowed other than that needed for drug dosing.
- b. Subjects should fast for at least 4 hours after administration of the test or reference treatment. All meals should be standardized during the study, and the same meals should be served during both phases of the study.
- c. No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
- d. Subjects should take no Rx medications, including oral contraceptives, or OTC medications beginning 2 weeks before drug administration and until after the study is completed.

Blood Sampling— Venous blood samples should be collected pre-dose (0 hours) and at 0.25, 0.50, 0.75, 1.0, 1.50, 2.0, 2.50, 3.0, 4.0, 6.0, 8.0, 12, 24, 36, 48, and 72 hours post-dose. Plasma should be separated promptly and immediately frozen until assayed. Following at least a minimum 1-week washout period, subjects should begin the second phase of the study.

Analytical Methods— Alprazolam should be assayed using a suitable method fully validated with respect to adequate sensitivity, specificity, linearity, recovery, and accuracy and precision (both within and between days). Stability of the samples under frozen conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be determined. Chromatograms of the analysis of the unknown samples, including all associated standard curve and quality control chromatograms, should be available for regulatory authorities. The sponsor should justify the rejection of any analytical data and provide a rationale for selection of the reported values.

Statistical Analysis of Pharmacokinetic Data (Plasma)— See Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design under General Guidances.

Clinical Report, Side Effects, and Adverse Reactions— Subject medical histories, physical examinations and laboratory reports, and all incidents of possible adverse reactions to the study formulations should be reported.

Retention of Samples— The laboratory conducting the bioequivalence testing should retain an appropriately identified reserve sample of both the test and reference products used to perform the in vivo bioequivalence study for approval of the application. Each reserve sample should consist of at least 200 dosage units and should be retained for a period of not less than 5 years following approval of the application. For more information on retention of bioequivalence samples, refer to 21 CFR 320.63.

in vitro testing requirements

Dissolution Testing— Conduct dissolution testing on 12 dosage units of the test product and the reference product, employing the biostudy lots. The current official USP dissolution method (see [Dissolution](#) (711)) should be followed and should be referenced by the sponsor. The following method and tolerances are currently recommended for this product:

Medium: phosphate buffer solution (pH 6.0); 500 mL.

Apparatus 1: 100 rpm.

Times: 10, 20, 30, and 45 minutes.

Procedure— Use USP if available, or other validated method (see [Validation of Compendial Procedures](#) (1225)).

Tolerances (Q): not less than 80% in 30 minutes.

The percentage of label claim dissolved at each specified testing interval should be reported for each individual dosage unit. The mean percentage dissolved, the range (highest, lowest) of dissolution, and the coefficient of variation (relative standard deviation) should be reported.

Content Uniformity Test— Content uniformity testing on the test product lots should be performed as described in USP.

waiver requirements

Waiver of in vivo bioequivalence study requirements for the 0.25-, 0.5-, and 2-mg strengths of the generic product may be granted pursuant to 21 CFR 320.22(d)(2), provided the following conditions are met:

1. The 0.25-, 0.5-, and 2-mg strengths are proportionally similar in both active and inactive ingredients to another strength (1 mg), which has been demonstrated to be bioequivalent to a reference (1 mg) product in vivo.
2. The 0.25-, 0.5-, and 2-mg strengths of the generic product meet dissolution requirements.
3. The drug product follows linear kinetics over its range of available strengths.

Bumetanide Tablets—In Vivo Bioequivalence and In Vitro Dissolution Testing<sup>4</sup>

Clinical Usage/Pharmacology— Bumetanide is a potent diuretic indicated for the treatment of edema associated with congestive heart failure and hepatic and renal disease, including the nephrotic syndrome. Bumetanide is contraindicated in anuria, hepatic coma, states of severe electrolyte depletion, and in patients hypersensitive to the drug.



The major site of action of bumetanide is the ascending limb of the loop of Henle, where it inhibits the sodium-potassium-2 chloride absorptive pump. The usual daily dosing range for bumetanide is 0.5 to 2.0 mg.

**Pharmacokinetics**— Bumetanide is reported to be readily absorbed from the gastrointestinal (GI) tract with a  $T_{max}$  of 0.5 to 2 hours, and a bioavailability of about 80% to 90%. Several pharmacokinetic studies have shown that bumetanide, administered orally, is eliminated rapidly in humans, with a half-life of between 1 and 2 hours. Plasma protein-binding is approximately 95%. Oral administration of bumetanide resulted in 36% to 60% recovery of the unchanged drug from urine. The volume of distribution ( $V_d$ ) is approximately 0.2 L per kg. Following oral administration of bumetanide, the onset of diuresis occurs in 30 to 60 minutes. Peak activity is reached between 0.5 and 3 hours.

#### in vivo bioequivalence studies<sup>5</sup>

##### Product Information—

FDA Designated Reference Product: Bumex® (Roche Laboratories) 2.0-mg tablets.

**Batch Size**— The test batch or lot must be manufactured under production conditions and must be of a size that is at least 10% of the largest lot planned for full production or a minimum of 100,000 units, whichever is larger.

**Potency**— The assayed potency of the reference product should not differ from that of the test product by more than 5%.

**Type of Study Required**— A single-dose, randomized, two-period, two-treatment, two-sequence crossover study under fasting conditions comparing equal doses of the test and reference products.

##### Recommended Protocol for Conducting a Single-Dose, Fasted Bioequivalence Study—

**Objective**— The objective is to compare the rate and extent of absorption of a generic formulation with that of a reference formulation when given as equal labeled doses.

**Design**— The study design is a single-dose, two-treatment, two-period, two-sequence crossover with at least a three-day washout period between Phase I and Phase II dosing. An equal number of subjects should be randomly assigned to the two possible dosing sequences. Before the study begins, the proposed protocol should be approved by an institutional review board.

**Facilities**— The clinical facilities and analytical laboratory used for the study should be identified along with the names, titles, and curriculum vitae of the medical and scientific or analytical directors.

**Selection of Subjects**— The sponsor should enroll a number of subjects sufficient to ensure statistical validity of the study. It is recommended that a minimum of 24 subjects be used in this study. Subjects should be healthy, preferably nonsmoking, volunteers aged 18 to 50 years and within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). Subjects should be selected on the basis of acceptable medical history, physical examination, and clinical laboratory test results. Female subjects must be given a pregnancy test prior to beginning the study. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. If smokers are included, they should be identified as such. Written, informed consent must be obtained from all study participants before they are accepted into the studies.

**Procedure**— Following an overnight fast of at least 10 hours, subjects should be administered a single dose (2.0 mg) of the test or reference product with 240 mL of water. The subjects should drink 240 mL of water at the following times: -2, -1, 0, 1, 2, 4, 6, 8, and 10 hours relative to dosing.

##### Restrictions— Study volunteers should observe the following restrictions:

- a. Subjects should fast for at least 4 hours after administration of the test or reference treatment. All meals should be standardized during the study, and the same meals should be served during both phases of the study.
- b. No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
- c. Subjects should take no Rx medications, including oral contraceptives, beginning 2 weeks and no OTC medications beginning 1 week before drug administration and until after the study is completed.

**Blood Sampling**— Venous blood samples in a volume sufficient for sample analysis and anticoagulated as appropriate should be collected pre-dose (0 hours) and at 0.25, 0.50, 0.75, 1.0, 1.25, 1.50, 1.75, 2.0, 2.50, 3, 4, 5, 6, 8, and 12 hours post-dose. Plasma should be separated promptly, immediately frozen, and stored at  $-20^{\circ}\text{C}$  until assayed. Following a minimum 1-week washout period, subjects should begin the second phase of the study.

**Urine Sampling**— Urine may be collected over the following time intervals: -2 to 0, 0 to 1, 1 to 2, 2 to 4, 4 to 6, 6 to 8, 8 to 10, and 10 to 12 hours post-dosing. The urine volume and pH should be noted. One 50-mL aliquot should be stored refrigerated for possible analysis of sodium and potassium. A 15-mL sample should be stored frozen at  $-20^{\circ}\text{C}$  for possible bumetanide assay.

**Analytical Methods**— High-performance liquid chromatographic methods have been reported for assaying bumetanide in human plasma and urine.

Bumetanide should be assayed using a suitable method fully validated with respect to stability, sensitivity, specificity, linearity, recovery, and accuracy and precision (both within and between days). Stability of the samples under frozen conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be determined. Chromatograms of the analysis of the unknown samples, including all associated standard curve and quality control chromatograms, should be available for regulatory authorities. The sponsor should justify the rejection of any analytical data and provide a rationale for selection of the reported values.

**Statistical Analysis of Pharmacokinetic Data (Plasma)**— See Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design under General Guidances.

**Diuretic Effects and Bumetanide Urinary Excretion Data**— Urine samples may be analyzed to determine the following parameters:

- a. The mean rate of excretion of sodium, potassium, water, and bumetanide for each collection period.
- b. The maximum excretion rate (excretion rate calculated for that time interval during which the rate is highest) for sodium, potassium, water, and bumetanide.
- c. Time to maximum excretion rate for sodium, potassium, water, and bumetanide.
- d. Cumulative excretion of sodium, potassium, water, and bumetanide over all sampling time intervals.

**Clinical Report, Side Effects, and Adverse Reactions**— Subject medical histories, physical examinations and laboratory reports, and all incidents of possible adverse reactions to the study formulations should be reported.

**Retention of Samples**— The laboratory conducting the bioequivalence testing should retain an appropriately identified reserve sample of both the test product and the reference standard used to perform the in vivo bioequivalence studies for approval of the application. Each reserve sample should consist of at least 200 dosage units and should be retained for a period of not less than 5 years following approval of the application. For more information on retention of bioequivalence samples, refer to 21 CFR 320.63.

#### in vitro testing requirements

**Dissolution Testing**— Conduct dissolution testing on 12 dosage units of the test product and the reference product, employing the biostudy lots. The current official USP dissolution method (see [Dissolution](#) (711)) should be followed and should be referenced by the sponsor. The following method and tolerances are currently recommended for this product: Medium: water; 900 mL.

Apparatus 2: 50 rpm.

Times: 10, 20, 30, and 45 minutes.

**Procedure**— Use USP if available, or other validated method (see [Validation of Compendial Procedures](#) (1225)).

Tolerances (Q): not less than 85% in 30 minutes.



The percentage of label claim dissolved at each specified testing interval should be reported for each individual dosage unit. The mean percentage dissolved, the range (highest, lowest) of dissolution, and the coefficient of variation (relative standard deviation) should be reported.

Content Uniformity Test—Content uniformity testing on the test product lots should be performed as described in USP.

waiver requirements

Waiver of in vivo bioequivalence study requirements for the 0.5- and 1.0-mg strengths of the generic product may be granted pursuant to 21 CFR 320.22(d)(2), provided the following conditions are met:

1. The 0.5- and 1-mg tablets are proportionally similar in both active and inactive ingredients to the 2-mg tablet, which has been demonstrated to be bioequivalent to the reference 2-mg product in vivo.
2. The 0.5- and 1-mg tablets of the generic product meet dissolution test requirements.

#### Buspirone Hydrochloride Tablets—In Vivo Bioequivalence and In Vitro Dissolution Testing<sup>4</sup>

**Clinical Pharmacology/Usage**— Buspirone hydrochloride is an anti-anxiety agent. Clinically it is effective in the management of anxiety disorders or short-term relief of symptoms of anxiety. Buspirone has no anticonvulsant or muscle relaxant activity and has little sedative effect. It does not substantially affect psychomotor function. There is no evidence that the drug causes either physical or psychological dependence. The mechanism of action of buspirone is not known. Some in vitro preclinical studies indicate that buspirone has a high affinity for serotonin (5-HT1A) receptors and a moderate affinity for brain D2 receptors.

For the management of anxiety disorders, the usual initial adult dosage of buspirone is 10 to 15 mg daily, usually in 2 or 3 divided doses. Dosage is increased, as necessary, in increments of 5 mg daily to achieve an optimal therapeutic response. The maximum daily dose should not exceed 60 mg per day.

**Pharmacokinetics**— Buspirone is rapidly and almost completely absorbed from the GI tract. The drug undergoes extensive first-pass metabolism, with about 4% of a dose reaching the systemic circulation unchanged following oral administration. Following oral administration of a single dose of 20 mg in healthy volunteers, peak plasma buspirone concentrations of 1 to 6 ng per mL have been observed to occur within 40 to 90 minutes. Plasma concentrations of unchanged buspirone are low and exhibit substantial interindividual variation with oral administration of the drug. Approximately 95% of buspirone is bound to plasma proteins.

Buspirone is rapidly metabolized by oxidation to produce several hydroxylated derivatives and a pharmacologically active metabolite, 1-pyrimidinylpiperazine (1-PP). Because of rapid metabolism, less than 1% of the parent drug is excreted unchanged in the urine. The pharmacologically active metabolite has about 20% to 25% of the anxiolytic activity of buspirone. In humans, blood concentrations of 1-PP remain low even after chronic administration of buspirone. The contribution of 1-PP to the pharmacologic and toxic effect thus remains to be elucidated fully.

The average elimination half-life of unchanged buspirone after single doses of 10 to 40 mg is reported to be 2 to 3 hours. Buspirone exhibits linear kinetics following administration of doses (10 to 40 mg) in the therapeutic range. Although food increases the bioavailability of buspirone by decreasing first-pass metabolism, the total amount of drug (changed and unchanged) in plasma is not affected.

#### in vivo bioequivalence studies<sup>5</sup>

Product Information—

FDA Designated Reference Product: Buspar® (Bristol-Meyers Squibb) 10-mg tablet.

**Batch Size**— The test batch or lot must be manufactured under production conditions and must be of a size at least 10% that of the largest lot planned for full production or a minimum of 100,000 units, whichever is larger.

**Potency**— The assayed potency of the reference product should not differ from that of the test product by more than 5%.

#### Types of Studies Required—

1. A single-dose, randomized, two-period, two-treatment, two-sequence crossover study under fasting conditions comparing equal doses of the test and reference products.
2. A single-dose, randomized, three-treatment, three-period, six-sequence crossover, limited food effects study comparing equal doses of the test and reference products when administered immediately following a standard high-fat breakfast.

#### Recommended Protocol for Conducting a Single-Dose, Fasted Bioequivalence Study—

**Objective**— The objective is to compare the rate and extent of absorption of buspirone and 1-PP from a generic formulation with that from a reference formulation when given as equal labeled doses.

**Design**— The study design is a single-dose, two-treatment, two-period, two-sequence crossover with a 1-week washout period between Phase I and Phase II dosing or a single-dose, two-treatment, four-period, four-sequence replicate design study with a 1-week washout period between each phase of dosing. An equal number of subjects should be randomly assigned to each of the two possible dosing sequences. Before the study begins, the proposed protocol should be approved by an institutional review board.

**Facilities**— The clinical facilities and analytical laboratory used for the study should be identified along with the names, titles, and curriculum vitae of the medical and scientific or analytical directors.

**Selection of Subjects**— The sponsor should enroll a number of subjects sufficient to ensure statistical validity of the study. Subjects should be healthy, preferably nonsmoking, volunteers aged 18 to 55 and within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). Subjects should be selected on the basis of acceptable medical history, physical examination, and clinical laboratory test results. Female subjects must be given a pregnancy test prior to beginning the study. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. If smokers are included, they should be identified as such. Written, informed consent must be obtained from all study participants before they are accepted into the study.

**Procedure**— Following an overnight fast of at least 10 hours, subjects should be administered four 10-mg tablets of the test or reference product with 240 mL of water.

**Restrictions**— Study volunteers should observe the following restrictions:

- a. Water may be taken, except for 1 hour before and after drug administration, when no liquid is allowed other than that needed for drug dosing.
- b. Subjects should fast for at least 4 hours after administration of the test or reference treatment. All meals should be standardized during the study, and the same meals should be served during both phases of the study.
- c. No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
- d. Subjects should take no Rx medications, including oral contraceptives, beginning 2 weeks and no OTC medications beginning 1 week before drug administration and until after the study is completed.

**Blood Sampling**— Venous blood samples should be collected pre-dose (0 hours) and at 0.25, 0.50, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 7, 8, 12, and 24 hours post-dose. Plasma should be separated promptly and immediately frozen until assayed. Following a minimum 1-week washout period, subjects should begin the second phase of the study.

**Subject Monitoring**— Blood pressure and pulse rate should be monitored hourly during the first 4 hours of the study. Subjects with a heart rate less than 45 bpm or greater than 110 bpm should have an electrocardiogram (lead II) performed and have their pulse monitored hourly. Subjects should report any unusual symptoms observed during the study. Subjects should be periodically questioned during each phase of the study for any unusual symptoms experienced after drug administration.

**Analytical Methods**— Buspirone and 1-PP should be assayed using a suitable method fully validated with respect to adequate sensitivity, specificity, linearity, recovery, accuracy, and precision (both within and between days). Stability of the samples under frozen conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be determined.



...chromatograms of the analysis of the unknown samples, including all associated standard curve and quality control chromatograms, should be available for regulatory authorities. The sponsor should justify the rejection of any analytical data and provide a rationale for selection of the reported values.

Statistical Analysis of Pharmacokinetic Data— See Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design under General Guidances.

**Limited Food Effects Study—**

**Objective**— The objective is to compare the rate and extent of absorption of buspirone and 1-PP from a generic formulation with that from a reference formulation under nonfasting conditions and to compare the rate and extent of absorption of the drug from a generic formulation under fasting and nonfasting conditions when given as equal labeled doses.

**Design**— The study design is a single-dose, three-treatment, three-period, six-sequence crossover with a 1-week washout period between Phase I and Phase II, and Phase II and Phase III.

The limited food effects study (with a minimum of 12 subjects) should be performed in the same manner as the single-dose study under fasting conditions, with the following exceptions:

**Procedure**— An equal number of subjects should be assigned to each of the 6 dosing sequences possible in a three-treatment, three-period study design. Each subject will receive the following treatments:

TREATMENT 1: Generic product, four 10-mg tablets administered after a standard high-fat breakfast.

TREATMENT 2: Reference product, four 10-mg Buspar® tablets administered after a standard high-fat breakfast.

TREATMENT 3: Generic product, four 10-mg tablets administered under fasting conditions.

Following a 10-hour fast, the subjects receiving treatments under fed conditions should be served a standard high-fat breakfast. The subjects should have 30 minutes to finish the entire breakfast and then should immediately receive Treatment 1 or Treatment 2, taken with 240 mL of water. Subjects receiving the treatment under fasting conditions should receive Treatment 3, taken with 240 mL of water only. The same lots of the test and reference products should be used in the fasted and fed studies. No food should be allowed for at least 4 hours post-dose, with water allowed after the first hour. Subjects should be served scheduled standardized meals throughout the study, and the same meals should be served during all phases of the study.

**Statistical Analysis**— In general, a comparable food effect will be assumed if the AUC0-T, AUC0-, and Cmax mean values for the test product administered under fed conditions are within 20% of the respective mean values obtained for the reference product administered under fed conditions.

**Clinical Report, Side Effects, and Adverse Reactions**— Subject medical histories, physical examinations and laboratory reports, and all incidents of possible adverse reactions and side effects to the study formulations should be reported.

**Retention of Samples**— The laboratory conducting the bioequivalence testing should retain appropriately identified reserve samples of both the test and reference products used to perform the in vivo bioequivalence studies for approval of the application. Each reserve sample should consist of at least 200 dosage units or 5 times the amount needed for complete release testing, whichever is greater, and should be retained for a minimum of 5 years following approval of the application. For more information on retention of bioequivalence samples, refer to 21 CFR 320.63.

**in vitro testing requirements**

**Dissolution Testing**— Conduct dissolution testing on 12 dosage units of the test and reference products, employing the biostudy lots. The current official USP dissolution method (see [Dissolution \(711\)](#)) should be followed and should be referenced by the sponsor. The following times should be used to determine the dissolution profile. Times: 10, 20, 30, and 45 minutes.

The percentage of label claim dissolved at each specified testing interval should be reported for each individual dosage unit. The mean percentage dissolved, the range of dissolution (highest, lowest), and the coefficient of variation (relative standard deviation) should be reported.

**Content Uniformity Test**— Content uniformity testing on the test and reference product lots should be performed as described in USP.

**waiver requirements**

Waiver of in vivo bioequivalence study requirements for the 5-mg strength of the generic product may be granted pursuant to 21 CFR 320.22(d)(2) provided the following conditions are met:

1. The 5-mg tablet is proportionally similar in both active and inactive ingredients to the firm's 10-mg tablet, which has been demonstrated to be bioequivalent to the reference product in vivo.
2. The 5-mg tablet of the generic product meets dissolution test requirements.

**Captopril Tablets—In Vivo Bioequivalence and In Vitro Dissolution Testing<sup>4</sup>**

**Clinical Usage/Pharmacology**— Captopril, an antihypertensive, inhibits the enzyme that converts angiotensin I, a relatively inactive decapeptide, to angiotensin II, a potent endogenous vasoconstrictor substance. This enzyme, angiotensin converting enzyme, or ACE, is a peptidyldipeptide carboxy hydrolase.

The recommended initial dosage for treatment of hypertension is 25 mg twice a day (b.i.d.) or three times a day (t.i.d.). This can be increased to 50 mg b.i.d. or t.i.d. after 1 or 2 weeks if the lower dose is ineffective. Doses of captopril higher than 50 mg b.i.d. are recommended only with concomitant administration of a thiazide diuretic.

**Pharmacokinetics**— Following oral administration, approximately 60% to 75% of the dose of captopril is rapidly absorbed from the gastrointestinal tract in fasting healthy adults or hypertensive patients. Peak blood levels of unchanged captopril occur about 1 hour after oral administration. Areas under the concentration-time curve and maximum blood concentrations after single oral doses of captopril appear to be dose-related over a range of 10 to 100 mg. Approximately 25% to 30% of the drug in the systemic circulation is bound to plasma proteins.

Because the presence of food in the GI tract is reported to reduce absorption of the drug by 30% to 40%, captopril is labeled to be dosed 1 hour before meals. Blood pressure reduction is usually maximal 60 to 90 minutes post-dose. The elimination half-life of captopril is reported to be about 2 hours.

About half the absorbed dose of captopril is rapidly metabolized, mainly to captopril-cysteine disulfide and to the disulfide dimer of captopril. Captopril and its metabolites are excreted in the urine. Renal excretion of unchanged captopril occurs via tubular secretion. In patients with normal renal function, more than 95% of the absorbed dose is excreted in the urine in 24 hours. About 40% to 50% of the drug excreted in the urine is unchanged captopril.

**in vivo bioequivalence study<sup>5</sup>**  
Product Information—

1. FDA Designated Reference Product: Capoten® (Squibb) 100-mg tablets.
2. **Batch Size**—The test batch or lot must be manufactured under production conditions and must be of a size at least 10% that of the largest lot planned for full production or a minimum of 100,000 units, whichever is larger.
3. **Potency**—The assayed potency of the reference product should not differ from that of the test product by more than 5%.

**Type of Study Required**— A single-dose, randomized, two-period, two-treatment, two-sequence crossover study under fasting conditions comparing 100-mg doses of the test and reference products is required.

**Recommended Protocol for Conducting a Single-Dose Bioequivalence Study Under Fasting Conditions**—

**Objective**— The objective is to compare the rate and extent of absorption of a generic formulation with that of a reference formulation when given as equal labeled doses.



Design—The study design is a single-dose, two-treatment, two-period, two-sequence crossover with a 1-week washout period between Phase I and Phase II dosing. An equal number of subjects should be randomly assigned to each of the two possible dosing sequences. Before the study begins, the proposed protocol should be approved by an institutional review board.

Facilities—The clinical facilities and analytical laboratory used for the study should be identified along with the names, titles, and curriculum vitae of the medical and scientific or analytical directors.

Selection of Subjects—The sponsor should enroll a number of subjects sufficient to ensure statistical validity of the study. It is recommended that a minimum of 24 subjects be used in this study. Subjects should be healthy volunteers aged 18 to 50 years and within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). Subjects should be selected on the basis of acceptable medical history, physical examination, and clinical laboratory test results. Female subjects must be given a pregnancy test prior to beginning the study. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. Written, informed consent must be obtained from all study participants before they are accepted into the studies.

Procedure—Following an overnight fast of at least 10 hours, subjects should be given a single 100-mg dose of the test or reference product with 240 mL of water. Subject blood pressure and pulse rate should be measured immediately before drug administration (pre-dose) and at 0.5, 1, 1.5, 2, 3, 4, 6, and 8 hours post-dose.

Restrictions—Study volunteers should observe the following restrictions:

- a. Water may be taken except for 1 hour before and after drug administration, when no liquid is allowed other than that needed for drug dosing.
- b. Subjects should fast for at least 4 hours after administration of the test or reference treatment. All meals should be standardized during the study, and the same meals should be served during both phases of the study.
- c. No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
- d. Subjects should take no Rx medications, including oral contraceptives, beginning 2 weeks and no OTC medications beginning 1 week before drug administration and until after the study is completed.

Blood Sampling—Venous blood samples should be collected pre-dose (0 hours) and at 0.25, 0.50, 0.75, 1, 1.25, 1.50, 1.75, 2, 2.50, 3, 3.50, 4, 4.50, 5, 6, 7, 8, and 10 hours post-dose. Whole blood or blood plasma may be used for the assay. In either case, the blood samples should be chemically stabilized immediately after collection (see Analytical Methods below).

If plasma is to be assayed, it should be separated promptly from the stabilized whole blood. The whole blood or blood plasma samples should then be immediately frozen until assayed. Following a minimum 1-week washout period, subjects should begin the second phase of the study.

Analytical Methods—Captopril should be measured in an appropriate biological matrix such as whole blood or blood plasma. Several assay methods are available. The method chosen should be fully validated with respect to adequate sensitivity, specificity, linearity, recovery, and accuracy and precision (both within and between days). Because the thiol group of captopril is readily oxidized, a chemical stabilizer must be added to the blood samples immediately after collection. Stability of the samples under frozen storage conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be determined. Chromatograms of the analysis of the unknown samples, including all associated standard curve and quality control chromatograms, should be available for regulatory authorities. The sponsor should justify the rejection of any analytical data and provide a rationale for selection of the reported values.

Statistical Analysis of Pharmacokinetic Data—See Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design under General Guidances.

Clinical Report, Side Effects, and Adverse Reactions—Subject medical histories, physical examinations and laboratory reports, and all incidents of possible adverse reactions to the study formulations should be reported.

Retention of Samples—The laboratory conducting the bioequivalence testing should retain an appropriately identified reserve sample of both the test and reference products used to perform the in vivo bioequivalence study for approval of the application. Each reserve sample should consist of at least 200 dosage units and should be retained for a period of not less than 5 years following approval of the application. For more information on retention of bioequivalence samples, refer to 21 CFR 320.63.

#### in vitro testing requirements

Dissolution Testing—Conduct dissolution testing on 12 dosage units of the test product versus 12 units of the reference product. The biostudy lots should be used for those product strengths tested in vivo. The current official USP dissolution method (see [Dissolution \(711\)](#)) should be followed and should be referenced by the sponsor. The following method and tolerances are currently recommended for this product:

Medium: 0.1 N hydrochloric acid; 900 mL.

Apparatus 1: 50 rpm.

Times: 10, 20, and 30 minutes.

Procedure—Use USP if available, or other validated method (see [Validation of Compendial Procedures \(1225\)](#)).

Tolerances (Q): not less than 80% in 20 minutes.

The percentage of label claim dissolved at each specified testing interval should be reported for each individual dosage unit. The mean percentage dissolved, the range (highest, lowest) of dissolution, and the coefficient of variation (relative standard deviation) should be reported.

Content Uniformity Test—Content uniformity testing on the test product lots should be performed as described in USP.

#### waiver requirements

Waiver of in vivo bioequivalence study requirements for the 12.5-, 25-, and 50-mg strengths of the generic product may be granted pursuant to 21 CFR 320.22(d)(2), provided the following conditions are met:

1. The lower strengths are proportionally similar in both active and inactive ingredients to the 100-mg strength, which has been demonstrated to be bioequivalent to the reference product in vivo.
2. The 12.5-, 25-, and 50-mg strengths of the generic product meet the dissolution test requirements.

#### Cefaclor Capsules and Suspensions—In Vivo Bioequivalence and In Vitro Dissolution Testing<sup>4</sup>

Clinical Usage/Pharmacology—Cefaclor is a cephalosporin antibiotic that inhibits bacterial cell-wall synthesis in a manner similar to that of penicillin. Cefaclor is used in the treatment of otitis media caused by susceptible *Streptococcus pneumoniae*, *Haemophilus influenzae* (including ampicillin-resistant strains), *staphylococci*, and group A  $\beta$ -hemolytic streptococci; of lower respiratory tract infections (including pneumonia) caused by susceptible *Streptococcus pneumoniae*, *Haemophilus influenzae*, or group A  $\beta$ -hemolytic streptococci; of upper respiratory tract infections (including pharyngitis and tonsillitis) caused by susceptible group A  $\beta$ -hemolytic streptococci; of urinary tract infections (including pyelonephritis and cystitis) caused by susceptible *Escherichia coli*, *Proteus mirabilis*, *Klebsiella*, or *staphylococci*; or of skin and skin structure infections caused by susceptible *Staphylococcus aureus* or group A  $\beta$ -hemolytic streptococci.

The usual adult dosage is 250 mg every 8 hours. For more severe infections (such as pneumonia), doses may be doubled.

Pharmacokinetics—Cefaclor is well absorbed after oral administration to fasting subjects. Total absorption is similar in the fasted and the fed states. When it is taken with food, the peak concentration achieved is 50% to 75% of that observed in fasting subjects and generally appears about 1 hour later.

Following administration of 250-mg, 500-mg, and 1-g doses to fasting subjects, average peak serum levels of approximately 7, 13, and 23  $\mu$ g per mL, respectively, were obtained within 30 to 60 minutes. Approximately 60% to 85% of the drug is excreted unchanged in urine within 8 hours, the major portion being excreted within the first 2 hours. The serum elimination half-life in subjects with normal renal function is 0.6 to 0.9 hour. In patients with severely reduced renal function, the plasma elimination half-life of the drug is 2.3 to 2.8



hours. Hemodialysis shortens the half-life by 25% to 30%.

[in vivo bioequivalence studies<sup>5</sup>](#)

Product Information—

1. FDA Designated Reference Product: Cedlor® (Eli Lilly) 500-mg capsules; or powder for oral suspension containing 375 mg per 5 mL (Eli Lilly).
2. Batch Size—The test batch or lot must be manufactured under production conditions and must be of a size at least 10% that of the largest lot planned for full production or a minimum of 100,000 capsules, whichever is larger.
3. Potency—The assayed potency of the reference product should not differ from that of the test product by more than 5%.

Types of Studies Required—

1. A single-dose, randomized, two-period, two-treatment, two-sequence crossover study under fasting conditions comparing 500-mg doses of the test and reference capsules and 375-mg-per-5-mL doses of the test and reference suspensions is required.
2. A single-dose, randomized, three-treatment, three-period, six-sequence, crossover, limited food effects study comparing equal doses of the test product under fasting conditions as well as the test and reference products when administered immediately following a standard high-fat breakfast<sup>6</sup> is required.

Recommended Protocol for Conducting a Single-Dose Bioequivalence Study under Fasting Conditions—

Objective— The objective is to compare the rate and extent of absorption of a generic formulation with that of a reference formulation when given as equal labeled doses.

Design— The study design is a single-dose, two-treatment, two-period, two-sequence crossover with a 1-week washout period between Phase I and Phase II dosing. An equal number of subjects should be randomly assigned to the two possible dosing sequences. Before the study begins, the proposed protocol should be approved by an institutional review board.

Facilities— The clinical facilities and analytical laboratory used for the study should be identified along with the names, titles, and curriculum vitae of the medical and scientific or analytical directors.

Selection of Subjects— The sponsor should enroll a number of subjects sufficient to ensure statistical validity of the study. It is recommended that a minimum of 24 subjects be used in this study. Subjects should be healthy volunteers aged 18 to 50 years and within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). Subjects should be selected on the basis of acceptable medical history, physical examination, and clinical laboratory test results. Female subjects must be given a pregnancy test prior to beginning the study. Subjects with any current or past medical condition, including sensitivity to cephalosporin or penicillin analogues, that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. Written, informed consent must be obtained from all study participants before they are accepted into the study.

Procedure— Following an overnight fast of at least 10 hours, subjects should be administered a single dose of the test or reference product with 240 mL of water.

Restrictions— Study volunteers should observe the following restrictions:

1. Water may be taken except for 1 hour before and after drug administration when no liquid is allowed other than that needed for drug dosing.
2. Subjects should fast for at least 4 hours after administration of the test or reference treatment. All meals should be standardized during the study, and the same meals should be served during both phases of the study.
3. No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
4. Subjects should take no Rx medications, including oral contraceptives, or OTC medications beginning 2 weeks before drug administration and until after the study is completed.

Blood Sampling— Venous blood samples should be collected pre-dose (0 hours) and at 0.25, 0.50, 0.75, 1.0, 1.25, 1.50, 2, 3, 4, 5, 6, and 8 hours post-dose. Due to the chemical instability of cefaclor, the blood samples should be analyzed as soon as they are drawn, or plasma samples should be promptly frozen at  $-70^{\circ}\text{C}$  until analysis. Following a minimum 1-week washout period, subjects should begin Phase II. For each subject, the sponsor should state the time elapsed between sample collection and its assay. An explanation should be given for any missing samples.

Analytical Methods— For the measurement of cefaclor in plasma samples, a microbiological assay, HPLC method, or other suitable method should be selected. The method used should be described in detail, and references, if any, should be cited. The method should include detailed calculation procedures for the assay results. The method chosen should be fully validated with respect to adequate sensitivity, specificity, linearity, recovery, and accuracy and precision (both within and between days). Stability of the analyte in plasma samples under frozen conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be determined. It should be noted that cefaclor is most stable in the acid pH range at a temperature of  $4^{\circ}\text{C}$ . For analytical work, cefaclor reference standard stock solutions should be prepared fresh daily in pH 4.5 buffer.

Chromatograms of the analysis of the unknown samples, including all associated standard curve and quality control chromatograms, should be available for regulatory authorities. The sponsor should justify the rejection of any analytical data and provide a rationale for selection of the reported values.

Statistical Analysis of Pharmacokinetic Data— See Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design under General Guidances.

Limited Food Effects Study— The limited food effects study should be performed in the same manner as the single-dose study under fasting conditions, with the following exceptions:

Procedure— An equal number of subjects should be assigned to each of the 6 dosing sequences possible in a three-treatment, three-period study design (total 18 subjects). Each subject will receive the following treatments:

TREATMENT 1: Generic product, a 500-mg cefaclor capsule or 375-mg-per-5-mL suspension administered after a standard high-fat breakfast.

TREATMENT 2: Reference product, a 500-mg Cedlor® capsule or 375-mg-per-5-mL suspension (Eli Lilly) administered after a standard high-fat breakfast.

TREATMENT 3: Generic product, a 500-mg cefaclor capsule or 375-mg-per-5-mL suspension administered under fasting conditions.

Following a 10-hour fast, the subjects receiving treatments under fed conditions should be served a standard high-fat breakfast. The subjects should have 30 minutes to finish the entire breakfast, and then should immediately receive Treatment 1 or Treatment 2, taken with 240 mL of water. Subjects receiving the treatment under fasting conditions should be dosed with Treatment 3, taken with 240 mL of water only. The same lots of the test and reference products should be used in the fasted and fed studies. No food should be allowed for at least 4 hours post-dose with water allowed after the first hour. Subjects should be served scheduled standardized meals throughout the study, and the same meals should be served during both phases of the study.

Statistical Analysis— In general, a comparable food effect will be assumed if the AUC<sub>0-T</sub>, AUC<sub>0- $\infty$</sub> , and C<sub>max</sub> mean values for the test product administered under fed conditions are within 20% of the respective mean values obtained for the reference product administered under fed conditions.

Clinical Report, Side Effects, and Adverse Reactions— Subject medical histories, physical examinations and laboratory reports, and all incidents of possible adverse reactions to the study formulations should be reported.

Retention of Samples— The laboratory conducting the bioequivalence testing should retain an appropriately identified reserve sample of both the test and reference products used to perform the in vivo bioequivalence study for approval of the application. Each reserve sample should consist of at least 200 dosage units and should be retained for a minimum of 5 years following approval of the application. For more information on retention of bioequivalence samples, refer to 21 CFR 320.63.

in vitro testing requirements

Dissolution Testing— Conduct dissolution testing on 12 dosage units of the test product versus 12 units of the reference product. The biostudy lots should be used for those product strengths tested in vivo. The current official USP dissolution method (see [Dissolution](#) 711) should be followed and should be referenced by the sponsor. The following method and tolerances are currently recommended for this product:



Medium: water; 900 mL.

Apparatus 2: 50 rpm.

Times: 10, 20, 30, and 45 minutes.

Procedure— Use USP if available, or other validated method (see [Validation of Compendial Procedures \(1225\)](#)).

Tolerance (Q) not less than 80% in 30 minutes.

The percentage of label claim dissolved at each specified testing interval should be reported for each individual dosage unit. The mean percentage dissolved, the range (highest, lowest) of dissolution, and the coefficient of variation (relative standard deviation) should be reported.

Content Uniformity Test— Content uniformity testing on the test product lots should be performed as described in USP.

waiver requirements

Waiver of in vivo bioequivalence study requirements for the 250-mg strength of the generic capsule may be granted pursuant to 21 CFR 320.22(d)(2), provided the following conditions are met:

1. The 250-mg capsule is proportionally similar in both active and inactive ingredients to the firm's 500-mg capsule, which has been demonstrated to be bioequivalent to the reference product *in vivo*.
2. The 250-mg capsule of the generic product meets dissolution test requirements.

Waiver of in vivo bioequivalence study requirements for the powder for reconstitution as a suspension for oral administration, 125-, 187-, and 250-mg-per-5-mL strengths of the generic product may be granted pursuant to 21 CFR 320.22(d)(2) provided the following conditions are met:

1. The 125-, 187-, and 250-mg-per-5-mL suspensions are proportionally similar in their active and inactive ingredients to the firm's 375-mg-per-5-mL suspension.
2. An acceptable in vivo bioequivalence study has been conducted for the 375-mg-per-5-mL suspension.

#### Cimetidine Tablets—In Vivo Bioequivalence and In Vitro Dissolution Testing<sup>4</sup>

Clinical Usage/Pharmacology— Cimetidine is a histamine H<sub>2</sub>-receptor antagonist, used for the treatment of endoscopically or radiographically confirmed duodenal ulcer, pathologic GI hypersecretory conditions (e.g., Zollinger-Ellison syndrome, systemic mastocytosis, and multiple endocrine adenomas), and active, benign, gastric ulcer.

Cimetidine inhibits competitively and selectively the interaction of histamine with H<sub>2</sub> receptors. It inhibits basal (fasting) and nocturnal acid secretion, acid secretion stimulated by food, sham feeding, fundic distention, and various pharmacological agents.

Effective cimetidine concentrations between 0.5 and 1.0 µg per mL are required to suppress gastric acid secretion under basal or stimulated conditions. However, no correlation to the therapeutic response from pharmacokinetic data has been established between plasma concentrations of cimetidine or any of the pharmacodynamic parameters and duodenal ulcer healing rate.

For treatment of active duodenal ulcer, the usual adult oral dosage of cimetidine is 800 mg daily at bedtime. For maintenance therapy following healing of acute duodenal ulcer, the usual oral dosage of cimetidine is 400 mg daily at bedtime. For the treatment of pathologic hypersecretory conditions, the usual adult oral dosage is 300 mg, 4 times daily with meals and at bedtime. For the treatment of active benign gastric ulcer, the usual adult oral dosage is 800 mg at bedtime or 300 mg, 4 times daily with meals and at bedtime.

Pharmacokinetics— Following intravenous administration, the plasma concentration profile follows multicompartmental characteristics. The total systemic clearance is high (500 to 600 mL per minute) and is mainly determined by renal clearance. The volume of distribution is of the order of 1 L per kg. Elimination half-life is approximately 2 hours. Following oral administration of cimetidine, 2 plasma concentration peaks are frequently observed at about 1 hour and after about 3 hours, probably due to discontinuous absorption in the intestine or individual variation in gastric emptying (but not enterohepatic recycling since the biliary excretion rate in humans is less than 2%). The absolute bioavailability is about 60% in healthy subjects and around 70% in peptic ulcer patients. Absorption and clearance of cimetidine are linear after 200- and 800-mg doses. When given with food, the extent of absorption of the drug remains unchanged, but the time to reach the maximum peak concentration is delayed with only 1 peak in the plasma concentration curve observed at about 2 hours following dosing. Plasma protein binding of cimetidine is 20% and does not significantly affect the pharmacokinetics of the drug. Cimetidine distributes extensively into the kidney, lung, and muscle tissue but less than 1% into the cerebrospinal fluid.

Following IV administration, about 50% to 80% of the dose is recovered in urine as unchanged cimetidine. This fraction is less after oral doses. Approximately 2% of cimetidine is excreted in the bile. About 25% to 40% of the cimetidine dose is eliminated as metabolites, formed mainly in the liver. The metabolites are sulfoxide and 5-hydroxymethyl derivatives, and possibly guanylurea, although this latter compound may result from in vitro degradation. Elimination of cimetidine is accelerated in the presence of phenobarbital due to induction of its metabolism. Clearance of the drug is higher in children, who have greater renal elimination rates. With increasing age, the volume of distribution of the drug decreases, total plasma clearance decreases as a function of decreasing renal clearance, and plasma half-life increases.

#### in vivo bioequivalence studies<sup>5</sup>

##### Product Information—

1. FDA Designated Reference Product: Tagamet® (SmithKline Beecham) 800-mg tablets.
2. Batch Size—The test batch or lot must be manufactured under production conditions and must be of a size that is at least 10% of the largest lot planned for full production or a minimum of 100,000 units, whichever is larger.
3. Potency—The assayed potency of the reference product should not differ from that of the test product by more than 5%.

#### Types of Studies Required—

1. A single-dose, randomized, fasting, two-period, two-treatment, two-sequence crossover study comparing the 800-mg strength of the test and reference products. This protocol may also be used for lower strength tablets if an 800-mg tablet is not being manufactured. In this case, equal doses of the test and reference products should be used.
2. A single-dose, two-way crossover, full food study comparing the 800-mg strength of the test and reference products. Due to the double-peak phenomenon, which is only observed under fasting conditions, the food study may be more reliable for determining the bioequivalency of the test product and therefore should be done with a full complement of subjects. This protocol may also be used for lower strength tablets if an 800-mg tablet is not being manufactured. In this case, equal doses of the test and reference products should be used.

#### Recommended Protocol for Conducting a Single-Dose Bioequivalence Study under Fasting Conditions—

Objective— The objective is to compare the rate and extent of absorption of a generic formulation with that of a reference formulation when given as equal labeled doses.

Design— The study design is a single-dose, two-treatment, two-period, two-sequence crossover with a 1-week washout period between Phase I and Phase II dosing. An equal number of subjects should be randomly assigned to the two possible dosing sequences. Before the study begins, the proposed protocol should be approved by an institutional review board.

Facilities— The clinical facilities and analytical laboratory used for the study should be identified along with the names, titles, and curriculum vitae of the medical and scientific or analytical directors.

Selection of Subjects— The sponsor should enroll a number of subjects sufficient to ensure statistical validity of the study. It is recommended that a minimum of 24 subjects be used in this study. Subjects should be healthy volunteers aged 18 to 50 years and within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). Subjects should be selected on the basis of acceptable medical history, physical examination, and clinical laboratory



test results. Female subjects must be given a pregnancy test prior to beginning the study. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. Written, informed consent must be obtained from all study participants before they are accepted into the study.

Procedure— Following an overnight fast of at least 10 hours, subjects should be administered a single 800-mg dose of the test or reference product with 240 mL of water.

Restrictions— Study volunteers should observe the following restrictions:

- a. Water may be taken except for 1 hour before and after drug administration, when no liquid is allowed other than that needed for drug dosing.
- b. Subjects should fast for at least 4 hours after administration of the test or reference treatment. All meals should be standardized during the study, and the same meals should be served during both phases of the study.
- c. No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
- d. Subjects should take no Rx medications, including oral contraceptives, or OTC medications beginning 2 weeks before drug administration until after the study is completed.

Blood Sampling— Venous blood samples should be collected pre-dose (0 hours) and at 0.25, 0.50, 0.75, 1.0, 1.50, 2.0, 2.50, 3.0, 3.50, 4.0, 5.0, 6.0, 8.0, 10, 12, 16, 20, and 24 hours post-dose. Plasma should be separated promptly and immediately frozen until assayed. Following at least a minimum 1-week washout period, subjects should begin the second phase of the study.

Analytical Methods— Cimetidine should be assayed using a suitable method fully validated with respect to adequate sensitivity, specificity, linearity, recovery, accuracy, and precision (both within and between days). Stability of the samples under frozen conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be determined.

Chromatograms of the analysis of the unknown samples, including all associated standard curve and quality control chromatograms, should be available for regulatory authorities. The sponsor should justify the rejection of any analytical data and provide a rationale for selection of the reported values.

Statistical Analysis of Pharmacokinetic Data: See Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design under General Guidances.

Clinical Report, Side Effects, and Adverse Reactions— Subject medical histories, physical examinations and laboratory reports, and all incidents of possible adverse reactions to the study formulations should be reported.

Retention of Samples— The laboratory conducting the bioequivalence testing should retain an appropriately identified reserve sample of both the test and reference products used to perform the in vivo bioequivalence study for approval of the application. Each reserve sample should consist of at least 200 dosage units and should be retained for a period of not less than 5 years following approval of the application. For more information on retention of bioequivalence samples, refer to 21 CFR 320.63.

Recommended Protocol for Conducting a Single-Dose Bioequivalence Study Under Postprandial Conditions— There should be at least 24 healthy volunteers. The postprandial study should be conducted in the same manner as described for the fasting study except under fed conditions. The subjects should be given a standard high-fat breakfast<sup>6</sup> after an overnight fast of approximately 10 hours. The dose should be given with 240 mL of water approximately 30 minutes after beginning breakfast. The plasma cimetidine data should be obtained and analyzed in the same manner as described for the fasting study. Due to the double-peal phenomenon, which is only observed under fasting conditions, the postprandial study may be more reliable for determining the bioequivalency of a test product.

#### in vitro testing requirements

Dissolution Testing— Conduct dissolution testing on 12 dosage units of the test product versus 12 units of the reference product. The biostudy lots should be used for those product strengths tested in vivo. The current official USP dissolution method (see [Dissolution](#) 711) should be followed and should be referenced by the sponsor. The following method and tolerances are currently recommended for this product:

Medium: deaerated water; 900 mL.

Apparatus 1: 100 rpm.

Times: 10, 15, and 30 minutes.

Procedure— Use USP if available, or other validated method (see [Validation of Compendial Procedures](#) 1225).

Tolerances (Q): not less than 75% in 15 minutes.

The percentage of label claim dissolved at each specified testing interval should be reported for each individual dosage unit. The mean percentage dissolved, the range (highest, lowest) of dissolution, and the coefficient of variation (relative standard deviation) should be reported.

Content Uniformity Test— Content uniformity testing on the test product lots should be performed as described in USP.

#### waiver requirements

Waiver of in vivo bioequivalence study requirements for the 200-, 300-, and 400-mg strengths of the generic product may be granted pursuant to 21 CFR 320.22(d)(2), provided the following conditions are met:

1. The 200-, 300-, and 400-mg strengths are proportionally similar in both active and inactive ingredients to another strength (800 mg), which has been demonstrated to be bioequivalent to a reference (800 mg) product in vivo.
2. The 200-, 300-, and 400-mg strengths of the generic product meet all applicable compendial requirements, including the specified dissolution and content uniformity testing requirements.
3. The drug product follows linear kinetics over its range of available strengths.

#### Diclofenac Sodium Delayed-Release Tablets—In Vivo Bioequivalence and In Vitro Dissolution Testing<sup>4</sup>

Clinical Usage/Pharmacology— Diclofenac sodium is an orally administered nonsteroidal anti-inflammatory drug (NSAID), which also has analgesic and antipyretic properties. Currently approved indications for diclofenac sodium are for the acute or chronic treatment of the signs and symptoms of rheumatoid arthritis (RA), osteoarthritis, and ankylosing spondylitis. Doses above 200 mg a day in 3 or 4 divided doses have not been studied in RA patients. Diclofenac sodium inhibits prostaglandin synthesis, which may be involved in its mechanism of action. Due to possible cross-reactivity, diclofenac sodium is contraindicated in patients in whom aspirin or other NSAID has produced asthma, urticaria, or allergic reactions.

Pharmacokinetics— Diclofenac sodium is rapidly absorbed following oral administration with reported time of maximum concentration (Tmax) mean values of 1 to 3 hours and ranges of 1 to 5 hours under fasting conditions in normal volunteers. After single oral doses of 25-, 50-, and 75-mg delayed-release tablets, reported maximum concentrations (Cmax) in normal fasting subjects were 0.5 to 1, 0.9 to 1.5, and 1.9 to 2  $\mu$ g per mL, respectively. Area under the plasma concentration-time curve (AUC) increases linearly over the dose range 25 to 150 mg; however, Cmax is less than dose-proportional with values of 1, 1.5, and 2  $\mu$ g per mL after doses of 25, 50, and 75 mg, respectively.

Diclofenac sodium undergoes first-pass metabolism to give a systemic availability of 50% to 60%. Food may markedly delay the rate of absorption from delayed-release tablets but does not appear to change AUC significantly. In two single-dose (50 mg) studies, the nonfasting mean Tmax values were 5.4 hours (N = 12, range 2.5 to 12 hours) and 9.7 hours (N = 6, range 8 to 10 hours). The volume of distribution (Vd) of diclofenac sodium is about 0.12 to 0.17 L per kg and it is greater than 99% bound to plasma proteins. After an IV dose, elimination of diclofenac sodium from plasma appears triphasic; after oral dosing, reported terminal  $\beta$ -phase half-life ( $t_{1/2}$ ) values are 1 to 2 hours with a range of 0.5 to 4.3 hours. The apparent elimination  $t_{1/2}$  of total radiolabeled compounds in patients with normal renal function is 25 to 33 hours.

The major route of elimination is hepatic clearance with 90% of a dose eliminated in 96 hours with about 65% of the dose in urine and 35% of the dose in bile. The dose is excreted as glucuronide and sulfate conjugates of unchanged drug and four metabolites. The principal metabolite (4'-hydroxy) has about 1/40 the activity of the parent drug in animal models of arthritis. It is possible that this metabolite may contribute to the overall activity because it accounts for 30% to 40% of the dose.

#### in vivo bioequivalence studies<sup>5</sup>



## Product Information—

FDA Designated Reference Product: Voltaren® (Geigy) 75-, 50-, or 25-mg delayed-release oral tablets.

Batch Size— The test batch or lot must be manufactured under production conditions and must be of a size that is at least 10% that of the largest lot planned for full production or a minimum of 100,000 units, whichever is larger.

Potency— The assayed potency of the reference product should not differ from that of the test product by more than 5%.

## Types of Studies Required—

1. A single-dose, randomized, two-period, two-treatment, two-sequence crossover study under fasting conditions comparing equal doses of the test and reference products.
2. A single-dose, randomized, three-treatment, three-period, six-sequence crossover, limited food effects study comparing equal doses of the test and reference products when administered immediately following a standard high-fat breakfast.<sup>6</sup>
3. As stated in 21 CFR 320.22(d)(2)(iv), no waiver may be granted for the 25- or 50-mg strengths based on the 75-mg strength. Separate fasting studies must be performed for all of the strengths.

## Recommended Protocol for Conducting a Single-Dose, Fasted Bioequivalence Study—

Objective— The objective is to compare the rate and extent of absorption of a generic formulation with that of a reference formulation when given as equal labeled doses.

Design— The study design is a single-dose, two-treatment, two-period, two-sequence crossover with a 2-week washout period between phases. An equal number of subjects should be randomly assigned to the two possible dosing sequences. Before the study begins, the proposed protocol should be approved by an institutional review board.

Facilities— The clinical facilities and analytical laboratory used for the study should be identified along with the names, titles, and curriculum vitae of the medical and scientific or analytical directors.

Selection of Subjects— The sponsor should enroll a number of subjects sufficient to ensure statistical validity of the study. It is recommended that a minimum of 36 subjects be used in this study. Subjects should be healthy, preferably nonsmoking, volunteers aged 18 to 50 years and within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). Subjects should be selected on the basis of acceptable medical history, physical examination, and clinical laboratory test results. Female subjects must be given a pregnancy test prior to beginning the study. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. If smokers are included, they should be identified as such. Written, informed consent must be obtained from all study participants before they are accepted into the study.

Procedure— Following an overnight fast of at least 10 hours, subjects should be administered a single dose of the test or reference product with 240 mL of water.

## Restrictions— Study volunteers should observe the following restrictions:

- a. Water may be taken except for 1 hour before and after drug administration when no liquid is allowed other than that needed for drug dosing.
- b. Subjects should fast for at least 4 hours after administration of the test or reference treatment. All meals should be standardized during the study, and the same meals should be served during both phases of the study.
- c. No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
- d. Subjects should take no Rx medication, including oral contraceptives, or OTC medication beginning 1 week before drug administration and until after the study is completed.

Blood Sampling— Venous blood samples should be collected pre-dose (0 hours) and at 0.50, 0.75, 1, 1.25, 1.50, 1.75, 2, 2.25, 2.50, 2.75, 3, 3.33, 3.67, 4, 5, 6, 8, 10, and 12 hours post-dose. Plasma serum should be separated promptly and immediately frozen until assayed. Following a 2-week washout period, subjects should begin the second phase of the study.

Analytical Methods— The active ingredient should be assayed using a suitable method fully validated with respect to adequate sensitivity, specificity, linearity, recovery, accuracy, and precision (both within and between days). Stability of the samples under frozen conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be determined. Chromatograms of the analysis of the unknown samples, including all associated standard curve and quality control chromatograms, should be available for regulatory authorities. The sponsor should justify the rejection of any analytical data and provide a rationale for selection of the reported values.

Subject Monitoring— Blood pressure and pulse rate should be monitored hourly during the first 4 hours of the study. Any subject with a heart rate less than 45 bpm or greater than 110 bpm should have an electrocardiogram (lead II) performed and have their pulse monitored hourly. Subjects should report any unusual symptoms observed during the study. Subjects should be periodically questioned during each phase of the study for any unusual symptoms experienced after drug administration.

Statistical Analysis of Pharmacokinetic Data— See Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design under General Guidances. In addition, the following parameters should be tabulated: lag time (Tlag), the time of the last zero concentration before the first non-zero concentration; and adjusted Tmax (Tmaxadj), which is the observed Tmax minus Tlag.

## Limited Food Effects Study—

Objective— The objective is to compare the rate and extent of absorption of diclofenac sodium from a generic formulation with that from a reference formulation under nonfasting conditions, and to compare the rate and extent of absorption of the drug form from a generic product under fasting and nonfasting conditions when given as equal labeled doses.

Design— The study design is a single-dose, three-treatment, three-period, six-sequence crossover with a 2-week washout period between Phase I and Phase II, and Phase II and Phase III of dosing.

In view of the wide range of reported values for Tmax in the presence of food, it is recommended that the sponsor perform a pilot study to determine appropriate sampling times for the limited food effects study.

The limited food effects study (12 to 18 subjects) should be performed in the same manner as the single-dose fasting study, with the following exceptions:

Procedure— An equal number of subjects should be assigned to each of the 6 dosing sequences possible in a three-treatment, three-period study design. Each subject will receive the following treatments:

TREATMENT 1: Generic product, a 75-mg tablet, administered after a standard high-fat breakfast.

TREATMENT 2: Reference product, a 75-mg Voltaren® tablet, administered after a standard high-fat breakfast.

TREATMENT 3: Generic product, a 75-mg tablet, administered under fasting conditions.

Following a 10-hour fast, the subjects receiving treatments under fed conditions should be served a standard high-fat breakfast. The subjects should have 30 minutes to finish the entire breakfast, and then should immediately receive Treatment 1 or Treatment 2, with 240 mL of water. Subjects receiving the treatment under fasting conditions should receive Treatment 3, with 240 mL of water. The same lots of the test and reference products should be used in the fasted and fed studies. No food should be allowed for at least 4 hours post-dose, with water allowed after the first hour. Subjects should be served scheduled standardized meals throughout the study, and the same meals should be served during all phases of the study.

Statistical Analysis— In general, a comparable food effect will be assumed if the AUC0- $\infty$ , AUC0- $\infty$ , and Cmax mean values for the test product administered under fed conditions are within 20% of the respective mean values for the reference product administered under fed conditions.

Clinical Report, Side Effects, and Adverse Reactions— Subject medical histories, physical examinations and laboratory reports, and all incidents of possible adverse reactions to the study formulations should be reported.

Retention of Samples— The laboratory conducting the bioequivalence testing should retain appropriately identified reserve samples of both the test and reference products used to perform the in vivo bioequivalence studies for approval of the application. Each reserve sample should consist of at least 200 dosage units, and should be retained for a minimum of 5 years following approval of the application. For more information on retention of bioequivalence samples, refer to 21 CFR 320.63.



#### in vitro testing requirements

There is currently no official USP dissolution method for a diclofenac sodium dosage form. A tentative method recommended by FDA is described below.

**Drug Release Testing**— Conduct dissolution testing on 12 dosage units of the test product and the reference product, employing the biostudy lots. The following method and tolerances are currently recommended for this product:

##### Method B (724) —

Medium: 2 hours in 0.1 N hydrochloric acid followed by 45 minutes in pH 6.8, 0.05 M phosphate buffer prepared as follows. Dissolve 76 g of tribasic sodium phosphate in water to obtain 1000 mL of solution. Mix 250 mL of this solution with 750 mL of 0.1 N hydrochloric acid and, if necessary, adjust with 2 N hydrochloric acid or 2 N sodium hydroxide to a pH of  $6.8 \pm 0.05$ ; 900 mL.

Apparatus 2: 50 rpm.

Times: acid stage: 30, 60, and 120 minutes; buffer stage: 5, 10, 20, 30, 45, and 60 minutes.

Procedure— Use USP if available, or other validated method (see [Validation of Compendial Procedures \(1225\)](#)).

Tolerances (Q): acid stage: not more than 10% in 120 minutes; buffer stage: not less than 75% in 45 minutes.

The percentage of label claim dissolved at each specified testing interval should be reported for each individual dosage unit. The mean percentage dissolved, the range (highest, lowest) of dissolution, and the coefficient of variation (relative standard deviation) should be reported.

**Content Uniformity Test**— Content uniformity testing on the test product lots should be performed as described in USP.

##### waiver requirements

As stated in 21 CFR 320.22(d)(2)(iv), no waiver may be granted for the 25- or 50-mg strengths based on the 75-mg strength. Separate studies must be performed for all of the strengths.

The limited food effects study requirement for the 50- and 25-mg strengths of the generic diclofenac sodium product may be waived if the following conditions are met:

1. The limited food study for the 75-mg strength demonstrates bioequivalence to the reference product.
2. The formulations for the 50- and 25-mg strengths are proportionally similar to the 75-mg strength.

#### Diltiazem Hydrochloride Tablets—In Vivo Bioequivalence and In Vitro Dissolution Testing<sup>4</sup>

**Clinical Pharmacology/Usage**— Diltiazem hydrochloride is classified as a cardiovascular preparation and an anti-anginal calcium channel blocker. It is currently approved for angina pectoris due to coronary artery spasm and for chronic stable angina (classic effort-associated angina). Its therapeutic effects are brought about by its ability to block calcium entry into cardiac and vascular smooth muscle cells. It is known to dilate coronary arteries as well as to increase the tolerance of angina sufferers to physical exertion by reducing the demand for myocardial oxygen.

The dosage is adjusted according to the needs of adult patients. The starting regimen of 30 mg four times daily is increased gradually until the optimum response is achieved with safety. Safety and efficacy of diltiazem is not established for pediatric use. Diltiazem is prescribed to a pregnant woman only if the potential benefit justifies any potential risk to the fetus. A nursing mother taking diltiazem should not breast-feed her infant. The most commonly observed adverse reactions are edema, headache, nausea, dizziness, rash, and asthenia (weakness, lethargy).

**Pharmacokinetics**— After oral administration of diltiazem, 80% to 90% of the dose is absorbed. Diltiazem undergoes extensive first-pass metabolism and has 40% to 44% oral bioavailability. Diltiazem is 70% to 80% bound to plasma proteins. The peak plasma diltiazem level is reached within 2 to 3 hours after a single oral dose. Elimination half-lives of 3 to 5 hours and 5 to 9 hours have been reported for diltiazem. Diltiazem is metabolized by three major pathways into various metabolites. These pathways are O-deacetylation, N-demethylation, and O-demethylation. Desacetyl diltiazem (DAD) and N-monodemethyl diltiazem (NMD) are active metabolites.

Until 1987, the measurement of DAD and NMD was found to be difficult in single-dose (IV and oral) studies. In 1989, however, Boyd et al. showed the possibility of measuring DAD and NMD following a single oral dose.

At one time, DAD was thought to be the major metabolite of diltiazem. It is 40% to 50% active compared to the parent compound and shows, on average, only 20% of the plasma levels produced by diltiazem. DAD is 68% bound to plasma proteins. Following a single oral dose, average half-lives of 7.5 hours and 19.5 hours have been reported.

Recent studies have demonstrated that N-monodemethylation, leading to the formation of NMD, which is less active than DAD, is the major metabolic pathway. Average plasma levels of NMD are about 40% of those of diltiazem. NMD is 77% bound to plasma proteins and has an average half-life of 8 to 10 hours.

Diltiazem hydrochloride has a therapeutic concentration range of 50 to 200 ng per mL and is toxic above 1200 ng per mL.

##### in vivo bioequivalence studies<sup>5</sup>

##### Product Information—

FDA Designated Reference Product: Cardizem® (Marion) 120-mg tablets.

**Batch Size**— The test batch or lot must be manufactured under production conditions and must be of a size at least 10% that of the largest lot planned for full production or a minimum of 100,000 units, whichever is larger.

**Potency**— The assayed potency of the reference product should not differ from that of the test product by more than 5%.

**Type of Study Required**— A single-dose, randomized, two-period, two-treatment, two-sequence crossover study under fasting conditions comparing equal doses of the test and reference products.

##### Recommended Protocol for Conducting a Single-Dose, Fasted Bioequivalence Study—

**Objective**— The objective is to compare the rate and extent of absorption of diltiazem hydrochloride from a generic formulation with that from a reference formulation when given as equal labeled doses.

**Design**— The study design is a single-dose, two-treatment, two-period, two-sequence crossover with a 1-week washout period between Phase I and Phase II dosing. An equal number of subjects should be randomly assigned to each of the two possible dosing sequences. Before the study begins, the proposed protocol should be approved by an institutional review board.

**Facilities**— The clinical facilities and analytical laboratory used for the study should be identified along with the names, titles, and curriculum vitae of the medical and scientific or analytical directors.

**Selection of Subjects**— The sponsor should enroll a number of subjects sufficient to ensure statistical validity of the study. It is recommended that a minimum of 24 subjects be used in this study. Subjects should be healthy, preferably nonsmoking, volunteers aged 18 to 55 and within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). Subjects should be selected on the basis of acceptable medical history, physical examination, and clinical laboratory test results. Female subjects must be given a pregnancy test prior to beginning the study. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. If smokers are included, they should be identified as such. Written, informed consent must be obtained from all study participants before they are accepted into the study.

**Procedure**— Following an overnight fast of at least 10 hours, subjects should be administered a single 120-mg dose of the test or reference product with 240 mL of water.



Restrictions— Study volunteers should observe the following restrictions:

- a. Water may be taken except for 1 hour before and after drug administration when no liquid is allowed other than that needed for drug dosing.
- b. Subjects should fast for at least 4 hours after administration of the test or reference treatment. All meals should be standardized during the study, and the same meals should be served during both phases of the study.
- c. No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
- d. Subjects should take no Rx medications, including oral contraceptives, beginning 2 weeks and no OTC medications beginning 1 week before drug administration and until after the study is completed.

Blood Sampling— Venous blood samples should be collected pre-dose (0 hours) and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 14, 16, 24, 30, 36, and 48 hours post-dose. Plasma should be separated promptly and immediately frozen until assayed. Following a minimum 1-week washout period, subjects should begin the second phase of the study.

Subject Monitoring— Blood pressure and pulse rate should be monitored hourly during the first 4 hours of the study. Subjects with a heart rate less than 45 bpm or greater than 110 bpm should have an electrocardiogram (lead II) performed and have their pulse monitored hourly. Subjects should report any unusual symptoms observed during the study. Subjects should be periodically questioned during each phase of the study for any unusual symptoms experienced after drug administration.

Analytical Methods— Diltiazem and its major metabolites, DAD and NMD, should be assayed using a suitable method fully validated with respect to adequate sensitivity, specificity, linearity, recovery, accuracy, and precision (both within and between days). Stability of the samples under frozen conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be determined. Chromatograms of the analysis of the unknown samples, including all associated standard curve and quality control chromatograms, should be available for regulatory authorities. The sponsor should justify the rejection of any analytical data and provide a rationale for selection of the reported values.

Statistical Analysis of Pharmacokinetic Data— See Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design under General Guidances.

Clinical Report, Side Effects, and Adverse Reactions— Subject medical histories, physical examinations and laboratory reports, and all incidents of possible adverse reactions and side effects to the study formulations should be reported.

Retention of Samples— The laboratory conducting the bioequivalence testing should retain appropriately identified reserve samples of both the test and reference products used to perform the *in vivo* bioequivalence studies for approval of the application. Each reserve sample should consist of at least 200 dosage units or 5 times the amount needed for complete release testing, whichever is greater, and should be retained for a minimum of 5 years following approval of the application. For more information on retention of bioequivalence samples refer to 21 CFR 320.63.

#### in vitro testing requirements

Dissolution Testing— Conduct dissolution testing on 12 dosage units of the test and reference products, employing the biostudy lots. The current official USP dissolution method (see [Dissolution \(711\)](#)) should be followed and should be referenced by the sponsor. The following times should be used to determine the dissolution profile:  
Times: 30, 60, 90, and 180 minutes.

The percentage of label claim dissolved at each specified testing interval should be reported for each individual dosage unit. The mean percentage dissolved, the range of dissolution (highest, lowest), and the coefficient of variation (relative standard deviation) should be reported.

Content Uniformity Test— Content uniformity testing on the test and reference product lots should be performed as described in USP.

#### waiver requirements

Waiver of *in vivo* bioequivalence study requirements for the immediate-release 30-, 60-, and 90-mg strength of the generic product may be granted pursuant to 21 CFR 320.22(d)(2) provided the following conditions are met:

1. The 30-, 60-, and 90-mg tablets are proportionally similar in both active and inactive ingredients to the firm's 120-mg tablet, which has been demonstrated to be bioequivalent to the reference product *in vivo*.
2. The 30-, 60-, and 90-mg tablets of the generic product meet dissolution test requirements.

#### Flurbiprofen Tablets—*In Vivo* Bioequivalence and *In Vitro* Dissolution Testing<sup>4</sup>

Clinical Usage/Pharmacology— Flurbiprofen is an orally administered nonsteroidal anti-inflammatory drug (NSAID) that also has analgesic and antipyretic properties. Labeled indications for flurbiprofen are for the acute or long-term treatment of the signs and symptoms of rheumatoid arthritis and osteoarthritis. Doses above 300 mg per day are not recommended. Flurbiprofen inhibits prostaglandin synthesis, which may be associated with its mechanism of action. Due to possible cross-reactivity, flurbiprofen is contraindicated in patients in whom aspirin or other NSAIDs have produced asthma, urticaria, or other allergic reactions.

Pharmacokinetics— Flurbiprofen is rapidly absorbed following oral administration, with reported time of maximum concentration values (T<sub>max</sub>) of 1 to 2 hours and ranges of 0.5 to 4 hours. After single oral doses of 50 and 100 mg, reported maximum concentrations (C<sub>max</sub>) in normal subjects were 5.7 to 7.4 µg per mL and 12.6 to 17.2 µg per mL, respectively.

Food may decrease C<sub>max</sub> by 27% to 32% by delaying absorption but does not significantly change area under the plasma concentration-time curve (AUC). In another study, flurbiprofen absorption was found to be bimodal (with double peaks in some cases) after administration of the tablet form in the fasted (water) or fed (apple juice) state. In this study, the lag time before the onset of the second absorption phase was dependent on the gastric emptying time. The volume of distribution (V<sub>d</sub>) of flurbiprofen is about 0.1 L per kg with values of V<sub>d</sub>/F (F = oral availability) of 7.0 to 9.1 L after a 100-mg dose. Flurbiprofen is ≥ 99% bound to plasma proteins (albumin). The binding has been reported to be minimally nonlinear over the concentration ranges encountered clinically. In one study, however, AUC<sub>0-∞</sub> increased linearly over the dose range of 100 to 300 mg. Flurbiprofen elimination from plasma appears biphasic, with terminal  $\beta$ -phase half-life (t<sub>1/2</sub>) values reported from 3 to 7 hours (mean = 5.7 hr; range 3 to 12 hours). The major route of elimination is hepatic clearance, with 95% of a daily dose excreted in urine in 24 hours, either as unchanged drug (20% to 25% of dose) or as the three metabolites—4'-hydroxy (40% to 47%), 3'-hydroxy-4'-methoxy (20% to 30%), and 3',4'-dihydroxy (5%). Other studies have reported 73% to 77% dose recovery in the urine up to 48 hours. Between 60% to 90% of flurbiprofen and its metabolites are present in urine as glucuronide and sulfate conjugates. The 4'-hydroxy metabolite demonstrates low pharmacological activity in animal models.

Virtually all of the anti-inflammatory activity of flurbiprofen is present in the S(+)-enantiomer. Following oral doses of either the pure R(-)- or S(+)-enantiomer, only the administered enantiomer was detectable in plasma or urine. The available evidence from human studies indicates that R-flurbiprofen does not undergo the *in vivo* irreversible inversion to the S-enantiomer that has been observed with other NSAIDs of the 2-arylpropionic acid class. In three studies, significant differences were noted between the enantiomers for AUC with lower values for the R-enantiomer and with increased CL/F (CL = clearance) or decreased t<sub>1/2</sub> for the R-enantiomer.

#### in vivo bioequivalence studies<sup>5</sup>

##### Product Information—

FDA Designated Reference Product: *Ansaid®* (Upjohn) 100-mg tablets.

Batch Size— The test batch or lot must be manufactured under production conditions and must be of a size at least 10% that of the largest lot planned for full production or a minimum of 100,000 units, whichever is larger.

Potency— The assayed potency of the reference product should not differ from that of the test product by more than 5%.

#### Types of Studies Required—

1. A single-dose, randomized, two-period, two-treatment, two-sequence crossover study under fasting conditions comparing equal doses of the test and reference products.
2. A single-dose, randomized, three-treatment, three-period, six-sequence crossover, limited food effects study comparing equal doses of the test and reference products when administered immediately following a standard high-fat breakfast.<sup>6</sup>

Recommended Protocol for Conducting a Single-Dose Bioequivalence Study under Fasting Conditions—



Objective— The objective is to compare the rate and extent of absorption of flurbiprofen from a generic formulation with that from a reference formulation when given as equal labeled doses.

Design— The study design is a single-dose, two-treatment, two-period, two-sequence crossover with a 1-week washout period between Phase I and Phase II dosing. An equal number of subjects should be randomly assigned to each of the two possible dosing sequences. Before the study begins, the proposed protocols should be approved by an institutional review board.

Facilities— The clinical and analytical laboratories used for the study should be identified along with the names, titles, and curriculum vitae of the medical and scientific or analytical directors.

Selection of Subjects— The sponsor should enroll a number of subjects sufficient to ensure statistical validity of the study. It is recommended that a minimum of 24 subjects should be used in this study. Subjects should be healthy, preferably nonsmoking, volunteers aged 18 to 55 years and within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). Subjects should be selected on the basis of acceptable medical history, physical examination, and clinical laboratory test results. Female subjects must be given a pregnancy test prior to beginning the study. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. If smokers are included, they should be identified as such. Written, informed consent must be obtained from all study participants before they are accepted into the study.

Procedure— Following an overnight fast of at least 10 hours, subjects should be administered a single dose of the test product or the reference product with 240 mL of water.

Restrictions— Study volunteers should be subject to the following restrictions:

- a. Water may be taken, except for 1 hour before and after drug administration, when no liquid is allowed other than that needed for drug dosing.
- b. Subjects should fast for at least 4 hours after administration of the test or reference treatment. All meals should be standardized during the study, and the same meals should be served during both phases of the study.
- c. No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
- d. Subjects should take no Rx medications, including oral contraceptives, beginning 2 weeks and no OTC medications beginning 1 week before drug administration and until after the study is completed.

Blood Sampling— Venous blood samples should be collected at pre-dose (0 hr), and at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, 18, 24, and 36 hours post-dose. Plasma should be separated promptly and immediately frozen until assayed. Following a minimum 1-week washout period, subjects should begin the second phase of the study.

Subject Monitoring— Blood pressure and pulse rate should be monitored hourly during the first 4 hours of the study. Subjects with a heart rate less than 45 bpm or greater than 110 bpm should have an electrocardiogram (lead II) performed and have their pulse monitored hourly. Subjects should report any unusual symptoms observed during the study. Subjects should be periodically questioned during each phase of the study for any unusual symptoms experienced after drug administration.

Analytical Methods— Flurbiprofen should be assayed using a suitable method fully validated with respect to adequate sensitivity, specificity, linearity, recovery, and accuracy and precision (both within and between days). Stability of the samples under frozen conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be determined. Chromatograms of the analysis of the unknown samples, including all associated standard curve and quality control chromatograms, should be available for regulatory authorities. The sponsor should justify the rejection of any analytical data and provide a rationale for selection of the reported values.

Statistical Analysis of Pharmacokinetic Data (Blood Plasma/Serum)— See Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design under General Guidances.

#### Limited Food Effects Study—

Objective— The objective is to compare the rate and extent of absorption of flurbiprofen from a generic formulation with that from a reference formulation under nonfasting conditions and to compare the rate and extent of absorption of the drug from a generic formulation under fasting and nonfasting conditions when given as equal labeled doses.

Design— The study design is a single-dose, three-treatment, three-period, six-sequence crossover with a 1-week washout period between Phase I and II, and Phases II and III. An equal number of subjects should be assigned to each of the 6 dosing sequences.

The limited food effects study should be performed in the same manner as the single-dose study under fasting conditions, with the following exceptions:

Procedures— An equal number of subjects should be assigned to each of the 6 dosing sequences possible in a three-treatment, three-period study design (minimum of 12 subjects).

Each subject will receive the following treatments:

TREATMENT 1: Generic product, a 100-mg tablet, administered after a standard high-fat breakfast.

TREATMENT 2: Reference product, a 100-mg Ansaid® tablet (Upjohn), administered after a standard high-fat breakfast.

TREATMENT 3: Generic product, a 100-mg tablet, administered under fasting conditions.

Following a 10-hour fast, the subjects receiving treatments under fed conditions should be served a standard high-fat breakfast. The subjects should have 30 minutes to finish the entire breakfast and then should immediately receive Treatment 1 or Treatment 2, taken with 240 mL of water. Subjects receiving the treatment under fasting conditions should receive Treatment 3, taken with 240 mL of water only. The same lots of the test and reference products should be used as in the fasted and fed studies. No food should be allowed for at least 4 hours post-dose, with water allowed after the first hour. Subjects should be served scheduled standardized meals throughout the study, and the same meals should be served during all phases of the study.

Statistical Analysis— In general, a comparable food effect will be assumed provided the AUC<sub>0-t</sub>, AUC<sub>0-∞</sub>, and C<sub>max</sub> mean values for the test product administered under fed conditions are within 20% of the respective mean values obtained for the reference product administered under fed conditions.

Clinical Report, Side Effects, and Adverse Reactions— Subject medical histories, physical examination reports, and all incidents of possible adverse reactions to the study formulations should be reported.

Retention of Samples— The laboratory conducting the bioequivalence testing should retain appropriately identified reserve samples of both the test and reference products used to perform the in vivo bioequivalence studies for approval of the application. Each reserve sample should consist of at least 200 dosage units or 5 times the amount needed for complete release testing, whichever is greater, and should be retained for a minimum of 5 years following approval of the application. For more information on retention of bioequivalence samples, refer to 21 CFR 320.63.

#### in vitro testing requirements

Dissolution Testing— Conduct dissolution testing on 12 dosage units of the test and reference products, employing the biostudy lots. The current official USP dissolution method in the monograph (see [Dissolution 711](#)) should be followed and should be referenced by the sponsor. The following times should be used to determine the dissolution profile: Times: 10, 20, 30, and 45 minutes.

The percentage of label claim dissolved at each specified testing interval should be reported for each individual dosage unit. The mean percentage dissolved, the range of dissolution (highest, lowest), and the coefficient of variation (relative standard deviation) should be reported.

Content Uniformity Test— Content uniformity testing on the test and reference product lots should be performed as described in USP.

#### waiver requirements

Waiver of in vivo bioequivalence study requirements for the 50-mg strength of the generic tablet may be granted pursuant to 21 CFR 320.22(d)(2) provided the following conditions are met:

1. The 50-mg tablet is proportionally similar in both active and inactive ingredients to the firm's 100-mg tablet, which has been demonstrated to be bioequivalent to the reference product.



2. The 50-mg tablet of the generic product meets dissolution test requirements.

#### Gemfibrozil Capsules and Tablets—In Vivo Bioequivalence and In Vitro Dissolution Testing<sup>4</sup>

**Clinical Pharmacology/Usage—** Gemfibrozil is used clinically as a lipid-regulatory agent, which lowers serum triglycerides and produces a variable reduction in total serum cholesterol.

The decrease occurs primarily in the very low-density lipoprotein (VLDL) and less frequently in the low-density lipoprotein (LDL). In addition, there is elevation of the high-density lipoprotein (HDL) concentration. Gemfibrozil is used in adult patients with all types of dyslipidemia (except type 1). The recommended dosage of gemfibrozil is 600 mg twice daily given 30 minutes before morning and evening meals.

The mechanism whereby gemfibrozil lowers plasma triglycerides and increases HDL cholesterol concentration is not well established. One mechanism by which the drug reduces circulating triglyceride concentration may be through suppression of free fatty acid mobilization from adipose tissue. Gemfibrozil also stimulates lipoprotein lipase activity.

**Gemfibrozil is not recommended for use in patients with hepatic or severe renal dysfunction, including primary biliary cirrhosis and pre-existing gallbladder disease. The main adverse reactions are dyspepsia and abdominal pain.** Gemfibrozil is metabolized to a number of compounds in humans. All the metabolites and the unchanged drug form glucuronide conjugates, which are excreted in urine. The metabolites have no lipid lowering activity.

**Pharmacokinetics—** Following oral administration of gemfibrozil, absorption is rapid and complete. After the administration of gemfibrozil (600 mg twice daily), mean peak plasma concentrations are about 10 to 15 µg per mL. Peak plasma concentrations are attained 1 to 2 hours after administration of single doses up to 2000 mg or after repeated doses up to 800 mg twice daily. Plasma drug concentration is directly proportional to dose and tends to rise during repeated administration, although steady state is achieved within 7 to 14 days with twice daily doses. The mean elimination half-life of gemfibrozil is 1.5 to 3.0 hours.

#### in vivo bioequivalence studies<sup>5</sup>

##### Product Information—

FDA Designated Reference Product: Lopid® (Parke-Davis Co.) 300-mg capsules and 600-mg tablets.

**Batch Size—** The test batch or lot must be manufactured under production conditions and must be of a size that is at least 10% that of the largest lot planned for full production or a minimum of 100,000 units, whichever is larger.

**Potency—** The assayed potency of the reference product should not differ from that of the test product by more than 5%.

**Type of Study Required—** A single-dose, randomized, two-period, two-treatment, two-sequence crossover study under fasting conditions comparing equal doses of the test and reference products.

#### Recommended Protocol for Conducting a Single-Dose, Fasted Bioequivalence Study—

**Objective—** The objective is to compare the rate and extent of absorption of a generic formulation with that of a reference formulation when given as equal labeled doses.

**Design—** The study design is a single-dose, two-treatment, two-period, two-sequence crossover with a 2-week washout period between Phase I and Phase II dosing. An equal number of subjects should be randomly assigned to each of the two possible dosing sequences. Before the study begins, the proposed protocol should be approved by an institutional review board.

**Facilities—** The clinical facilities and analytical laboratory used for the study should be identified along with the names, titles, and curriculum vitae of the medical and scientific or analytical directors.

**Selection of Subjects—** The sponsor should enroll a number of subjects sufficient to ensure statistical validity of the study. It is recommended that a minimum of 24 subjects be used in this study. Subjects should be healthy, preferably nonsmoking, volunteers aged 18 to 55 and within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). Subjects should be selected on the basis of acceptable medical history, physical examination, and clinical laboratory test results. Female subjects must be given a pregnancy test prior to beginning the study. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. If smokers are included, they should be identified as such. Written, informed consent must be obtained from all study participants before they are accepted into the study.

**Procedure—** Following an overnight fast of at least 10 hours, subjects should be administered a single 600-mg dose (two 300-mg capsules or one 600-mg tablet) of the test or reference product with 240 mL of water.

**Restrictions—** Study volunteers should observe the following restrictions:

- a. Water may be taken except for 1 hour before and after drug administration when no liquid is allowed other than that needed for drug dosing.
- b. Subjects should fast for at least 4 hours after administration of the test or reference treatment. All meals should be standardized during the study, and the same meals should be served during both phases of the study.
- c. No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
- d. Subjects should take no Rx medications, including oral contraceptives, beginning 2 weeks and no OTC medications beginning 1 week before drug administration and until after the study is completed.

**Blood Sampling—** Venous blood samples should be collected pre-dose (0 hours) and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12, and 14 hours post-dose. Plasma should be separated promptly, stored in light resistant containers, and immediately frozen until assayed. Following a minimum 2-week washout period, subjects should begin the second phase of the study.

**Subject Monitoring—** Blood pressure and pulse rate should be monitored hourly during the first 4 hours of the study. Subjects with a heart rate less than 45 bpm or greater than 110 bpm should have an electrocardiogram (lead II) performed and have their pulse monitored hourly. Subjects should report any unusual symptoms observed during the study. Subjects should be periodically questioned during each phase of the study for any unusual symptoms experienced after drug administration.

**Analytical Methods—** Gemfibrozil should be assayed using a suitable method fully validated with respect to adequate sensitivity, specificity, linearity, recovery, accuracy, and precision (both within and between days). Stability of the samples under frozen conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be determined.

**Chromatograms of the analysis of the unknown samples, including all associated standard curve and quality control chromatograms, should be available for regulatory authorities. The sponsor should justify the rejection of any analytical data and provide a rationale for selection of the reported values.**

**Statistical Analysis of Pharmacokinetic Data—** See Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design under General Guidances.

**Clinical Report, Side Effects, and Adverse Reactions—** Subject medical histories, physical examinations and laboratory reports, and all incidents of possible adverse reactions and side effects to the study formulations should be reported.

**Retention of Samples—** The laboratory conducting the bioequivalence testing should retain appropriately identified reserve samples of both the test and reference products used to perform the in vivo bioequivalence studies for approval of the application. Each reserve sample should consist of at least 200 dosage units or 5 times the amount needed for complete release testing and should be retained for a minimum of 5 years following approval of the application. For more information on retention of bioequivalence samples refer to 21 CFR 320.63.

#### in vitro testing requirements

**Dissolution Testing—** Conduct dissolution testing on 12 dosage units of the test product and the reference product, employing the biostudy lots. The current official USP dissolution method in the monograph (see [Dissolution](#) 711) should be followed and should be referenced by the sponsor. The following times should be used to determine the dissolution profile:

Times: 15, 30, 45, and 60 minutes for capsules; and 10, 20, 30, and 45 minutes for tablets.

The percentage of label claim dissolved at each specified testing interval should be reported for each individual dosage unit. The mean percentage dissolved, the range of dissolution (highest, lowest), and the coefficient of variation (relative standard deviation) should be reported.



Content Uniformity Test—Content uniformity testing on the test and reference product lots should be performed as described in USP.

#### Glipizide Tablets—In Vivo Bioequivalence and In Vitro Dissolution Testing<sup>4</sup>

**Clinical Usage/Pharmacology**—Glipizide is an oral antidiabetic agent, which lowers blood glucose levels. It is approved for controlling blood glucose in noninsulin-dependent diabetes mellitus (NIDDM) patients whose blood glucose cannot be controlled by diet alone. Although the precise mechanism of hypoglycemic action of glipizide has not been clearly established, it appears to lower the blood glucose concentration mainly by stimulating endogenous insulin release from beta cells of the pancreas.

To achieve maximum reduction in the postprandial blood glucose concentration, glipizide should be administered 30 minutes before a meal. The recommended initial adult dose of glipizide is 5 mg daily, and the maximum recommended single daily dose is 15 mg. The optimum dosing regimen for each patient is obtained by titration. Glucose (urinary and blood) and blood glycosylated hemoglobin (Hb A1c) are used as the indicators of effective therapy. The most commonly observed adverse effects of glipizide are dizziness, sweating, tremors, and lightheadedness.

**Pharmacokinetics**—Glipizide is rapidly and completely absorbed after oral administration with a bioavailability of approximately 95%. After oral administration, the drug is 98.4% bound to plasma proteins, reaches Cmax in 1 to 3 hours, has an elimination half-life of 2 to 5 hours, and has a volume of distribution (Vd) of 11 L. Based on the mean plasma profiles, glipizide appears to exhibit either single compartment disposition or a very short distribution phase. The primary metabolites of glipizide are hydroxylation products and polar conjugates that are inactive and are excreted in both urine (70%) and feces. Biliary excretion is estimated to be approximately 30%. Less than 5% to 10% of the administered drug is excreted intact in urine. The extent of absorption of an oral dose of glipizide is unaffected by food in normal volunteers, but absorption is delayed by about 40 minutes. There are conflicting reports about the influence of food on Tmax. Plasma levels of glipizide in the range of 20 to 90 ng per mL have been reported as therapeutically effective.

#### in vivo bioequivalence studies<sup>5</sup>

##### Product Information—

FDA Designated Reference Product: Glucotrol® (Roerig) 10-mg tablets.

**Batch Size**—The test batch or lot must be manufactured under production conditions and must be of a size that is at least 10% of the largest lot planned for full production or a minimum of 100,000 units, whichever is larger.

**Potency**—The assayed potency of the reference product should not differ from that of the test product by more than 5%.

#### Types of Studies Required—

1. A single-dose, randomized, two-period, two-treatment, two-sequence crossover study under fasting conditions comparing equal doses of the test and reference products.
2. A single-dose, randomized, three-treatment, three-period, six-sequence, crossover, limited food effects study comparing equal doses of the test product under fasting conditions as well as the test and reference products when administered immediately following a standard high-fat breakfast.<sup>6</sup>

#### Recommended Protocol for Conducting a Single-Dose, Bioequivalence Study under Fasting Conditions—

**Objective**—The objective is to compare the rate and extent of absorption of a generic formulation with that of a reference formulation when given in equal labeled doses.

**Design**—The study design is a single-dose, two-treatment, two-period, two-sequence crossover with a 1-week washout period between Phase I and Phase II dosing. An equal number of subjects should be randomly assigned to each of the two possible dosing sequences. Before the study begins, the proposed protocol should be approved by an institutional review board.

**Facilities**—The clinical facilities and analytical laboratory used for the study should be identified along with the names, titles, and curriculum vitae of the medical and scientific or analytical directors.

**Selection of Subjects**—The sponsor should enroll a number of subjects sufficient to ensure statistical validity of the study. It is recommended that a minimum of 24 subjects be used in this study. Subjects should be healthy, preferably nonsmoking, volunteers aged 18 to 50 years and within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). Subjects should be selected on the basis of an acceptable medical history, physical examination, and clinical laboratory test results. Female subjects must be given a pregnancy test prior to beginning the study. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. If smokers are included, they should be identified as such. Written, informed consent must be obtained from all study participants before they are accepted into the study.

**Procedure<sup>7</sup>**—Following an overnight fast of at least 10 hours, subjects should be administered a single dose (10 mg) of the test or reference product with 240 mL of a 20% glucose solution in water. After dosing, subjects should receive 60 mL of a 20% glucose solution in water every 15 minutes for 4 hours.

**Restrictions**—Study volunteers should observe the following restrictions:

- a. No additional water or fluids, except for the glucose solution, are allowed from 1 hour pre-dose to 1 hour post-dose.
- b. Subjects should fast for at least 4 hours after administration of the test or reference treatment. All meals should be standardized during the study, and the same meals should be served during both phases of the study.
- c. No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
- d. Subjects should take no Rx medication, including oral contraceptives, or OTC medication beginning 2 weeks before drug administration and until after the study is completed.

**Blood Sampling**—Venous blood samples should be collected pre-dose (0 hours) and at 0.50, 1, 1.50, 2, 3, 4, 5, 6, 8, 10, 12, 16, 24, and 36 hours post-dose. Plasma should be separated promptly and immediately frozen until assayed. Following a 1-week washout period, subjects should begin the second phase of the study. For each subject the sponsor should state the time elapsed between sample collection and its assay. An explanation should be given for any missing samples.

**Subject Monitoring**—Blood pressure and pulse rate should be monitored hourly during the first 4 hours of the study. Subjects with a heart rate less than 45 bpm or greater than 110 bpm should have an electrocardiogram (lead II) performed and have their pulse monitored hourly. Subjects should report any unusual symptoms observed during the study. Subjects should be periodically questioned during each phase of the study for any unusual symptoms experienced after drug administration.

**Analytical Methods**—The active ingredient should be assayed using a suitable method fully validated with respect to adequate sensitivity, specificity, linearity, recovery, accuracy, and precision (both within and between days). Stability of the samples under frozen conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be determined. Chromatograms of the analysis of the unknown samples, including all associated standard curve and quality control chromatograms, should be available for regulatory authorities. The sponsor should justify the rejection of any analytical data and provide a rationale for selection of the reported values.

**Statistical Analysis of Pharmacokinetic Data**—See Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design under General Guidances.

**Limited Food Effects Study**—The limited food effects study should be performed in the same manner as the single-dose fasting study, with the following exceptions:

**Procedure**—An equal number of subjects should be assigned to each of the six dosing sequences possible in a three-treatment, three-period study design. Each subject will receive the following treatments:

TREATMENT 1: Generic product, a 10-mg glipizide tablet administered after a standard high-fat breakfast.

TREATMENT 2: Reference product, a 10-mg Glucotrol® tablet administered after a standard high-fat breakfast.

TREATMENT 3: Generic product, a 10-mg glipizide tablet administered under fasting conditions.

Following a 10-hour fast, the subjects receiving treatments under nonfasting conditions should be served a standard high-fat breakfast. The subjects should have 30 minutes to finish the entire breakfast, and then should immediately receive Treatment 1 or Treatment 2, with 240 mL of a 20% glucose solution in water. Subjects receiving the treatment under fasting conditions should be dosed with Treatment 3, taken with 240 mL of a 20% glucose solution in water. The same lots of the test and reference products should be used in the fasting and nonfasting studies. After dosing, each subject in all 3 treatments should receive 60 mL of a 20% glucose solution in water every 15 minutes for 4 hours. No food should be allowed for at least 4 hours post-dose, and no additional water or fluids should be allowed from 1 hour pre-dose to 1 hour post-dose. Subjects should be served scheduled, standardized meals



throughout the study, and the same meals should be served during both phases of the study.

**Statistical Analysis**— In general, a comparable food effect will be assumed if the AUC0-t, AUC0-, and Cmax mean values for the test product administered under nonfasting conditions are within 20% of the respective mean values for the reference product administered under nonfasting conditions.

**Clinical Report, Side Effects, and Adverse Reactions**— Subject medical histories, physical examinations and laboratory reports, and all incidents of possible adverse reactions to the study formulations should be reported.

**Retention of Samples**— The laboratory conducting the bioequivalence testing should retain appropriately identified reserve samples of both the test and reference products used to perform the in vivo bioequivalence studies for approval of the application. Each reserve sample should consist of at least 200 dosage units and should be retained for a minimum of 5 years after approval of the application. For more information on retention of bioequivalence samples, refer to 21 CFR 320.63.

#### in vitro testing requirements

**Dissolution Testing**— Conduct dissolution testing on 12 dosage units of the test and reference products, employing the biostudy lots. The current official USP dissolution method in the monograph should be followed and referenced by the sponsor. The following times should be used to determine the dissolution profile:

Times: 15, 30, 45, and 60 minutes.

**Content Uniformity Test** (905) — Content uniformity testing on the test and reference product lots should be performed as described in USP.

#### waiver requirements

Waiver of in vivo bioequivalence study requirements for the 5-mg strength of the generic product may be granted pursuant to 21 CFR 320.22(d)(2), provided the following conditions are met:

1. The 5-mg tablet is proportionally similar in both active and inactive ingredients to the 10-mg tablet, which has been demonstrated to be bioequivalent to the reference product in vivo.
2. The 5-mg tablet of the generic product meets the dissolution testing requirements.

#### Glyburide Tablets—In Vivo Bioequivalence and In Vitro Dissolution Testing<sup>4</sup>

**Clinical Usage/Pharmacology**— Glyburide is an oral antidiabetic agent, which lowers the blood glucose level. It is currently approved for controlling blood glucose in non-insulin-dependent diabetes mellitus (NIDDM) patients whose blood glucose cannot be controlled by diet alone. Glyburide acts mainly by stimulating endogenous insulin release from beta cells of the pancreas.

Glyburide is usually administered as a single daily dose each morning with breakfast or with the first main meal. The recommended initial adult dose is 2.5 to 5 mg daily for nonmicronized glyburide and 1.5 to 3 mg daily for micronized glyburide. The maximum recommended single daily dose is 10 mg for nonmicronized glyburide (up to 20 mg a day may be given in two divided doses) and 12 mg for micronized glyburide. The optimum dosing regimen of glyburide for each patient is obtained by titration. Glucose (urinary and blood) and blood glycosylated hemoglobin (Hb A1c) are used as the indicators of effective therapy. The most commonly observed adverse effects after administration of glyburide are dizziness or lightheadedness, which are due to hypoglycemia.

**Pharmacokinetics**— Glyburide is rapidly and completely absorbed after oral administration. As there is no significant first pass metabolism, 100% of the oral dose is bioavailable. After oral administration, nonmicronized glyburide reaches peak plasma or serum concentrations within 4 hours and exhibits an elimination half-life of 10 hours (more recently, half-lives of 1.4 to 5 hours have been reported). Micronized glyburide reaches peak concentrations within 2 to 3 hours and has a reported elimination half-life of about 4 hours. Glyburide concentration-time curves in plasma exhibit biphasic elimination. The primary metabolites of glyburide are hydroxylation products (4-trans-hydroxy and 3-cis-hydroxy derivatives) that are inactive and are excreted in urine (50%) and in feces (50%) via bile. Plasma levels of glyburide in the range of 20 to 90 ng per mL have been reported as therapeutically effective.

#### in vivo bioequivalence studies<sup>5</sup>

##### Product Information—

FDA Designated Reference Product: Micronase® (Pharmacia & Upjohn) 5-mg tablets, or Glynase® (Pharmacia & Upjohn) 6-mg tablets.

**Batch Size**— The test batch or lot must be manufactured under production conditions and must be of a size that is at least 10% of the largest lot planned for full production or a minimum of 100,000 units, whichever is larger.

**Potency**— The assayed potency of the reference product should not differ from that of the test product by more than 5%.

#### Types of Studies Required—

1. A single-dose, randomized, two-period, two-treatment, two-sequence crossover study under fasting conditions comparing equal 5-mg or 6-mg doses of the test and reference products.
2. A single-dose, randomized, three-treatment, three-period, six-sequence, crossover, limited food effects study comparing equal doses of the test product under fasting conditions as well as the test and reference products when administered immediately following a standard high-fat breakfast.<sup>6</sup>

#### Recommended Protocol for Conducting a Single-Dose Bioequivalence Study under Fasting Conditions—

**Objective**— The objective is to compare the rate and extent of absorption of a generic formulation with that of a reference formulation when given as equal labeled doses.

**Design**— The study design is a single-dose, two-treatment, two-period, two-sequence crossover with a 1-week washout period between Phase I and Phase II dosing. An equal number of subjects should be randomly assigned to each of the two possible dosing sequences. Before the study begins, the proposed protocol should be approved by an institutional review board.

**Facilities**— The clinical facilities and analytical laboratory used for the study should be identified along with the names, titles, and curriculum vitae of the medical and scientific or analytical directors.

**Selection of Subjects**— The sponsor should enroll a number of subjects sufficient to ensure statistical validity of the study. It is recommended that a minimum of 24 subjects be used in this study. Subjects should be healthy, preferably nonsmoking, volunteers aged 18 to 50 years and within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). Subjects should be selected on the basis of an acceptable medical history, physical examination, and clinical laboratory test results. Female subjects must be given a pregnancy test prior to beginning the study. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. If smokers are included, they should be identified as such. Written, informed consent must be obtained from all study participants before they are accepted into the study.

**Procedure**— Following an overnight fast of at least 10 hours, subjects should be administered a single dose (5 mg or 6 mg) of the test or reference product with 240 mL of a 20% glucose solution in water. After dosing, the subjects should be given 60 mL of a 20% glucose solution in water, every 15 minutes for 4 hours.

**Restrictions**— Study volunteers should observe the following restrictions:

- a. No additional water or fluids, except the glucose solution, are allowed from 1 hour pre-dose to 1 hour post-dose.
- b. Subjects should fast for at least 4 hours after administration of the test or reference treatment. All meals should be standardized during the study, and the same meals should be served during both phases of the study.
- c. No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
- d. Subjects should take no Rx medications, including oral contraceptives, beginning 2 weeks, and no OTC medications beginning 1 week, before drug administration and until after the study is completed.



Blood Sampling— Venous blood samples should be collected predose (0 hours) and at 0.50, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, 24, 30, and 36 hours post-dose. Plasma should be separated promptly and immediately frozen until assayed. Following a 1-week washout period, subjects should begin the second phase of the study. For each subject, the sponsor should state the time elapsed between sample collection and its assay. An explanation should be given for any missing samples.

Subject Monitoring— Blood pressure and pulse rate should be monitored hourly during the first 4 hours of the study. Subjects with a heart rate less than 45 bpm or greater than 110 bpm should have an electrocardiogram (lead II) performed and have their pulse monitored hourly. Subjects should report any unusual symptoms observed during the study. Subjects should be periodically questioned during each phase of the study for any unusual symptoms experienced after drug administration.

Analytical Methods— The active ingredient should be assayed using a suitable method fully validated with respect to adequate sensitivity, specificity, linearity, recovery, and accuracy and precision (both within and between days). Stability of the samples under frozen conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be determined. Chromatograms of the analysis of the unknown samples, including all associated standard curve and quality control chromatograms, should be available for regulatory authorities. The sponsor should justify the rejection of any analytical data and provide a rationale for selection of the reported values.

Statistical Analysis of Pharmacokinetic Data— See Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design under General Guidances.

Limited Food Effects Study— The limited food effects study should be performed in the same manner as the single-dose fasting study, with the following exceptions:

Procedure— An equal number of subjects should be assigned to each of the six dosing sequences possible in a three-treatment, three-period study design. Each subject will receive the following treatments:

TREATMENT 1: Generic product, a 5-mg glyburide tablet or a 6-mg micronized glyburide tablet, administered after a standard high-fat breakfast.

TREATMENT 2: Reference product, a 5-mg Micronase® tablet or a 6-mg Glynase® tablet, administered after a standard high-fat breakfast.

TREATMENT 3: Generic product, a 5-mg glyburide tablet or a 6-mg micronized glyburide tablet, administered under fasting conditions.

Following a 10-hour fast, the subjects receiving treatments under nonfasting conditions should be served a standard high-fat breakfast. The subjects should have 30 minutes to finish the entire breakfast, and then should immediately receive Treatment 1 or Treatment 2, with 240 mL of a 20% glucose solution in water. Subjects receiving the treatment under fasting conditions should receive Treatment 3, with 240 mL of a 20% glucose solution in water. The same lots of the test and reference products should be used in the fasting and nonfasting studies. After dosing, each subject in all 3 treatments should receive 60 mL of a 20% glucose solution in water every 15 minutes for 4 hours. No food should be allowed for at least 4 hours post-dose, and no additional water or fluids should be allowed from 1 hour pre-dose to 1 hour post-dose. Subjects should be served scheduled, standardized meals throughout the study, and the same meals should be served during both phases of the study.

Statistical Analysis— In general, a comparable food effect will be assumed if the AUC0-t, AUC0-∞, and Cmax mean values for the test product administered under nonfasting conditions are within 20% of the respective mean values for the reference product administered under nonfasting conditions.

Clinical Report, Side Effects, and Adverse Reactions— Subject medical histories, physical examinations and laboratory reports, and all incidents of possible adverse reactions to the study formulations should be reported.

Retention of Samples— The laboratory conducting the bioequivalence testing should retain appropriately identified reserve samples of both the test and reference products used to perform the in vivo bioequivalence studies for approval of the application. Each reserve sample should consist of at least 200 dosage units, and should be retained for a minimum of 5 years after approval of the application. For more information on retention of bioequivalence samples, refer to 21 CFR 320.63.

#### in vitro testing requirements

There is currently no official monograph for a glyburide dosage form in USP.

Content Uniformity Test (905)— Content uniformity testing on the test product lots should be performed as described in USP.

#### waiver requirements

Waiver of in vivo bioequivalence study requirements for the 1.25- and 2.5-mg strengths of the generic product may be granted pursuant to 21 CFR 320.22(d)(2), provided the following conditions are met:

1. The 1.25- and 2.5-mg tablets are proportionally similar in both active and inactive ingredients to the 5-mg tablet, which has been demonstrated to be bioequivalent to the reference product in vivo.
2. The 1.25- and 2.5-mg tablets of the generic product meet the dissolution testing requirements.

Waiver of in vivo bioequivalence requirements for the 1.5- and 3-mg strengths of the generic micronized product may be granted pursuant to 21 CFR 320.22(d)(2), provided the following conditions are met:

1. The 1.5- and 3-mg tablets are proportionally similar in both active and inactive ingredients to the 6-mg tablet, which has been demonstrated to be bioequivalent to the reference product in vivo.
2. The 1.5- and 3-mg tablets of the generic product meet the dissolution testing requirements.

#### Guanabenz Acetate—In Vivo Bioequivalence and In Vitro Dissolution Testing<sup>4</sup>

Clinical Pharmacology/Usage— Guanabenz acetate is a centrally acting antihypertensive agent. It appears to stimulate  $\alpha_2$ -adrenergic receptors in the CNS and cause inhibition of sympathetic outflow from the brain.

Guanabenz acetate is indicated in the treatment of hypertension and may be employed alone or in combination with a thiazide diuretic. It is contraindicated in patients with known hypersensitivity to the drug and should be used with caution in patients with hepatic or renal impairment, with cardiovascular diseases, during pregnancy, and in geriatric patients.

The side effects of guanabenz acetate are generally mild and may include a dry mouth, drowsiness or sedation, dizziness, weakness, and a headache. Abrupt withdrawal of guanabenz acetate (especially at doses  $\geq 32$  mg per day) may cause a rapid increase in serum catecholamine concentrations and systolic and diastolic blood pressures.

The initial dosage is 4 mg twice daily and may be gradually increased to a maximum of 32 mg twice daily.

Pharmacokinetics— Following an oral dose of guanabenz acetate, about 75% of the drug is absorbed. Because of extensive first-pass metabolism, the bioavailability is low—20% to 30% in monkeys—but the extent has not been established in man. A food effect on the drug absorption has not been determined.

Guanabenz acetate is rapidly and extensively distributed into the CNS and various organs. Following a 16-mg dose to fasting healthy individuals, peak plasma levels of unchanged drug are only 2.4 to 2.7 ng per mL at 2 to 5 hours with about 90% bound to proteins. For hepatically impaired patients, the Cmax is higher, 7.8 ng per mL. From the limited studies in humans, the concentration-time curves for the drug are best fit to a one- or two-compartment open model with a first-order absorption process and a lag time. The apparent steady-state volume of distribution is about 93 and 147 L per kg after 16- and 32-mg oral doses, respectively.

Therapeutic effects begin within 1 hour and last over 10 hours with a peak around 2 to 4 hours. At usual clinical doses, effects appear to be linearly related to dose.

The elimination half-life of guanabenz acetate averages 4 to 9 hours in healthy males. For liver impaired or hypertensive patients, the half-life averages 12 to 14 hours.

Guanabenz acetate metabolites—mainly from the liver—are excreted in urine (70% to 80% within 72 hours) and feces (10% to 30% in 6 days). One and four-tenths percent of the drug is excreted unchanged in the urine. Urinary metabolites include (E)-p-hydroxyguanabenz (11%), its glucuronide conjugate (25%), and the Z-guanabenz acetate (1.1%—the only active metabolite with about 25% parent drug activity).

in vivo bioequivalence studies<sup>5</sup>

## Product Information—

FDA Designated Reference Product: Wytenstir® (Wyeth-Ayerst) 8-mg tablet.

Batch Size— The test batch or lot must be manufactured under production conditions and must be of a size that is at least 10% that of the largest lot planned for full production or a minimum of 100,000 units, whichever is larger.

Potency— The assayed potency of the reference product should not differ from that of the test product by more than 5%.

Type of Study Required— A single-dose, randomized, two-period, two-treatment, two-sequence crossover study under fasting conditions comparing equal doses of the test and reference products.

## Recommended Protocol for Conducting a Single-Dose, Fasted Bioequivalence Study—

Objective— The objective is to compare the rate and extent of absorption of guanabenz acetate from a generic formulation with that of a reference formulation when given as equal labeled doses.

Design— The study design is a single-dose, two-treatment, two-period, two-sequence crossover with a 1-week washout period between Phase I and Phase II dosing. An equal number of subjects should be randomly assigned to each of the two possible dosing sequences. Before the study begins, the proposed protocol should be approved by an institutional review board.

Facilities— The clinical facilities and analytical laboratory used for the study should be identified along with the names, titles, and curriculum vitae of the medical and scientific or analytical directors.

Selection of Subjects— The sponsor should enroll a number of subjects sufficient to ensure statistical validity of the study. It is recommended that a minimum of 24 subjects be used in this study. Subjects should be healthy, preferably nonsmoking, volunteers aged 18 to 50 and within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). Subjects should be selected on the basis of acceptable medical history, physical examination, and clinical laboratory test results. Female subjects must be given a pregnancy test prior to beginning the study. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. If smokers are included, they should be identified as such. Written, informed consent must be obtained from all study participants before they are accepted into the study.

Procedure— Following an overnight fast of at least 10 hours, subjects should be administered one 8-mg tablet of the test or reference product with 240 mL of water.

## Restrictions— Study volunteers should observe the following restrictions:

- a. Water may be taken except for 1 hour before and after drug administration when no liquid is allowed other than that needed for drug dosing.
- b. Subjects should fast for at least 4 hours after administration of the test or reference treatment. All meals should be standardized during the study, and the same meals should be served during both phases of the study.
- c. No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
- d. Subjects should take no Rx medications, including oral contraceptives, beginning 2 weeks and no OTC medications beginning 1 week before drug administration and until after the study is completed.

Blood Sampling— Venous blood samples should be collected pre-dose (0 hours) and at 0.50, 1, 1.50, 2, 2.50, 3, 3.50, 4, 6, 8, 12, 15, 24, 30, and 36 hours post-dose. Plasma should be separated promptly and immediately frozen until assayed. Following a minimum 1-week washout period, subjects should begin the second phase of the study.

Subject Monitoring— Blood pressure and pulse rate should be monitored hourly during the first 4 hours of the study. Subjects with a heart rate less than 45 bpm or greater than 110 bpm should have an electrocardiogram (lead II) performed and have their pulse monitored hourly. Subjects should report any unusual symptoms observed during the study. Subjects should be periodically questioned during each phase of the study for any unusual symptoms experienced after drug administration.

Analytical Methods— Guanabenz acetate should be assayed using a suitable method fully validated with respect to adequate sensitivity, specificity, linearity, recovery, accuracy, and precision (both within and between days). Stability of the samples under frozen conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be determined. Chromatograms of the analysis of the unknown samples, including all associated standard curve and quality control chromatograms, should be available for regulatory authorities. The sponsor should justify the rejection of any analytical data and provide a rationale for selection of the reported values.

Statistical Analysis of Pharmacokinetic Data— See Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design under General Guidances.

Clinical Report, Side Effects, and Adverse Reactions— Subject medical histories, physical examinations and laboratory reports, and all incidents of possible adverse reactions and side effects to the study formulations should be reported.

Retention of Samples— The laboratory conducting the bioequivalence testing should retain appropriately identified reserve samples of both the test and reference products used to perform the in vivo bioequivalence studies for approval of the application. Each reserve sample should consist of at least 200 dosage units or 5 times the amount needed for complete release testing and should be retained for a minimum of 5 years following approval of the application. For more information on retention of bioequivalence samples, refer to 21 CFR 320.63.

## in vitro testing requirements

Dissolution Testing— Conduct dissolution testing on 12 dosage units of the test product and the reference product, employing the biostudy lots. [The current official USP dissolution method (see [Dissolution](#) 711) should be followed and should be referenced by the sponsor.] The following method and tolerances are currently recommended for this product: Medium: water; 1000 mL.

Apparatus 2: 50 rpm.

Times: 15, 30, 45, and 60 minutes.

Procedure— Use USP if available, or other validated method (see [Validation of Compendial Procedures](#) 1225).

Tolerance (Q): not less than 75% in 60 minutes.

The percentage of label claim dissolved at each specified testing interval should be reported for each individual dosage unit. The mean percentage dissolved, the range (highest, lowest) of dissolution, and the coefficient of variation (relative standard deviation) should be reported.

Content Uniformity Test— Content uniformity testing on the test and reference product lots should be performed as described in USP.

## waiver requirements

Waiver of in vivo bioequivalence study requirements for the 4-mg strength of the generic product may be granted pursuant to 21 CFR 320.22(d)(2), provided the following conditions are met:

1. The 4-mg tablet is proportionally similar in both active and inactive ingredients to the firm's 8-mg tablet, which has been demonstrated to be bioequivalent to the reference product in vivo.
2. The 4-mg tablet of the generic product meets dissolution test requirements.

Hydroxychloroquine Sulfate Tablets—In Vivo Bioequivalence and In Vitro Dissolution Testing<sup>4</sup>

Clinical Usage/Pharmacology— Hydroxychloroquine sulfate is indicated for the suppressive treatment and treatment of acute attacks of malaria due to *Plasmodium vivax*, *P. malariae*, *P. ovale*, and susceptible strains of *P. falciparum*. It is also indicated for the treatment of discoid and systemic lupus erythematosus and rheumatoid arthritis. Use of this drug is



contraindicated in the presence of retinal or visual field changes attributable to any 4-aminoquinoline compound.

The exact mechanism of antimalarial activity of hydroxychloroquine and the mechanism of action of the drug in the treatment of rheumatoid arthritis and lupus erythematosus have not been determined. However, the actions of the drug are presumably the same as those of chloroquine. Dosage and administration are dependent on the indications.

**Pharmacokinetics**— Information in literature on the pharmacokinetics of hydroxychloroquine is scarce. The mean bioavailability of hydroxychloroquine after oral administration is 74%. A 200-mg dose given orally resulted in a Cmax in whole blood of 120 to 170 ng per mL and a Tmax of 3 to 4 hours. Terminal half-life ranged from 19 to 28 days. After intravenous administration, hydroxychloroquine was found to have a large volume of distribution (5,500 L from blood, 44,000 L from plasma) and a terminal elimination half-life of approximately 40 days. It may require 6 months to achieve a steady-state blood level at a 200 mg per day dosing regimen.

#### in vivo bioequivalence studies<sup>5</sup>

##### Product Information—

FDA Designated Reference Product: Plaquenil® (Sanofi Winthrop) 200-mg tablet.

**Batch Size**— The test batch or lot must be manufactured under production conditions and must be of a size that is at least 10% of the largest lot planned for full production or a minimum of 100,000 dosage units, whichever is larger.

**Potency**— The assayed potency of the reference product should not differ from that of the test product by more than 5%.

##### Types of Studies Required—

1. A single-dose, randomized, two-period, two-treatment, two-sequence, crossover study under fasting conditions comparing equal doses of the test and reference products.
2. A single-period, parallel study comparing equal doses of the test and reference products.

##### Recommended Protocol for Conducting a Single-Dose Bioequivalence Study under Fasting Conditions—

**Objective**— The objective is to compare the rate and extent of absorption of a generic formulation with that of the reference formulation when given as equal labeled doses.

**Design**— The study design is a single-dose, two-treatment, two-period, two-sequence, crossover study with a washout period of at least 4 months. An equal number of subjects should be randomly assigned to the two possible dosing sequences. As an alternative, the design may be a single-dose, randomized, two-treatment, one-period, parallel study with a blood sampling time of approximately 3 months. Before the study begins, the proposed protocol should be approved by an institutional review board.

**Facilities**— The clinical facilities and analytical laboratory used for the study should be identified along with the names, titles, and curriculum vitae of medical and scientific or analytical directors.

**Selection of Subjects**— The sponsor should enroll a number of subjects sufficient to ensure statistical validity of the study. It is recommended that a minimum of 48 subjects be used for crossover studies or 96 subjects for parallel studies. Subjects should be healthy, preferably nonsmoking, volunteers aged 18 to 50 years and within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). Subjects should be selected on the basis of acceptable medical history, physical examination, and clinical laboratory test results. Female subjects must be given a pregnancy test prior to beginning the study. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. If smokers are included, they should be identified as such. Written, informed consent must be obtained from all study participants before they are accepted into the study.

**Procedure**— Following an overnight fast of at least 10 hours, subjects should be administered a single dose of the test or reference product with 240 mL of water.

##### Restrictions— Study volunteers should observe the following restrictions:

- a. The subjects should fast for 5 hours post-dose. Water may be taken except for 2 hours after drug administration.
- b. No xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until 24 hours post-dose.
- c. No alcoholic beverages should be consumed for 48 hours prior to and during the study period.
- d. Subjects should take no Rx medications, including oral contraceptives, beginning 2 weeks and no OTC medications beginning 1 week before drug administration and until after the study is completed.

**Blood Sampling**— Venous blood samples should be collected into tubes containing an anti-coagulant pre-dose (0 hours), and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12, 24, 36, 48, 72, 96, 120 hours and at 8, 12, 15, 19, 22, 26, 29, 33, 36, 43, 50, 57, 64, 70, 80, and 90 days. Blood should be immediately frozen until assayed.

**Subject Monitoring**— Blood pressure and pulse rate should be monitored hourly during the first 4 hours of the study. Subjects with a heart rate less than 45 bpm or greater than 110 bpm should have an electrocardiogram (lead II) performed and have their pulse monitored hourly. Subjects should report any unusual symptoms observed during the study. Subjects should be periodically questioned during each phase of the study for any unusual symptoms experienced after drug administration. A physical examination and clinical laboratory tests should be repeated after completing the blood level study.

**Analytical Methods**— Hydroxychloroquine in whole blood should be assayed using a suitable method fully validated with respect to adequate sensitivity, specificity, linearity, recovery, accuracy, and precision (both within and between days). The method to be used should be specific enough to measure the parent drug without interferences from metabolites and endogenous or exogenous components in the blood. Stability of samples under frozen conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be determined. Chromatograms of the analysis of the unknown samples, including all associated standard curves and quality control chromatograms, should be available for regulatory authorities. The sponsor should justify the rejection of any analytical data and provide a rationale for selection of the reported values.

**Pharmacokinetic Analysis**— From the blood drug concentration-time data, the following pharmacokinetic parameters should be determined for hydroxychloroquine:

- a. Area under the concentration-time curve from time zero to time t (AUC<sub>0-t</sub>), calculated by the trapezoidal rule, where t is the last time point with measurable non-zero concentration.
- b. Area under the concentration-time curve from time zero to time infinity (AUC<sub>0-∞</sub>), where AUC<sub>0-∞</sub> = AUC<sub>0-t</sub> + Ct / λ<sub>z</sub>, Ct is the last measurable non-zero concentration, and λ<sub>z</sub> is the terminal elimination rate constant calculated using an appropriate method.
- c. The terminal phase elimination rate constant, λ<sub>z</sub>, and the elimination half-life are calculated using an appropriate method.
- d. Peak drug concentration, C<sub>max</sub>, and the time to peak drug concentration, T<sub>max</sub>, are obtained directly from the data without interpolation.

**Statistical Analysis of Pharmacokinetic Data**— See Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment, Crossover Design under General Guidances; and an appropriate model should be chosen for the parallel study.

**Clinical Report, Side Effects, and Adverse Reactions**— Subject medical histories, physical examinations and laboratory reports, and all incidents of possible adverse reactions to the study formulations should be reported.

**Retention of Samples**— The laboratory conducting the bioequivalence testing should retain an appropriately identified reserve sample of both the test and reference products used to perform the in vivo bioequivalence study for approval of the application. Each reserve sample should consist of at least 200 dosage units or 5 times the amount needed for complete release testing and should be retained for a minimum of 5 years following approval of the application. For more information on retention of bioequivalence samples, refer to 21 CFR 320.63.

##### in vitro testing requirements

**Dissolution Testing**— Conduct dissolution testing on 12 dosage units of the test and reference products, employing the biostudy lots. The current official USP dissolution method should be followed and should be referenced by the sponsor. The following times should be used to determine the dissolution profile:

Times: 15, 30, 45, and 60 minutes.

The percentage of label claim dissolved at each specified sampling interval should be reported for each individual dosage unit. The mean percentage dissolved, the range of dissolution (highest and lowest), and the coefficient of variation (relative standard deviation) should be reported.



Content Uniformity Tests [905](#) — Content uniformity testing on the test and reference product lots should be performed as described in USP.

#### Indapamide Tablets— In Vivo Bioequivalence and In Vitro Dissolution Testing<sup>4</sup>

**Clinical Pharmacology/Usage—** Indapamide is indicated for the treatment of hypertension, alone or in combination with other antihypertensive drugs. It is also indicated for the treatment of salt and fluid retention associated with congestive heart failure. The recommended daily dose of indapamide is 2.5 mg, to be taken in the morning, which may be increased to 5.0 mg taken once daily.

Like other thiazide diuretics, indapamide works in the cortical diluting segment of the distal renal tubule. It acts on the luminal surface of the tubule, probably by inhibiting the Na<sup>+</sup>-Cl<sup>-</sup> co-transporter. Diuretic effects of indapamide are linearly related to dose in the range of 0.5 to 20 mg per day. The mechanism of antihypertensive action of indapamide is not established, and the magnitude of change in blood pressure does not correlate directly with change in extracellular fluid or plasma volume. A variety of actions of indapamide have been reported that may contribute to its antihypertensive activity. These effects include a decrease in the inward calcium current in the vascular smooth muscle, antipressor activity, and release of vasodilator prostaglandins such as prostaglandin E2 and prostacyclin. The net effect of indapamide may depend upon which of the various effects is dominant and may exhibit inter-patient variation.

**Pharmacokinetics—** The absorption of indapamide in healthy volunteers following oral administration is rapid and complete. The bioavailability of indapamide from oral solution and tablets is similar and is unaffected when the drug is given with food or antacids. The peak blood concentration, which exhibits linearity following administration of indapamide in doses of 2.5- to 10-mg, occurs 0.5 to 2 hours after drug administration. Steady-state blood concentration of indapamide is attained after 4 daily doses of 2.5 or 5.0 mg. Indapamide is readily taken up by erythrocytes. In human volunteers, a blood to plasma indapamide ratio of 5.7:1 at peak concentration has been reported, and its volumes of distribution estimated from blood and plasma concentrations are approximately 25 L per kg (V1) and 110 L per kg (V2), respectively. Indapamide is approximately 76% to 79% protein bound. Blood concentrations of indapamide exhibit biexponential decay with a rapid  $\alpha$ -phase and a prolonged  $\beta$ -phase. The terminal-phase elimination half-life of indapamide is in the range of 13 to 18 hours.

In humans, the major route of indapamide elimination is the urine, where 60% to 70% of an orally administered dose may be excreted. The estimated total systemic clearance of indapamide is in the range of 20 to 23.4 mL per minute. Because renal clearance of indapamide is only 1.71 mL per minute, hepatic clearance (23.8 mL per minute) may be responsible for a major portion of systemic clearance. Fecal elimination accounts for 16% to 23% of an orally administered dose. Indapamide is extensively metabolized in the liver. Up to 7% of the administered dose is excreted unchanged; the remainder as metabolites. In human volunteers, anywhere from 5 to 19 metabolic products of indapamide have been reported.

#### in vivo bioequivalence studies<sup>5</sup>

##### Product Information—

FDA Designated Reference Product: Lozol® (Rhône-Poulenc Rorer) 2.5-mg tablets.

**Batch Size—** The test batch or lot must be manufactured under production conditions and must be of a size that is at least 10% that of the largest lot planned for full production or a minimum of 100,000 units, whichever is larger.

**Potency—** The assayed potency of the reference product should not differ from that of the test product by more than 5%.

**Type of Study Required—** A single-dose, randomized, two-period, two-treatment, two-sequence crossover study under fasting conditions comparing equal doses of the test and reference products.

#### Recommended Protocol for Conducting a Single-Dose Bioequivalence Study under Fasting Conditions—

**Objective—** The objective is to compare the rate and extent of absorption of a generic formulation with that of a reference formulation when given as equal labeled doses.

**Design—** The study design is a single-dose, two-treatment, two-period, two-sequence crossover with a 2-week washout period between Phase I and Phase II dosing. An equal number of subjects should be randomly assigned to each of the two possible dosing sequences. Before the study begins, the proposed protocol should be approved by an institutional review board.

**Facilities—** The clinical facilities and analytical laboratory used for the study should be identified along with the names, titles, and curriculum vitae of the medical and scientific or analytical directors.

**Selection of Subjects—** The sponsor should enroll a number of subjects sufficient to ensure statistical validity of the study. It is recommended that a minimum of 24 subjects be used in this study. Subjects should be healthy, preferably nonsmoking, volunteers aged 18 to 50 and within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). Subjects should be selected on the basis of an acceptable medical history, physical examination, and clinical laboratory test results. Female subjects must be given a pregnancy test prior to beginning the study. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. If smokers are included, they should be identified as such. Written, informed consent must be obtained from all study participants before they are accepted into the study.

**Procedure—** Following an overnight fast of at least 10 hours, subjects should be administered two 2.5-mg tablets of the test or reference product with 240 mL of water.

**Restrictions—** Study volunteers should observe the following restrictions:

- a. Water may be taken, except for 1 hour before and after drug administration when no liquid is allowed other than that needed for drug dosing.
- b. Subjects should fast for at least 4 hours after administration of the test or reference treatment. All meals should be standardized during the study, and the same meals should be served during both phases of the study.
- c. No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
- d. Subjects should take no Rx medications, including oral contraceptives, beginning 2 weeks and no OTC medications beginning 1 week before drug administration and until after the study is completed.

**Blood Sampling—** Because indapamide binds preferentially to erythrocytes, its concentration as determined using plasma may not reflect true amounts of drug in the body if the hematocrit is altered within or between treatments. Therefore, whole blood concentrations of indapamide should be assayed. Venous blood samples should be collected pre-dose (0 hours) and at 0.50, 1, 1.50, 2, 2.50, 3, 4, 6, 8, 10, 12, 16, 24, 36, 48, 72 and 96 hours post-dose. Blood should be immediately frozen until assayed. Following a minimum 2-week washout period, subjects should begin the second phase of the study.

**Subject Monitoring—** Blood pressure and pulse rate should be monitored hourly during the first 4 hours of the study. Subjects with a heart rate less than 45 bpm or greater than 110 bpm should have an electrocardiogram (lead II) performed and have their pulse monitored hourly. Subjects should report any unusual symptoms observed during the study. Subjects should be periodically questioned during each phase of the study for any unusual symptoms experienced after drug administration.

**Analytical Methods—** Indapamide should be assayed using a suitable method fully validated with respect to adequate sensitivity, specificity, linearity, recovery, accuracy, and precision (both within and between days). Stability of the samples under frozen conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be determined. Chromatograms of the analysis of the unknown samples, including all associated standard curve and quality control chromatograms, should be available for regulatory authorities. The sponsor should justify the rejection of any analytical data and provide a rationale for selection of the reported values.

**Statistical Analysis of Pharmacokinetic Data—** See Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design under General Guidances.

**Clinical Report, Side Effects, and Adverse Reactions—** Subject medical histories, physical examinations and laboratory reports, and all incidents of possible adverse reactions and side effects to the study formulations should be reported.

**Retention of Samples—** The laboratory conducting the bioequivalence testing should retain appropriately identified reserve samples of both the test and reference products used to perform the in vivo bioequivalence studies for approval of the application. Each reserve sample should consist of at least 200 dosage units or 5 times the amount needed for complete release testing and should be retained for a minimum of 5 years following approval of the application. For more information on retention of bioequivalence samples, refer to 21 CFR



320.63.

## in vitro testing requirements

Dissolution Testing—Conduct dissolution testing on 12 dosage units of the test product versus 12 units of the reference product. The biostudy lots should be used for those product strengths tested in vivo. The current official USP dissolution method should be followed and should be referenced by the sponsor.

Times: 15, 30, 45, and 60 minutes.

Content Uniformity Test (905)—Content uniformity testing on the test and reference product lots should be performed as described in USP.

## waiver requirements

Waiver of in vivo bioequivalence study requirements for the 1.25-mg strength of the generic product may be granted pursuant to 21 CFR 320.22(d)(2), provided the following conditions are met:

1. The 1.25-mg tablet is proportionally similar in both active and inactive ingredients to the firm's 2.5-mg tablet, which has been demonstrated to be bioequivalent to the reference product in vivo.
2. The 1.25-mg tablet of the generic product meets dissolution test requirements.

Ketoprofen Capsules—In Vivo Bioequivalence and In Vitro Dissolution Testing<sup>4</sup>

Clinical Usage/Pharmacology—Ketoprofen is a nonsteroidal anti-inflammatory drug with analgesic and antipyretic properties. It is indicated for the acute or long-term treatment of the signs and symptoms of rheumatoid arthritis and osteoarthritis. Ketoprofen is contraindicated in patients who are hypersensitive to aspirin and other nonsteroidal anti-inflammatory drugs. Monitoring of renal function is indicated in patients taking concomitant diuretics.

Ketoprofen curtails the inflammatory mechanism in rheumatoid arthritis and other disease states by reversibly inhibiting cyclooxygenase and lipoxygenase, enzymes involved in the synthesis of prostaglandins and leukotrienes. It is also a powerful inhibitor of bradykinin, a pain and inflammation mediator, and prevents the release of lysosomal enzymes, which can cause tissue destruction in inflammatory reactions.

For rheumatoid arthritis and osteoarthritis in adults, an initial dose of 75 mg 3 times or 50 mg 4 times a day is recommended. In patients who tolerate ketoprofen well, a maximum total daily dose of 300 mg can be used. Ketoprofen should be taken with food or milk.

Pharmacokinetics—Ketoprofen is rapidly and completely absorbed from the gastrointestinal tract with peak plasma concentration occurring in 0.5 to 2 hours. The mean plasma elimination half-life ranges from 2 to 4 hours. The absolute bioavailability of the 100-mg capsule of ketoprofen as compared to an intravenous formulation is  $\geq 92\%$ . Concurrent ingestion of food appears to affect the rate but not the extent of absorption of ketoprofen.

Ketoprofen is more than 95% bound to plasma albumin with a relatively small apparent volume of distribution ( $V_d = 0.1-0.2 \text{ L per kg}$ ). Substantial concentrations of ketoprofen have been found in synovial fluid, which is the proposed primary site of action for NSAIDS in rheumatoid arthritis. Ketoprofen exhibits a longer  $T_{max}$ , a lower  $C_{max}$ , and a 2.5-fold greater elimination half-life in synovial fluid than in plasma.

Two studies in humans have shown that 90% of an oral dose of tritiated ketoprofen given to healthy subjects was recovered in the urine over several days; an additional 1% to 8% of the labeled dose appeared in the feces. Virtually all of the material eliminated into urine after an oral dose is in the form of ketoprofen conjugates; less than 1% is the unchanged drug.

Enterohepatic recirculation appeared to be negligible in humans. Ketoprofen metabolites are not active.

in vivo bioequivalence studies<sup>5</sup>

## Product Information—

FDA Designated Reference Product: Orudis® (Wyeth Ayerst) 75-mg capsules.

Batch Size—The test batch or lot must be manufactured under production conditions and must be of a size that is at least 10% of the largest lot planned for full production or a minimum of 100,000 units, whichever is larger.

Potency—The assayed potency of the reference product should not differ from that of the test product by more than 5%.

## Types of Studies Required—

1. A single-dose, randomized, two-period, two-treatment, two-sequence crossover study under fasting conditions comparing equal doses of the test and reference products.
2. A single-dose, randomized, three-treatment, three-period, six-sequence crossover, limited food effects study comparing equal doses of the test and reference products when administered immediately following a standard high-fat breakfast.<sup>6</sup>

## Recommended Protocol for Conducting a Single-Dose Bioequivalence Study under Fasting Conditions—

Objective—The objective is to compare the rate and extent of absorption of a generic formulation with that of a reference formulation when given as equal labeled doses.

Design—The study design is a single-dose, two-treatment, two-period, two-sequence crossover with a 1-week washout period between Phase I and Phase II dosing. An equal number of subjects should be randomly assigned to each of the two possible dosing sequences. Before the study begins, the proposed protocol should be approved by an institutional review board.

Facilities—The clinical facilities and analytical laboratory used for the study should be identified along with the names, titles, and curriculum vitae of the medical and scientific or analytical directors.

Selection of Subjects—The sponsor should enroll a number of subjects sufficient to ensure statistical validity of the study. It is recommended that a minimum of 24 subjects be used in this study. Subjects should be healthy, preferably nonsmoking, volunteers 18 to 50 years of age and within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). Subjects should be selected on the basis of an acceptable medical history, physical examination, and clinical laboratory test results. Female subjects must be given a pregnancy test prior to beginning the study. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. If smokers are included, they should be identified as such. Written, informed consent must be obtained from all study participants before they are accepted into the studies.

Procedure—Following an overnight fast of at least 10 hours, subjects should be administered a single 75-mg tablet of the test or reference product with 240 mL of water.

Restrictions—Study volunteers should observe the following restrictions:

- a. Water may be taken, except for 1 hour before and after drug administration, when no liquid is allowed other than that needed for drug dosing.
- b. Subjects should fast for at least 4 hours after administration of the test or reference treatment. All meals should be standardized during the study, and the same meals should be served during both phases of the study.
- c. No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
- d. Subjects should take no Rx medications, including oral contraceptives, beginning 2 weeks and no OTC medications beginning 1 week before drug administration and until after the study is completed.

Blood Sampling—Venous blood samples should be collected pre-dose (0 hours) and at 10, 20, 30, and 45 minutes and at 1, 1.25, 1.50, 2, 2.50, 3, 4, 6, 8, 10, 12, 16, and 24 hours post-dose. Plasma should be separated promptly and immediately frozen until assayed. Following a minimum 1-week washout period, subjects should begin the second phase of the study.

Analytical Methods—The active ingredient should be assayed using a suitable method fully validated with respect to adequate sensitivity, specificity, linearity, recovery, and accuracy and precision (both within and between days). Stability of the samples under frozen conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be



determined. Chromatograms of the analysis of the unknown samples, including all associated standard curve and quality control chromatograms, should be available for regulatory authorities. The sponsor should justify the rejection of any analytical data and provide a rationale for selection of the reported values.

**Subject Monitoring**—Blood pressure and pulse rate should be monitored hourly during the first 4 hours of the study. Subjects with a heart rate less than 45 bpm or greater than 110 bpm should have an electrocardiogram (lead II) performed and have their pulse monitored hourly. Subjects should report any unusual symptoms observed during the study. Subjects should be periodically questioned during each phase of the study for any unusual symptoms experienced after drug administration.

**Statistical Analysis of Pharmacokinetic Data**—See Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design under General Guidances.

#### Limited Food Effects Study—

**Objective**—The objective is to compare the rate and extent of absorption of ketoprofen from a generic formulation with that from a reference formulation under nonfasting conditions and to compare the rate and extent of absorption of the drug from a generic formulation under fasting and nonfasting conditions when given as equal labeled doses.

**Design**—The study design is a single-dose, three-treatment, three-period, six-sequence crossover with a 1-week washout period between Phase I and Phase II, and Phase II and Phase III. An equal number of subjects should be assigned to each of the six dosing sequences.

The limited food effects study should be performed in the same manner as the single-dose study under fasting conditions, with the following exceptions:

**Procedure**—An equal number of subjects (12 to 18) should be assigned to each of the six dosing sequences possible in a three-treatment, three-period study design. Each subject will receive the following treatments:

TREATMENT 1: Generic product, a 75-mg capsule administered after a standard high-fat breakfast.

TREATMENT 2: Reference product, a 75-mg Orudis® capsule administered after a standard high-fat breakfast.

TREATMENT 3: Generic product, a 75-mg capsule administered under fasting conditions.

Following a 10-hour fast, the subjects receiving treatments under fed conditions should be served a standard high-fat breakfast. The subjects should have 30 minutes to finish the entire breakfast, and then should immediately receive Treatment 1 or Treatment 2, taken with 240 mL of water. Subjects receiving the treatment under fasting conditions should receive Treatment 3, taken with 240 mL of water only. The same lots of the test and reference products should be used in the fasting and nonfasting studies. No food should be allowed for at least 4 hours post-dose with water allowed after the first hour. Subjects should be served scheduled standardized meals throughout the study, and the same meals should be served during all phases of the study.

**Statistical Analysis**—In general, a comparable food effect will be assumed if the AUC0–t, AUC0–, and Cmax mean values for the test product administered under nonfasting conditions are within 20% of the respective mean values obtained for the reference product administered under nonfasting conditions.

**Clinical Report, Side Effects, and Adverse Reactions**—Subject medical histories, physical examinations and laboratory reports, and all incidents of possible adverse reactions and side effects to the study formulations should be reported.

**Retention of Samples**—The laboratory conducting the bioequivalence testing should retain appropriately identified reserve samples of both the test and reference products used to perform the in vivo bioequivalence studies for approval of the application. Each reserve sample should consist of at least 200 dosage units and should be retained for a minimum of 5 years following approval of the application. For more information on retention of bioequivalence samples, refer to 21 CFR 320.63.

#### in vitro testing requirements

**Dissolution Testing** (711)—Conduct dissolution testing on 12 dosage units of the test product and reference products, employing the biostudy lots. The following method and tolerances are currently recommended for this product:

Medium: 0.05 M pH 7.4 phosphate buffer; 1000 mL.

Apparatus 2: 50 rpm.

Times: 10, 20, and 30 minutes.

**Procedure**—Use USP if available, or other validated method (see [Validation of Compendial Procedures](#) (1225)).

Tolerances (Q): not less than 80% in 30 minutes.

The percentage of label claim dissolved at each specified testing interval should be reported for each individual dosage unit. The mean percentage dissolved, the range (highest, lowest) of dissolution, and the coefficient of variation (relative standard deviation) should be reported.

**Content Uniformity Test** (905)—Content uniformity testing on the test and reference product lots should be performed as described in USP.

#### waiver requirements

Waiver of in vivo bioequivalence study requirements for the 25- and 50-mg strengths of the generic capsule may be granted pursuant to 21 CFR 320.22(d)(2), provided the following conditions are met:

1. The 25- and 50-mg capsules are proportionally similar in both active and inactive ingredients to the firm's 75-mg capsule, which has been demonstrated to be bioequivalent to the reference product in vivo.
2. The 25- and 50-mg capsules meet the dissolution test requirements.

#### Nadolol Tablets—In Vivo Bioequivalence and In Vitro Dissolution Testing<sup>4</sup>

**Clinical Pharmacology/Usage**—Nadolol is a long-acting, synthetic, nonselective beta-adrenergic receptor blocker. Currently approved indications include treatment of essential hypertension and treatment of angina pectoris.

For the treatment of hypertension, the usual initial dose is 40 mg of nadolol once daily. The dosage may be gradually increased in 40- to 80-mg increments until optimum blood pressure reduction is achieved. The usual maintenance dose is 40 or 80 mg once daily. Doses of up to 240 or 320 mg administered once daily may be needed.

For the treatment of angina pectoris, the usual initial dose is 40 mg of nadolol once daily. The dosage may be gradually increased in 40- to 80-mg increments at three- to five-day intervals until optimum clinical response is obtained or there is pronounced slowing of the heart rate. The usual maintenance dose is 40 or 80 mg once daily. Doses of up to 160 or 240 mg administered once daily may be needed.

Nadolol specifically competes with beta-adrenergic receptor agonists for available beta receptor sites; it inhibits both the beta-1 receptors located chiefly in the cardiac muscle and the beta-2 receptors located chiefly in the bronchial and vascular musculature, proportionately inhibiting the chronotropic, inotropic, and vasodilator responses to beta-adrenergic stimulation. Nadolol is contraindicated in cases of bronchial asthma, sinus bradycardia and greater than first degree conduction block, cardiogenic shock, and overt cardiac failure.

**Pharmacokinetics**—The absorption of nadolol from the GI tract is variable following oral dosing, averaging about 30%. About 20% to 30% of the drug is reversibly bound to plasma proteins. Peak blood serum concentrations are reached within 1 to 4 hours following oral administration. The elimination half-life is in the range of 12 to 24 hours, permitting once-daily administration. Steady-state serum levels are reached in 6 to 9 days with once-daily dosing. Because nadolol is excreted unchanged predominately in the urine, its half-life increases in patients with renal impairment.

Pharmacokinetically, the drug is described by an open, two-compartment model. The approved labeling for the drug states that the presence of food in the GI tract does not affect the rate or extent of absorption and that nadolol may be dosed without regard to meals.

#### in vivo bioequivalence studies<sup>5</sup>



## Product Information—

FDA Designated Reference Products: Corgard® (Bristol Laboratories) 40- and 160-mg tablets.

**Batch Size**— The test batch or lot must be manufactured under production conditions and must be of a size at least 10% that of the largest lot planned for full production or a minimum of 100,000 units, whichever is larger.

**Potency**— The assayed potency of the reference product should not differ from that of the test product by more than 5%.

## Types of Studies Required—

1. Two single-dose, randomized, two-period, two-treatment, two-sequence crossover studies under fasting conditions comparing equal doses of the 40- and 160-mg test and reference products.
2. A single-dose, randomized, three-treatment, three-period, six-sequence crossover, limited food effects study comparing equal doses of the 40-mg test and reference products when administered immediately following a standard high-fat breakfast.<sup>§</sup>

## Recommended Protocols for Conducting Single-Dose, Fasted Bioequivalence Studies—

**Objective**— The objective of the two studies is to compare the rate and extent of absorption of nadolol from generic formulations with that from reference formulations when given as equal labeled doses.

**Design**— The study design is a single-dose, two-treatment, two-period, two-sequence crossover with a 2-week washout period between Phase I and Phase II dosing. An equal number of subjects should be randomly assigned to each of the two possible dosing sequences. Before the study begins, the proposed protocol should be approved by an institutional review board.

**Facilities**— The clinical facilities and analytical laboratory used for the study should be identified along with the names, titles, and curriculum vitae of the medical and scientific or analytical directors.

**Selection of Subjects**— The sponsor should enroll a number of subjects sufficient to ensure statistical validity of the study. Subjects should be healthy, preferably nonsmoking, volunteers aged 18 to 55 and within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). Subjects should be selected on the basis of acceptable medical history, physical examination, and clinical laboratory test results. Subjects should have a resting pulse rate of at least 65 bpm. Female subjects must be given a pregnancy test prior to beginning the study. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. If smokers are included, they should be identified as such. Written, informed consent must be obtained from all study participants before they are accepted into the study.

**Procedure**— Following an overnight fast of at least 10 hours, subjects should be administered a single dose (40 or 160 mg) of the test or reference product with 240 mL of water.

## Restrictions— Study volunteers should observe the following restrictions:

- a. Water may be taken, except for 1 hour before and after drug administration, when no liquid is allowed other than that needed for drug dosing.
- b. Subjects should fast for at least 4 hours after administration of the test or reference treatment. All meals should be standardized during the study, and the same meals should be served during both phases of the study.
- c. No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
- d. Subjects should take no Rx medications, including oral contraceptives, beginning 2 weeks and no OTC medications beginning 1 week before drug administration and until after the study is completed.

**Blood Sampling**— Venous blood samples should be collected pre-dose (0 hours) and at 0.50, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, 18, 24, 36, 48, and 72 hours post-dose. Plasma should be separated promptly and immediately frozen until assayed. Following a minimum 2-week washout period, subjects should begin the second phase of the study.

**Subject Monitoring**— Blood pressure and pulse rate should be monitored hourly during the first 4 hours of the study. Subjects with a heart rate less than 45 bpm or greater than 110 bpm should have an electrocardiogram (lead II) performed and have their pulse monitored hourly. Subjects should report any unusual symptoms observed during the study. Subjects should be periodically questioned during each phase of the study for any unusual symptoms experienced after drug administration.

**Analytical Methods**— Nadolol should be assayed using a suitable method fully validated with respect to adequate sensitivity, specificity, linearity, recovery, accuracy, and precision (both within and between days). Stability of the samples under frozen conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be determined.

Chromatograms of the analysis of the unknown samples, including all associated standard curve and quality control chromatograms, should be available for regulatory authorities. The sponsor should justify the rejection of any analytical data and provide a rationale for selection of the reported values.

**Statistical Analysis of Pharmacokinetic Data**— See Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design under General Guidances.

## Limited Food Effects Study—

**Objective**— The objective is to compare the rate and extent of absorption of nadolol from a generic formulation with that from a reference formulation under nonfasting conditions and to compare the rate and extent of absorption of the drug from a generic formulation under fasting and nonfasting conditions when given as equal labeled doses.

**Design**— The study design is a single-dose, three-treatment, three-period, six-sequence crossover with a 2-week washout period between Phase I and Phase II and Phase II and Phase III.

The limited food effects study (minimum 12 subjects) should be performed in the same manner as the single-dose study under fasting conditions, with the following exceptions:

**Procedure**— An equal number of subjects should be assigned to each of the 6 dosing sequences possible in a three-treatment, three-period study design. Each subject will receive the following treatments:

TREATMENT 1: Generic product, a 40-mg tablet administered after a standard high-fat breakfast.

TREATMENT 2: Reference product, a 40-mg Corgard® tablet administered after a standard high-fat breakfast.

TREATMENT 3: Generic product, a 40-mg tablet administered under fasting conditions.

Following a 10-hour fast, the subjects receiving treatments under fed conditions should be served a standard high-fat breakfast. The subjects should have 30 minutes to finish the entire breakfast and then should immediately receive Treatment 1 or Treatment 2, taken with 240 mL of water. Subjects receiving the treatment under fasting conditions should receive Treatment 3, taken with 240 mL of water only. The same lots of the test and reference products should be used in the fasted and fed studies. No food should be allowed for at least 4 hours post-dose, with water allowed after the first hour. Subjects should be served scheduled standardized meals throughout the study, and the same meals should be served during all phases of the study.

**Statistical Analysis**— In general, a comparable food effect will be assumed if the AUC<sub>0-t</sub>, AUC<sub>0-∞</sub>, and C<sub>max</sub> mean values for the test product administered under fed conditions are within 20% of the respective mean values obtained for the reference product administered under fed conditions.

**Clinical Report, Side Effects, and Adverse Reactions**— Subject medical histories, physical examinations and laboratory reports, and all incidents of possible adverse reactions and side effects to the study formulations should be reported.

**Retention of Samples**— The laboratory conducting the bioequivalence testing should retain appropriately identified reserve samples of both the test and reference products used to perform the in vivo bioequivalence studies for approval of the application. Each reserve sample should consist of at least 200 dosage units or 5 times the amount needed for complete release testing, whichever is greater, and should be retained for a minimum of 5 years following approval of the application. For more information on retention of bioequivalence samples, refer to 21 CFR 320.63.

## in vitro testing requirements

**Dissolution Testing**— Conduct dissolution testing on 12 dosage units of the test and reference products, employing the biostudy lots. The current official USP dissolution method (see



[Dissolution](#) (711) should be followed and referenced by the sponsor. The following times should be used to determine the dissolution profile:  
Times: 10, 20, 30, 40, 50, and 60 minutes.

The percentage of label claim dissolved at each specified testing interval should be reported for each individual dosage unit. The mean percentage dissolved, the range of dissolution (highest, lowest), and the coefficient of variation (relative standard deviation) should be reported.

Content Uniformity Test—Content uniformity testing on the test and reference product lots should be performed as described in USP.

#### waiver requirements

Waiver of in vivo bioequivalence study requirements for the 20-, 80- and 120-mg strengths of the generic product may be granted pursuant to 21 CFR 320.22(d)(2) provided the following conditions are met:

1. The 20-mg tablet is proportionally similar in both active and inactive ingredients to the firm's 40-mg tablet, which has been demonstrated to be bioequivalent to the reference product in vivo. The 80- and 120-mg tablets are proportionally similar in both active and inactive ingredients to the firm's 160-mg tablet, which has been demonstrated to be bioequivalent to the reference product in vivo.
2. The 20-mg tablet of the generic product meets dissolution test requirements. The 80- and 120-mg tablets of the generic product meet dissolution test requirements.

#### Naproxen Tablets—In Vivo Bioequivalence and In Vitro Dissolution Testing<sup>4</sup>

**Clinical Pharmacology/Usage**— Naproxen is an orally administered nonsteroidal anti-inflammatory drug (NSAID), which also has analgesic and antipyretic properties. Currently approved indications for naproxen are (1) treatment of rheumatoid arthritis, osteoarthritis, juvenile arthritis, ankylosing spondylitis, tendinitis, bursitis, and acute gout; (2) relief of mild to moderate pain; and (3) treatment of primary dysmenorrhea. For adult arthritis and ankylosing spondylitis, the recommended dosage is 250-500 mg bid (morning and evening), up to 1500 mg per day for limited periods when a higher degree of anti-inflammatory activity is required and the dose is well tolerated. For juvenile arthritis, the recommended total daily dose of naproxen is 10 mg per kg in two divided doses. For acute gout, the recommended starting dose is 750 mg followed by 250 mg every 8 hours until the attack subsides. For the other indications, the recommended starting dose is 500 mg followed by 250 mg every 6 to 8 hours as required, not to exceed 1250 mg per day.

**At plasma pH**, naproxen exists predominantly as the naproxen ion that reversibly binds the enzyme cyclooxygenase and inhibits prostaglandin synthesis, affecting conditions where overproduction of prostaglandins occurs. Other mechanisms of action may be possible. Due to possible cross-reactivity, naproxen is contraindicated in patients in whom aspirin or other NSAID agents induce the syndrome of asthma, rhinitis, and nasal polyps, and in patients who have had any symptoms of allergic reactions to NSAIDs. The major adverse reaction is gastrointestinal irritation, upset, and dyspepsia, but serious gastrointestinal toxicity (ulceration, bleeding, and perforation) can occur with chronic use.

**Pharmacokinetics**— Naproxen is rapidly and completely absorbed following oral administration with a reported time of maximum concentration (T<sub>max</sub>) values of 2 hours and 2 to 4 hours. Because naproxen is insoluble in water at low pH but freely soluble at physiologic pH, the rate of gastric emptying and pH of the gastric and intestinal contents are more likely to be rate-limiting for naproxen absorption than dissolution in gastrointestinal fluids. After single oral doses of 100, 200, and 300 mg, reported maximum concentrations (C<sub>max</sub>) values were 12, 25, and 42 µg per mL, respectively. A 500-mg dose produces a C<sub>max</sub> of about 55 µg per mL. Food may delay absorption by decreasing the rate of gastric emptying, but does not significantly change C<sub>max</sub> or area under the plasma concentration-time curve (AUC). In general, the advantages of reduced gastrointestinal irritation are thought to outweigh any clinical effects of slowed absorption rate.

The volume of distribution of naproxen is about 0.09 to 0.16 L per kg. The drug distributes into synovial fluid to reach about 50% of plasma levels 3 to 4 hours after dosing. Naproxen is ≥ 99% bound to plasma proteins (albumin), and this binding is known to decrease with increasing plasma drug concentrations (99.6% bound at total plasma concentrations of 23 to 40 µg per mL; 97.4% bound at 473 µg per mL). Disproportional increments in naproxen AUC at doses >500 mg per day are attributed to nonlinear plasma protein binding. For this poorly extracted drug, the increased free fraction of naproxen at higher doses results in more unbound naproxen available for biotransformation and urinary excretion. Despite nonlinear disposition, the half-life of naproxen is independent of dosage or plasma concentration after both single or multiple doses; reported values are 12 to 16 hours. In humans, naproxen is metabolized to naproxen glucuronide (40%), an unknown conjugate (20%), and 6-desmethylnaproxen (28%). The latter moiety is itself conjugated with glucuronide (12%). Less than 10% of naproxen is excreted unchanged in the urine. About 95% of a dose appears in the urine after 5 days, with less than 5% fecal excretion.

#### in vivo bioequivalence studies<sup>5</sup>

##### Product Information—

FDA Designated Reference Product: Naprosyn® (Syntex) 500-mg tablet.

**Batch Size**— The test batch or lot must be manufactured under production conditions and must be of a size that is at least 10% that of the largest lot planned for full production or a minimum of 100,000 units, whichever is larger.

**Potency**— The assayed potency of the reference product should not differ from that of the test product by more than 5%.

#### Types of Studies Required—

1. A single-dose, randomized, two-period, two-treatment, two-sequence crossover study under fasting conditions comparing equal doses of the test and reference products.
2. A single-dose, randomized, three-treatment, three-period, six-sequence crossover, limited food effects study comparing equal doses of the test and reference products when administered immediately following a standard high-fat breakfast.<sup>6</sup>

#### Recommended Protocol for Conducting a Single-Dose Bioequivalence Study under Fasting Conditions—

**Objective**— The objective is to compare the rate and extent of absorption of a generic formulation with that from a reference formulation when given as equal labeled doses.

**Design**— The study design is a single-dose, two-treatment, two-period, two-sequence crossover with a 1-week washout period between Phase I and Phase II dosing. An equal number of subjects should be randomly assigned to each of the 2 possible dosing sequences. Before the study begins, the proposed protocol should be approved by an institutional review board.

**Facilities**— The clinical facilities and analytical laboratory used for the study should be identified along with the names, titles, and curriculum vitae of the medical and scientific or analytical directors.

**Selection of Subjects**— The sponsor should enroll a number of subjects sufficient to ensure statistical validity of the study. It is recommended that a minimum of 24 subjects be used in this study. Subjects should preferably be healthy, preferably nonsmoking, volunteers aged 18 to 55 within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). If smokers are included, they should be identified as such. Subjects should be selected on the basis of acceptable medical history, physical examination, and clinical laboratory test results. Female subjects must be given a pregnancy test prior to beginning the study. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. Written, informed consent must be obtained from all study participants before they are accepted into the study.

**Procedure**— Following an overnight fast of at least 10 hours, subjects should be administered a single 500-mg tablet of the test or reference product with 240 mL of water.

**Restrictions**— Study volunteers should observe the following restrictions:

- a. Water may be taken except for 1 hour before and after drug administration when no liquid is allowed other than that needed for drug dosing.
- b. Subjects should fast for at least 4 hours after administration of the test or reference treatment. All meals should be standardized during the study, and the same meals should be served during both phases of the study.
- c. No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
- d. Subjects should take no Rx medications, including oral contraceptives, beginning 2 weeks and no OTC medications beginning 1 week before drug administration and until after the study is completed.



...od Sampling—Venous blood samples should be collected pre-dose (0 hours) and at 0.50, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 12, 18, 24, 36, 48, and 72 hours post-dose. Plasma should be separated promptly and immediately frozen until assayed. Following a minimum 1-week washout period, subjects should begin the second phase of the study.

Subject Monitoring—Blood pressure and pulse rate should be monitored hourly during the first 4 hours of the study. Subjects with a heart rate less than 45 bpm or greater than 110 bpm should have an electrocardiogram (lead II) performed and have their pulse monitored hourly. Subjects should report any unusual symptoms observed during the study. Subjects should be periodically questioned during each phase of the study for any unusual symptoms experienced after drug administration.

Analytical Methods—Naproxen should be assayed using a suitable method fully validated with respect to adequate sensitivity, specificity, linearity, recovery, accuracy, and precision (both within and between days). Stability of the samples under frozen conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be determined. Chromatograms of the analysis of the unknown samples, including all associated standard curve and quality control chromatograms, should be available for regulatory authorities. The sponsor should justify the rejection of any analytical data and provide a rationale for selection of the reported values.

Statistical Analysis of Pharmacokinetic Data—See Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design under General Guidances.

#### Limited Food Effects Study—

Objective—The objective is to compare the rate and extent of absorption of naproxen from a generic formulation with that from a reference formulation under nonfasting conditions and to compare the rate and extent of absorption of the drug from a generic formulation under fasting and nonfasting conditions when given as equal labeled doses.

Design—The study design is a single-dose, three-treatment, three-period, six-sequence crossover with a 1-week washout period between Phase I and Phase II, and Phase II and Phase III. An equal number of subjects should be assigned to each of the 6 dosing sequences.

The limited food effects study should be performed in the same manner as the single-dose study under fasting conditions, with the following exceptions:

Procedure—An equal number of subjects should be assigned to each of the 6 dosing sequences possible in a three-treatment, three-period study design (minimum 12 subjects). Each subject will receive the following treatments:

TREATMENT 1: Generic product, a 500-mg tablet administered after a standard high-fat breakfast.

TREATMENT 2: Reference product, a 500-mg Naprosyn® tablet (Syntex) administered after a standard high-fat breakfast.

TREATMENT 3: Generic product, a 500-mg tablet administered under fasting conditions.

Following a 10-hour fast, the subjects receiving treatments under fed conditions should be served a standard high-fat breakfast. The subjects should have 30 minutes to finish the entire breakfast and then should immediately receive Treatment 1 or Treatment 2, taken with 240 mL of water. Subjects receiving the treatment under fasting conditions should receive Treatment 3, taken with 240 mL of water only. The same lots of the test and reference products should be used in the fasted and fed studies. No food should be allowed for at least 4 hours post-dose, with water allowed after the first hour. Subjects should be served scheduled standardized meals throughout the study, and the same meals should be served during all phases of the study.

Statistical Analysis—In general, a comparable food effect will be assumed if the AUC<sub>0-t</sub>, AUC<sub>0-</sub>, C<sub>max</sub> mean values for the test product administered under fed conditions are within 20% of the respective mean values obtained for the reference product administered under fed conditions.

Clinical Report, Side Effects, and Adverse Reactions—Subject medical histories, physical examinations and laboratory reports, and all incidents of possible adverse reactions and side effects to the study formulations should be reported.

Retention of Samples—The laboratory conducting the bioequivalence testing should retain appropriately identified reserve samples of both the test and reference products used to perform the *in vivo* bioequivalence studies for approval of the application. Each reserve sample should consist of at least 200 dosage units or 5 times the amount needed for complete release testing and should be retained for a minimum of 5 years following approval of the application. For more information on retention of bioequivalence samples refer to 21 CFR 320.63.

#### in vitro testing requirements

Dissolution Testing—Conduct dissolution testing on 12 dosage units of the test product and the reference product, employing the biostudy lots. The current official USP dissolution method (see [Dissolution](#) 711) should be followed and should be referenced by the sponsor. The following times should be used to determine the dissolution profile. Times: 10, 20 30, 45, and 60 minutes.

The percentage of label claim dissolved at each specified testing interval should be reported for each individual dosage unit. The mean percentage dissolved, the range (highest, lowest) of dissolution, and the coefficient of variation (relative standard deviation) should be reported.

Content Uniformity Test—Content uniformity testing on the test and reference product lots should be performed as described in USP.

#### waiver requirements

Waiver of *in vivo* bioequivalence study requirements for the 375- and 250-mg strengths of the generic product may be granted pursuant to 21 CFR 320.22(d)(2) provided the following conditions are met:

1. The 375- and 250-mg tablets are proportionally similar in both active and inactive ingredients to the firm's 500-mg tablet, which has been demonstrated to be bioequivalent to the reference product *in vivo*.
2. The 375- and 250-mg tablets of the generic product meet dissolution test requirements.

#### Pentoxifylline Extended-Release Tablets—*In Vivo* Bioequivalence and *In Vitro* Dissolution Testing<sup>4</sup>

Clinical Usage/Pharmacology—Pentoxifylline is a hemorrhheologic agent that improves the flow properties of blood by decreasing its viscosity and improving erythrocyte flexibility. These actions increase blood flow and enhance tissue oxygenation in patients with chronic peripheral arterial disease. It is indicated for the treatment of patients with intermittent claudication on the basis of chronic occlusive arterial disease of the limbs. The usual dosage is one tablet three times a day with meals.

Pharmacokinetics—Urinary recovery data suggests virtually complete absorption of pentoxifylline from the extended-release dosage form. The absolute bioavailability at steady state (every 8 hours for 6 days) is estimated to be 19.4%, indicating extensive first-pass metabolism. Pentoxifylline undergoes both oxidation and reduction metabolism with greater than 90% of a dose being eliminated renally as metabolites. Both pentoxifylline and its reduction metabolite 1-(5-hydroxyhexyl)-3,7-dimethylxanthine (M1) and its oxidation metabolite 1-(3-carboxypropyl)-3,7-dimethylxanthine (M5) have similar pharmacological effects.

Administration of single doses of 400-mg pentoxifylline extended-release tablets results in T<sub>max</sub> values of 2 to 4 hours and mean C<sub>max</sub> values of 55 to 300 ng per mL. The apparent mean half-life of the parent drug is about 3.4 hours, demonstrating the absorption-limited elimination rate of the extended-release dosage form. After multiple dosing of pentoxifylline extended-release tablets (400 mg every 8 hours for 6 to 7 days), mean steady-state values of pentoxifylline C<sub>max</sub> were 189 to 248 ng per mL and T<sub>max</sub> was 0.9 to 2 hours. Reported mean values for M1 (400-mg extended-release tablet) from single-dose studies are as follows: C<sub>max</sub>, 143 to 343 ng per mL; T<sub>max</sub>, 3.2 hours; and t<sub>1/2</sub>, 3.4 hours. Mean steady-state values for M1 from multiple-dose studies were reported as follows: C<sub>max</sub>, 562 to 576 ng per mL; and T<sub>max</sub>, 2 to 2.8 hours. For M5, the corresponding values were 943 ng per mL and 1.4 hours, respectively. Drug absorption is slower when pentoxifylline is taken with food resulting in a significant decrease in C<sub>max</sub> and increase in T<sub>max</sub> for parent drug and metabolites. However, AUC is not changed significantly.

#### in vivo bioequivalence studies<sup>5</sup>

##### Product Information—

FDA Designated Reference Product: Trental® (Hoechst Marion Roussel) 400-mg extended-release tablets.

Batch Size—The test batch or lot must be manufactured under production conditions and must be of a size that is at least 10% of the largest lot planned for full production or a minimum of 100,000 units, whichever is larger.



Potency— The assayed potency of the reference product should not differ from that of the test product by more than 5%.

Types of Studies Required—

1. A single-dose, randomized, two-treatment, two-period, two-sequence crossover study under fasting conditions comparing equal doses of the test and reference products.
2. A multiple-dose, steady-state, randomized, two-treatment, two-period, two-sequence crossover study under fasting conditions comparing equal doses of the test and reference products.
3. A single-dose, randomized, three-treatment, three-period, six-sequence crossover, limited food effects study comparing equal doses of the test and reference products when administered immediately following a standard high-fat breakfast.<sup>6</sup>

Recommended Protocol for Conducting a Single-Dose Bioequivalence Study under Fasting Conditions—

Objective— The objective is to compare the rate and extent of absorption of a generic formulation with that of the reference formulation when given as equal labeled doses.

Design— The study design is a single dose, two-treatment, two-period, two-sequence crossover with a 1-week washout period between Phase I and Phase II dosing. An equal number of subjects should be randomly assigned to each of the two possible dosing sequences. Before the study begins, the proposed protocol should be approved by an institutional review board.

Facilities— The clinical facilities and analytical laboratory used for the study should be identified along with the names, titles, and curriculum vitae of the medical and scientific or analytical directors.

Selection of Subjects— The sponsors should enroll a number of subjects sufficient to ensure statistical validity of the study. It is recommended that a minimum of 24 subjects be used in this study. Subjects should be healthy, preferably nonsmoking, volunteers aged 18 to 50 years and within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). Subjects should be selected on the basis of acceptable medical history, physical examination, and clinical laboratory test results. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. Female subjects must be given a pregnancy test prior to beginning the study. If smokers are included, they should be identified as such. Written, informed consent must be obtained from all study participants before they are accepted into the study.

Procedure— Following an overnight fast of at least 10 hours, subjects should be administered a single dose of the test or reference product with 240 mL of water.

Restrictions— Study volunteers should observe the following restrictions:

- a. Water may be taken, except for 1 hour before and after drug administration when no liquid is allowed other than that needed for drug dosing.
- b. Subjects should fast for at least 4 hours after administration of the test or reference treatment. All meals should be standardized during the study, and the same meals should be served during both phases of the study.
- c. No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
- d. Subjects should take no Rx medications, including oral contraceptives, beginning 2 weeks and no OTC medications beginning 1 week before drug administration and until after the study is completed.

Blood Sampling— Venous blood samples should be collected pre-dose (0 hours) and at 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 8, 10, 12, 16, 24, and 30 hours post-dose. Plasma or serum should be separated promptly and immediately frozen until assayed. Following a 1-week washout period, subjects should begin the second phase of the study.

Subject Monitoring— Blood pressure and pulse rate should be monitored hourly during the first 4 hours of the study. Subjects with a heart rate less than 45 bpm or greater than 110 bpm should have an electrocardiogram (lead II) performed and have their pulse monitored hourly. Subjects should report any unusual symptoms observed during the study. Subjects should be periodically questioned during each phase of the study for any unusual symptoms experienced after drug administration.

Analytical Methods— Pentoxyline and two of its active metabolites 1-(5-hydroxyhexyl)-3,7-dimethylxanthine (M1) and 1-(3-carboxypropyl)-3,7-dimethylxanthine (M5) should be assayed using a suitable method fully validated with respect to adequate sensitivity, specificity, linearity, recovery, and accuracy and precision (both within and between days). Stability of the samples under frozen conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be determined. Chromatograms of the analysis of the unknown samples, including all associated standard curve and quality control chromatograms, should be available for regulatory authorities. The sponsor should justify the rejection of any analytical data and provide a rationale for selection of the reported values.

Statistical Analysis of Pharmacokinetic Data— See Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design under General Guidances.

Recommended Protocol for Conducting a Multiple-Dose, Steady-State, Bioequivalence Study under Fasting Conditions—

Objective— The objective is to compare the steady-state rate and extent of absorption of a generic extended-release pentoxyline formulation with that of the reference formulation when given as equal labeled doses.

Design— The study design is a multiple-dose, steady-state, two-treatment, two-period, two-sequence crossover with a 1-week washout period between Phase I and Phase II dosing. An equal number of subjects should be randomly assigned to the two possible dosing sequences. Before the study begins, the proposed protocols should be approved by an institutional review board.

The multiple-dose, steady-state, bioequivalence study should be performed in the same manner as directed under Recommended Protocol for Conducting a Single-Dose Bioequivalence Study under Fasting Conditions, with the following exceptions:

Procedure— Subjects should begin a regimen in which consecutive doses of the test or reference products are given every 8 hours with 240 mL of water until steady-state blood serum and plasma levels are attained by all. After reaching steady state, subjects should be administered the final dose of the test or the reference product with 240 mL of water at the next dosing interval.

Restrictions— Item "b" should state: Meals should be scheduled at least 2 hours post-dose for all doses. After the final dose of each treatment is administered and intensive sampling begins, fasting should be continued for 4 hours. All meals should be standardized during the study, and the same meals should be served during both phases of the study.

Blood Sampling— The pre-dose blood sampling must include at least three trough level samples on three consecutive days (collected at the same time of day) to demonstrate that steady-state blood plasma and serum levels are achieved prior to the final Phase I dose. Adequate blood samples should be collected at appropriate times during a dosing interval at a steady state to permit estimation of AUCinterdose. Additional samples should then be collected to permit estimation of the terminal elimination rate constant  $\lambda_{z}$ . Following a 1-week washout period, subjects should begin the second phase of the study.

Limited Food Effects Study—

Objective— The objective is to compare the rate and extent of absorption of pentoxyline from a generic formulation with that from a reference formulation under nonfasting conditions and to compare the rate and extent of absorption of the drug from a generic formulation under fasting and nonfasting conditions when given as equal labeled doses.

Design— The study design is a single-dose, randomized, three-treatment, three-period, six-sequence crossover with a 1-week washout period between Phase I and Phase II, and Phase II and Phase III. An equal number of subjects should be assigned to each of the six dosing sequences.

The limited food effects study should be performed in the same manner as directed under Recommended Protocol for Conducting a Single-Dose Bioequivalence Study under Fasting Conditions, with the following exceptions:

Procedure— An equal number of subjects should be assigned to each of the six dosing sequences possible in a three-treatment, three-period study design (minimum 18 subjects).

Each subject will receive the following treatments:

TREATMENT 1: Generic product, a 400-mg extended-release pentoxyline tablet administered after a standard high-fat breakfast.

TREATMENT 2: Reference product, a 400-mg extended-release Trental® tablet administered after a standard high-fat breakfast.

TREATMENT 3: Generic product, a 400-mg extended-release pentoxyline tablet administered under fasting conditions.



following a 10-hour fast, the subjects receiving treatments under nonfasting conditions should be served a standard high-fat breakfast. The subjects should have 30 minutes to finish the entire breakfast, then should immediately receive Treatment 1 or Treatment 2, taken with 240 mL of water. Subjects receiving treatment under fasting conditions should receive Treatment 3, taken with 240 mL of water only. The same lots of the test and reference products should be used in the fasting and nonfasting studies. No food should be allowed for at least 4 hours post-dose, with water allowed after the first hour. Subjects should be served scheduled standardized meals throughout the study, and the same meals should be served during all phases of the study.

**Statistical Analysis**— In general, a comparable food effect will be assumed if the AUC<sub>0-t</sub>, AUC<sub>0-∞</sub>, and C<sub>max</sub> mean values for the test product administered under nonfasting conditions are within 20% of the respective mean values obtained for the reference product administered under nonfasting conditions.

**Clinical Report, Side Effects, and Adverse Reactions**— Subject medical histories, physical examinations and laboratory reports, and all incidents of possible adverse reactions to the study formulations should be reported.

**Retention of Samples**— The laboratory conducting the bioequivalence testing should retain appropriately identified reserve samples of both the test and reference product used to perform the *in vivo* bioequivalence studies for approval of the application. Each reserve sample should consist of at least 200 dosage units or 5 times the amount needed for complete release testing and should be retained for a minimum of 5 years following approval of the application. For more information on retention of bioequivalence samples, refer to 21 CFR 320.63.

#### in vitro testing requirements

**Drug Release Testing** (724) — Drug release testing should be conducted on 12 individual dosage units of batches of the test and reference products employing the biostudy lots. The potential for pH dependence of drug release from an extended-release product is well recognized. Drug release profiles should, therefore, be generated in aqueous media at the following pH ranges: 1–1.5, 4–4.5, and 6.0–6.8. See *In Vitro Drug Release for Quality Control Preapproval under General Guidances*. The following times should be used to determine the drug release profile.

Times: 1, 2, and 4 hours and every 2 hours until 80% of drug is released.

The percentage of label claim dissolved at each specified testing interval should be reported for each individual dosage unit. The mean percentage dissolved, the range (highest, lowest) of dissolution, and the coefficient of variation (relative standard deviation) should be reported.

**Content Uniformity Test** (905) — Content uniformity testing on the test and reference product lots should be performed as described in USP.

#### Pindolol Tablets—*In Vivo* Bioequivalence and *In Vitro* Dissolution Testing<sup>4</sup>

**Clinical Pharmacology/Usage**— Pindolol is a nonselective beta-adrenergic antagonist with intrinsic sympathomimetic activity in therapeutic dosage ranges. Pindolol has low membrane stabilizing activity. The drug is indicated for the management of hypertension. It may be used alone or concomitantly with other antihypertensive agents, particularly with a thiazide type diuretic.

In the management of hypertension, the recommended initial dose is 5 mg twice a day administered alone or in combination with other antihypertensives. The dosage may be adjusted in increments of 10 mg per day, up to a maximum dosage of 60 mg per day.

**Pharmacokinetics**— Following intravenous administration of pindolol, the plasma concentration-time data can be described by a biexponential equation. The disposition of pindolol after oral administration has also been reported to be monophasic with an elimination half-life of approximately 3 to 4 hours in healthy subjects or hypertensive patients with normal renal function.

Pindolol is rapidly absorbed, achieving peak plasma concentrations within 1 to 2 hours of drug administration. It has a stated bioavailability of greater than 95%. Earlier studies also reported oral bioavailability of 90% to 100%. One study, however, reported an oral absolute bioavailability of only 53%. The bioavailability of pindolol is not significantly affected by co-administration with food. Food, however, increases the rate of absorption. Pindolol has a negligible first-pass effect. The blood concentrations are linearly proportional to the administered dose in the range of 5 to 20 mg.

The lipid solubility of pindolol is considered intermediate in comparison with other beta blockers. The apparent volume of distribution has been reported to be 1.2 to 2 L per kg. Pindolol is about 40% to 60% bound to plasma proteins and is evenly distributed between plasma and red cells.

Approximately 35% to 40% of the administered dose is excreted unchanged in the urine and 60% to 65% is metabolized, primarily to hydroxy-metabolites, which are excreted as glucuronides and ether sulfates. The polar metabolites are excreted with a half-life of approximately 8 hours. About 6% to 9% of an administered intravenous dose is excreted from the bile into the feces.

#### in vivo bioequivalence studies<sup>5</sup>

##### Product Information—

FDA Designated Reference Product: Visken® (Sandoz) 10-mg tablet.

**Batch Size**— The test batch or lot must be manufactured under production conditions and must be of a size that is at least 10% that of the largest lot planned for full production or a minimum of 100,000 units, whichever is larger.

**Potency**— The assayed potency of the reference product should not differ from that of the test product by more than 5%.

**Type of Study Required**— A single-dose, randomized, two-period, two-treatment, two-sequence crossover study under fasting conditions comparing equal doses of the test and reference products.

##### Recommended Protocol for Conducting a Single-Dose Study under Fasting Conditions—

**Objective**— The objective is to compare the rate and extent of absorption of pindolol from a generic formulation with that of a reference formulation when given as equal labeled doses.

**Design**— The study design is a single-dose, two-treatment, two-period, two-sequence crossover with a 1-week washout period between Phase I and Phase II dosing. An equal number of subjects should be randomly assigned to each of the two possible dosing sequences. Before the study begins, the proposed protocol should be approved by an institutional review board.

**Facilities**— The clinical facilities and analytical laboratory used for the study should be identified along with the names, titles, and curriculum vitae of the medical and scientific or analytical directors.

**Selection of Subjects**— The sponsor should enroll a number of subjects sufficient to ensure statistical validity of the study. It is recommended that a minimum of 24 subjects be used in this study. Subjects should be healthy nonsmoking volunteers aged 18 to 50 and within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). Subjects should be selected on the basis of acceptable medical history, physical examination, and clinical laboratory test results. Female subjects must be given a pregnancy test prior to beginning the study. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. Written, informed consent must be obtained from all study participants before they are accepted into the study.

**Procedure**— Following an overnight fast of at least 10 hours, subjects should be administered two 10-mg tablets of the test or reference product with 240 mL of water.

**Restrictions**— Study volunteers should observe the following restrictions:

- a. Water may be taken except for 1 hour before and after drug administration when no liquid is allowed other than that needed for drug dosing.
- b. Subjects should fast for at least 4 hours after administration of the test or reference treatment. All meals should be standardized during the study, and the same meals should be served during both phases of the study.
- c. No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
- d. Subjects should take no Rx medications, including oral contraceptives, beginning 2 weeks and no OTC medications beginning 1 week before drug administration and until after



the study is completed.

Blood Sampling— Venous blood samples should be collected pre-dose (0 hours) and at 0.25, 0.50, 0.75, 1, 1.50, 2, 3, 4, 6, 8, 10, 12, 16, and 24 hours post-dose. Plasma or serum should be separated promptly and immediately frozen until assayed. Following a minimum 1-week washout period, subjects should begin the second phase of the study.

Subject Monitoring— Blood pressure and pulse rate should be monitored hourly during the first 4 hours of the study. Subjects with a heart rate less than 45 bpm or greater than 110 bpm should have an electrocardiogram (lead II) performed and have their pulse monitored hourly. Subjects should report any unusual symptoms observed during the study. Subjects should be periodically questioned during each phase of the study for any unusual symptoms experienced after drug administration.

Analytical Methods— Pindolol should be assayed using a suitable method fully validated with respect to adequate sensitivity, specificity, linearity, recovery, accuracy, and precision (both within and between days). Stability of the samples under frozen conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be determined.

Chromatograms of the analysis of the unknown samples, including all associated standard curve and quality control chromatograms, should be available for regulatory authorities. The sponsor should justify the rejection of any analytical data and provide a rationale for selection of the reported values.

Statistical Analysis of Pharmacokinetic Data— See Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design under General Guidances.

Clinical Report, Side Effects, and Adverse Reactions— Subject medical histories, physical examinations and laboratory reports, and all incidents of possible adverse reactions and side effects to the study formulations should be reported.

Retention of Samples— The laboratory conducting the bioequivalence testing should retain appropriately identified reserve samples of both the test and reference products used to perform the in vivo bioequivalence studies for approval of the application. Each reserve sample should consist of at least 200 dosage units or 5 times the amount needed for complete release testing and should be retained for a minimum of 5 years following approval of the application. For more information on retention of bioequivalence samples, refer to 21 CFR 320.63.

#### in vitro testing requirements

Dissolution Testing— Conduct dissolution testing on 12 dosage units of the test product versus 12 units of the reference product. The biostudy lots should be used for those product strengths tested in vivo. The current official USP dissolution method (see [Dissolution](#) 711) should be followed and should be referenced by the sponsor. The following method and tolerances are currently recommended for this product:

Medium: 0.1 N hydrochloric acid; 500 mL.

Apparatus 2: 50 rpm.

Times: 5, 10, and 15 minutes.

Procedure— Use USP if available, or other validated method (see [Validation of Compendial Procedures](#) 1225).

Tolerance (Q): not less than 80% in 15 minutes.

The percentage of label claim dissolved at each specified testing interval should be reported for each individual dosage unit. The mean percentage dissolved, the range (highest, lowest) of dissolution, and the coefficient of variation (relative standard deviation) should be reported.

Content Uniformity Test— Content uniformity testing on the test and reference product lots should be performed as described in USP.

#### waiver requirements

Waiver of in vivo bioequivalence study requirements for the 5-mg strength of the generic product may be granted pursuant to 21 CFR 320.22(d)(2), provided the following conditions are met:

1. The 5-mg tablet is proportionally similar in both active and inactive ingredients to the firm's 10-mg tablet, which has been demonstrated to be bioequivalent to the reference product in vivo.
2. The 5-mg tablet of the generic product meets dissolution test requirements.

#### Piroxicam Capsules—In Vivo Bioequivalence and In Vitro Dissolution Testing<sup>4</sup>

Clinical Pharmacology/Usage— Piroxicam is a nonsteroidal anti-inflammatory drug (NSAID). It is indicated for acute or long-term use in the relief of signs and symptoms of osteoarthritis and rheumatoid arthritis. It is recommended that a single 20-mg dose be given daily for these conditions. The daily dose may be divided into two 10-mg capsules.

Like other nonsteroidal anti-inflammatory drugs, piroxicam inhibits prostaglandin synthesis by blocking cyclooxygenase; it has no effect on lipoxygenase. Piroxicam has been reported to have a greater effect than other nonsteroidal anti-inflammatory drugs in inhibiting rheumatoid factor production, in increasing phytohemagglutinin response, and in increasing the percentage of suppressor T-cells in peripheral blood. The significance of these effects on its anti-inflammatory and antiarthritic action is not clear.

Piroxicam should not be used in patients with the syndrome comprised of bronchospasm, nasal polyps, and angioedema precipitated by aspirin or other NSAIDs. The major adverse reaction associated with piroxicam is GI irritation.

Pharmacokinetics— Following oral administration, piroxicam is rapidly absorbed. Peak plasma levels occur in 2 to 5 hours with a terminal elimination half-life ranging between 30 and 60 hours. Two or more peaks are usually observed in the piroxicam plasma concentration-time profile between 3 and 12 hours after dosing. Enterohepatic recirculation of piroxicam has been cited as the possible explanation for these multiple peaks. However, a preliminary study in healthy volunteers did not reveal any biliary secretion of piroxicam. The prolonged half-life of piroxicam results in the maintenance of relatively stable plasma concentrations throughout the day on a once daily regimen. With repeated daily doses of 20 mg, steady-state plasma levels of 3 to 5 µg per mL are attained in 7 to 12 days.

Piroxicam is extensively bound (99%) to plasma proteins. Despite very high plasma binding, piroxicam readily penetrates into the synovial fluid of patients with rheumatoid arthritis, osteoarthritis, and reactive synovitis, where piroxicam concentrations are about 40% of those in plasma. This concentration difference of unbound piroxicam across the synovial membrane may be explained by ion trapping of piroxicam in plasma, assuming a pH for synovial fluid significantly lower than 7.4. The very high plasma binding of piroxicam limits its distribution mainly to extracellular space. As a result, the Vd of piroxicam is small, usually about 0.14 L per kg, which is a value typical for most NSAIDs.

Piroxicam is eliminated mainly by biotransformation with less than 5% of the dose excreted unchanged in urine. Hydroxylation and subsequent conjugation with glucuronic acid is the major metabolic pathway in man. This piroxicam metabolite has little or no anti-inflammatory activity.

#### in vivo bioequivalence studies<sup>5</sup>

##### Product Information—

FDA Designated Reference Product: Feldene® (Pfizer Co.) 20-mg capsules.

Batch Size— The test batch or lot must be manufactured under production conditions and must be of a size that is at least 10% that of the largest lot planned for full production or a minimum of 100,000 units, whichever is larger.

Potency— The assayed potency of the reference product should not differ from that of the test product by more than 5%.

#### Types of Studies Required—

1. A single-dose, randomized, two-period, two-treatment, two-sequence crossover study under fasting conditions comparing equal doses of the test and reference products.
2. A single-dose, randomized, three-treatment, three-period, six-sequence crossover, limited food effects study comparing equal doses of the test and reference products when administered immediately following a standard high-fat breakfast.<sup>6</sup>



#### Recommended Protocol for Conducting a Single-Dose, Fasted Bioequivalence Study—

**Objective**— The objective is to compare the rate and extent of absorption of a generic formulation with that of a reference formulation when given as equal labeled doses.

**Design**— The study design is a single-dose, two-treatment, two-period, two-sequence crossover with a 2-week washout period between Phase I and Phase II dosing. An equal number of subjects should be randomly assigned to each of the two possible dosing sequences. Before the study begins, the proposed protocol should be approved by an institutional review board.

**Facilities**— The clinical facilities and analytical laboratory used for the study should be identified along with the names, titles, and curriculum vitae of the medical and scientific or analytical directors.

**Selection of Subjects**— The sponsor should enroll a number of subjects sufficient to ensure statistical validity of the study. It is recommended that a minimum of 24 subjects be used in this study. Subjects should be healthy, preferably nonsmoking, volunteers aged 18 to 55 and within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). Subjects should be selected on the basis of acceptable medical history, physical examination, and clinical laboratory test results. Female subjects must be given a pregnancy test prior to beginning the study. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. If smokers are included, they should be identified as such. Written, informed consent must be obtained from all study participants before they are accepted into the study.

**Procedure**— Following an overnight fast of at least 10 hours, subjects should be administered a single dose (20-mg) of the test or reference product with 240 mL of water.

#### Restrictions— Study volunteers should observe the following restrictions:

- a. Water may be taken except for 1 hour before and after drug administration when no liquid is allowed other than that needed for drug dosing.
- b. Subjects should fast for at least 4 hours after administration of the test or reference treatment. All meals should be standardized during the study, and the same meals should be served during both phases of the study.
- c. No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
- d. Subjects should take no Rx medications, including oral contraceptives, beginning 2 weeks and no OTC medications beginning 1 week before drug administration and until after the study is completed.

**Blood Sampling**— Venous blood samples should be collected pre-dose (0 hours) and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 24, 36, 48, 72, 96, 120, 144, and 168 hours post-dose. Plasma should be separated promptly and immediately frozen until assayed. Following a minimum 2-week washout period, subjects should begin the second phase of the study.

**Subject Monitoring**— Blood pressure and pulse rate should be monitored hourly during the first 4 hours of the study. Subjects with a heart rate less than 45 bpm or greater than 110 bpm should have an electrocardiogram (lead II) performed and have their pulse monitored hourly. Subjects should report any unusual symptoms observed during the study. Subjects should be periodically questioned during each phase of the study for any unusual symptoms experienced after drug administration.

**Analytical Methods**— Piroxicam should be assayed using a suitable method fully validated with respect to adequate sensitivity, specificity, linearity, recovery, accuracy, and precision (both within and between days). Stability of the samples under frozen conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be determined.

Chromatograms of the analysis of the unknown samples, including all associated standard curve and quality control chromatograms, should be available for regulatory authorities. The sponsor should justify the rejection of any analytical data and provide a rationale for selection of the reported values.

**Statistical Analysis of Pharmacokinetic Data**— See Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design under General Guidances.

#### Limited Food Effects Study—

**Objective**— The objective is to compare the rate and extent of absorption of piroxicam from a generic formulation with that from a reference formulation under nonfasting conditions and to compare the rate and extent of absorption of the drug from a generic formulation under fasting and nonfasting conditions when given as equal labeled doses.

**Design**— The study design is a single-dose, three-treatment, three-period, six-sequence crossover with a 2-week washout period between Phase I and Phase II, and Phase II and Phase III. An equal number of subjects should be assigned to each of the 6 dosing sequences.

The limited food effects study should be performed in the same manner as the single-dose study under fasting conditions, with the following exceptions:

**Procedure**— An equal number of subjects should be assigned to each of the 6 dosing sequences possible in a three-treatment, three-period study design (minimum 12 subjects). Each subject will receive the following treatments:

TREATMENT 1: Generic product, a 20-mg capsule administered after a standard high-fat breakfast.

TREATMENT 2: Reference product, a 20-mg Feldene® capsule (Pfizer) administered after a standard high-fat breakfast.

TREATMENT 3: Generic product, a 20-mg capsule administered under fasting conditions.

Following a 10-hour fast, the subjects receiving treatments under fed conditions should be served a standard high-fat breakfast. The subjects should have 30 minutes to finish the entire breakfast and then should immediately receive Treatment 1 or Treatment 2, taken with 240 mL of water. Subjects receiving the treatment under fasting conditions should receive Treatment 3, taken with 240 mL of water only. The same lots of the test and reference products should be used in the fasted and fed studies. No food should be allowed for at least 4 hours post-dose with water allowed after the first hour. Subjects should be served scheduled standardized meals throughout the study, and the same meals should be served during all phases of the study.

**Statistical Analysis**— In general, a comparable food effect will be assumed if the AUC<sub>0-t</sub>, AUC<sub>0-∞</sub>, and C<sub>max</sub> mean values for the test product administered under fed conditions are within 20% of the respective mean values obtained for the reference product administered under fed conditions.

**Clinical Report, Side Effects, and Adverse Reactions**— Subject medical histories, physical examinations and laboratory reports, and all incidents of possible adverse reactions and side effects to the study formulations should be reported.

**Retention of Samples**— The laboratory conducting the bioequivalence testing should retain appropriately identified reserve samples of both the test and reference products used to perform the in vivo bioequivalence studies for approval of the application. Each reserve sample should consist of at least 200 dosage units or 5 times the amount needed for complete release testing and should be retained for a minimum of 5 years following approval of the application. For more information on retention of bioequivalence samples refer to 21 CFR 320.63.

#### in vitro testing requirements

**Dissolution Testing**— Conduct dissolution testing on 12 dosage units of the test product and the reference product, employing the biostudy lots. The current official USP dissolution method (see [Dissolution](#) (711)) should be followed and should be referenced by the sponsor. The following times should be used to determine the dissolution profile: Times: 15, 30, 45, and 60 minutes.

The percentage of label claim dissolved at each specified testing interval should be reported for each individual dosage unit. The mean percentage dissolved, the range (highest, lowest) of dissolution, and the coefficient of variation (relative standard deviation) should be reported.

**Content Uniformity Test**— Content uniformity testing on the test and reference product lots should be performed as described in USP.

#### waiver requirements

Waiver of in vivo bioequivalence study requirements for the 10-mg capsule of the generic product may be granted pursuant to 21 CFR 320.22(d)(2) provided the following conditions are met:

1. The 10-mg capsule is proportionally similar in both active and inactive ingredients to the firm's 20-mg capsule, which has been demonstrated to be bioequivalent to the reference product *in vivo*.



2. The 10-mg capsule of the generic product meets dissolution test requirements.

#### Ranitidine Hydrochloride Tablets—In Vivo Bioequivalence and In Vitro Dissolution Testing<sup>4</sup>

**Clinical Usage/Pharmacology**— Ranitidine hydrochloride is a histamine H<sub>2</sub>-receptor antagonist, which inhibits competitively and reversibly the interaction of histamine with H<sub>2</sub>-receptors. It reduces gastric acid secretion elicited by histamine and gastrin and stimulated by betazole and food. Ranitidine hydrochloride also inhibits basal and nocturnal acid secretion.

Ranitidine hydrochloride is indicated in active and maintenance therapies of duodenal ulcer. It is also used in treatments of active, benign gastric ulcer, pathological hypersecretory conditions, and gastroesophageal reflux disease. Serum concentrations of 36 to 94 ng per mL of ranitidine hydrochloride have been shown to inhibit 50% of stimulated gastric acid secretion. The recommended oral dosage of ranitidine hydrochloride tablets for active duodenal ulcer is 150 mg twice a day, or 300 mg at bedtime.

**Pharmacokinetics**— An absolute oral bioavailability of 50% has been reported for 150-mg ranitidine hydrochloride tablets. Mean values of 60% and 52% have also been reported as the absolute bioavailability of two other formulations of 150- and 100-mg ranitidine hydrochloride tablets, respectively.

A linear relationship has been found between the dose and the area under the serum concentration-time curve following the administration of oral doses of 100, 150, 250, and 400 mg of ranitidine hydrochloride tablets.

A secondary peak in the plasma concentration-time curve has been reported for ranitidine hydrochloride following oral administration of 100- and 150-mg tablets in fasting studies. The mean values reported for the peaks were 1.5 hours for the first peak and 3.9 hours for the second.

The reported elimination half-life of ranitidine hydrochloride is 2.5 to 3.0 hours. Approximately 30% of ranitidine hydrochloride is excreted unchanged in urine within 24 hours following the administration of an oral dose. The major urinary metabolite of ranitidine hydrochloride in man is N-oxide, which represents less than 4% of the dose. S-oxide (1%) and desmethyl ranitidine (1%) are the other metabolites of ranitidine hydrochloride.

Multiple-dose studies in which ranitidine hydrochloride was administered in doses of 150 and 200 mg twice daily for 28 days showed that ranitidine hydrochloride did not accumulate in the body, and the values of the pharmacokinetic parameters were similar for both single- and multiple-dose studies.

Administration of food or antacid reportedly does not change the absorption of ranitidine hydrochloride tablets significantly. However, concomitant administration of high doses of antacids with the 150-mg tablets reduced its absorption.

#### in vivo bioequivalence studies<sup>5</sup>

##### Product Information—

FDA Designated Reference Product: Zantac® (Glaxo) 300-mg tablets.

**Batch Size**— The test batch or lot must be manufactured under production conditions and must be of a size at least 10% that of the largest lot planned for full production or a minimum of 100,000 units, whichever is larger.

**Potency**— The assayed potency of the reference product should not differ from that of the test product by more than 5%.

**Type of Study Required**— A single-dose, randomized, two-period, two-treatment, two-sequence crossover study under fasting conditions comparing equal doses of the test and reference products.

#### Recommended Protocol for Conducting a Single-Dose, Fasted Bioequivalence Study—

**Objective**— The objective is to compare the rate and extent of absorption of a generic formulation of ranitidine hydrochloride tablet with that of a reference formulation when given as equal labeled doses.

**Design**— The study design is a single-dose, two-treatment, two-period, two-sequence crossover with a 1-week washout period between Phase I and Phase II dosing. An equal number of subjects should be randomly assigned to each of the two possible dosing sequences. Before the study begins, the proposed protocol should be approved by an institutional review board.

**Facilities**— The clinical facilities and analytical laboratory used for the study should be identified along with the names, titles, and curriculum vitae of the medical and scientific or analytical directors.

**Selection of Subjects**— The sponsor should enroll a number of subjects sufficient to ensure statistical validity of the study. It is recommended that a minimum of 24 subjects be used in this study. Subjects should be healthy, preferably nonsmoking, volunteers 18 to 50 years of age and within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). Subjects should be selected on the basis of acceptable medical history, physical examination, and clinical laboratory test results. Female subjects must be given a pregnancy test prior to beginning the study and between Phase I and Phase II. Nursing mothers should be excluded from the study. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. If smokers are included, they should be identified as such. Written, informed consent must be obtained from all study participants before they are accepted into the studies.

**Procedure**— Following an overnight fast of at least 10 hours, subjects should be administered a single 300-mg tablet dose of the test or reference product with 240 mL of water.

#### Restrictions— Study volunteers should observe the following restrictions:

- Water may be taken except for 1 hour before and after drug administration, when no liquid is allowed other than that needed for drug dosing.
- Subjects should fast for at least 4 hours after administration of the test or reference treatment. All meals should be standardized during the study, and the same meals should be served during both phases of the study.
- No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
- Subjects should take no Rx medications, including oral contraceptives, beginning 2 weeks and no OTC medications beginning 1 week before drug administration and until after the study is completed.

**Blood Sampling**— Venous blood samples should be collected pre-dose (0 hours) and at 0.33, 0.50, 0.67, 1, 1.33, 1.50, 1.67, 2, 2.50, 3, 3.50, 4, 5, 6, 8, 10, 12, 16, and 24 hours post-dose. Plasma should be separated promptly and immediately frozen until assayed. Following a minimum 1-week washout period, subjects should begin the second phase of the study.

**Analytical Methods**— Ranitidine hydrochloride should be assayed using a suitable method fully validated with respect to adequate sensitivity, specificity, linearity, recovery, and accuracy and precision (both within and between days). Stability of the samples under frozen conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be determined. Chromatograms of the analysis of the unknown samples, including all associated standard curve and quality control chromatograms, should be available for regulatory authorities. The sponsor should justify the rejection of any analytical data and provide a rationale for selection of the reported values.

**Statistical Analysis of Pharmacokinetic Data**— See Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design under General Guidances.

**Clinical Report, Side Effects, and Adverse Reactions**— Subject medical histories, physical examinations and laboratory reports, and all incidents of possible adverse reactions to the study formulations should be reported.

**Retention of Samples**— The laboratory conducting the bioequivalence testing should retain an appropriately identified reserve sample of both the test and reference products used to perform the in vivo bioequivalence study for approval of the application. Each reserve sample should consist of at least 200 dosage units and should be retained for a period of not less than 5 years following approval of the application. For more information on retention of bioequivalence samples, refer to 21 CFR 320.63.

#### in vitro testing requirements

**Dissolution Testing**— Conduct dissolution testing on 12 dosage units of the test product and the reference product, employing the biostudy lots. The current official USP dissolution method (see [Dissolution](#) 711) should be followed and should be referenced by the sponsor. The following method and tolerances are currently recommended for this product:



Medium: deaerated water; 900 mL.

Apparatus 2: 50 rpm.

Times: 10, 20, 30, and 45 minutes.

Procedure— Use USP if available, or other validated method (see [Validation of Compendial Procedures \(1225\)](#)).

Tolerances (Q): not less than 80% is dissolved in 45 minutes.

The percentage of label claim dissolved at each specified testing interval should be reported for each individual dosage unit. The mean percentage dissolved, the range (highest, lowest) of dissolution, and the coefficient of variation (relative standard deviation) should be reported.

Content Uniformity Test (905)— Content uniformity testing on the test product lots should be performed as described in USP.

waiver requirements

Waiver of in vivo bioequivalence study requirements for the 150-mg strength of the generic product may be granted pursuant to 21 CFR 320.22(d)(2) provided the following conditions are met:

1. The 150-mg tablet is proportionally similar in both active and inactive ingredients to the 300-mg tablet, which has been demonstrated to be bioequivalent to the reference product *in vivo*.
2. The 150-mg tablet of the generic product meets the dissolution testing requirements.

#### Selegiline Hydrochloride Tablets—*In Vivo* Bioequivalence and *In Vitro* Dissolution Testing<sup>4</sup>

**Clinical Usage/Pharmacology**— Selegiline hydrochloride is an irreversible inhibitor of monoamine oxidase (MAO) with greater affinity for MAO Type B (predominant form in the brain) compared to Type A (predominant intestinal form). Type A MAO is not inhibited by selegiline at therapeutic doses; therefore, the ability of MAO Type A (GI tract and liver) to prevent hypertensive effects from exogenous amines absorbed from the gut is not compromised. Selegiline can be used safely without dietary restrictions and with concomitant drug use only at doses that selectively inhibit MAO Type B ( $\leq$  10 mg per day). In addition to its inhibitory actions on MAO Type B, selegiline may also prevent indirect acting sympathomimetics from displacing norepinephrine from adrenergic neurons. By blocking the metabolism of dopamine in the brain, selegiline increases the amount of dopamine available to dopamine receptors that might be beneficial to treatment of Parkinsonism. Selegiline is indicated as an adjunct in the management of Parkinsonian patients being treated with levodopa and carbidopa who exhibit deterioration in the quality of their response to this therapy. Dosing is 10 mg per day; in an effort to minimize adverse effects, such as nausea and dizziness, that may follow a single 10-mg dose, divided doses of 5 mg are administered at breakfast and lunch.

**Pharmacokinetics**— Much of the original pharmacokinetic data reported for selegiline were obtained after administration of 14C-selegiline. The following results represent the total 14C-label (parent drug plus metabolites): 1) After oral administration of 5 mg 14C-selegiline, C<sub>max</sub> ranged from 33 to 45 ng per mL with a T<sub>max</sub> of 0.5 to 2 hours. 2) The elimination half-life of 14C-selegiline from plasma has been approximated at 39 hours (range, 16 to 69 hours). Of the three principal metabolites of selegiline—desmethylselegiline (DES), methamphetamine (MA), and amphetamine (A)—only DES is thought to contribute to the overall pharmacological effect. Due to a large volume of distribution and extensive first-pass metabolism, selegiline plasma concentrations after a 10-mg dose are difficult to detect. In a single-dose pilot study (N = 4) using this assay, the mean plasma elimination half-lives of selegiline and DES were 1.9 hours and 2.2 hours, respectively. The effects of food on selegiline absorption have not been reported.

*in vivo* bioequivalence studies<sup>5</sup>

Product Information—

FDA Designated Reference Product: Eldepryl® (Somerset) 5-mg tablets.

**Batch Size**— The test batch or lot must be manufactured under production conditions and must be of a size that is at least 10% of the largest lot planned for full production or a minimum of 100,000 units, whichever is larger.

**Potency**— The assayed potency of the reference product should not differ from that of the test product by more than 5%.

#### Types of Studies Required—

1. A single-dose, randomized, two-period, two-treatment, two-sequence crossover study under fasting conditions comparing equal doses (two 5-mg tablets, total dose 10-mg) of the test and reference products.
2. A single-dose, randomized, three-treatment, three-period, six-sequence crossover, limited food effects study comparing equal doses (two 5-mg tablets, total dose 10 mg) of the test and reference products when administered immediately following a high-fat breakfast.<sup>6</sup>

#### Recommended Protocol for Conducting a Single-Dose Bioequivalence Study under Fasting Conditions—

**Objective**— The objective is to compare the rate and extent of absorption of a generic formulation with that of the reference formulation when given as equal labeled doses.

**Design**— The study design is a single-dose, two-treatment, two-period, two-sequence crossover with a 2-week washout period between Phase I and Phase II dosing. An equal number of subjects should be randomly assigned to each of the two possible dosing sequences. Before the study begins, the proposed protocol should be approved by an institutional review board.

**Facilities**— The clinical facilities and analytical laboratory used for the study should be identified along with the names, titles, and curriculum vitae of the medical and scientific or analytical directors.

**Selection of Subjects**— The sponsor should enroll a number of subjects sufficient to ensure statistical validity of the study. It is recommended that a minimum of 24 subjects be used in this study. Subjects should be healthy, preferably nonsmoking, volunteers aged 18 to 50 years and within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). Subjects should be selected on the basis of acceptable medical history, physical examination, and clinical laboratory test results. Female subjects must be given a pregnancy test prior to beginning the study. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. If smokers are included, they should be identified as such. Written, informed consent must be obtained from all study participants before they are accepted into the study.

**Procedure**— Following an overnight fast of at least 10 hours, subjects should be administered two 5-mg tablets (10 mg total dose) of the test or reference product with 240 mL of water.

**Restrictions**— Study volunteers should observe the following restrictions:

- a. Water may be taken, except for 1 hour before and after drug administration when no liquid is allowed other than that needed for drug dosing.
- b. Subjects should fast for at least 4 hours after administration of the test or reference treatment. All meals should be standardized during the study, and the same meals should be served during both phases of the study.
- c. No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
- d. Subjects should take no Rx medications, including oral contraceptives, beginning 2 weeks and no OTC medications beginning 1 week before drug administration and until after the study is completed.

**Blood Sampling**— Venous blood samples should be collected pre-dose (0 hours) and at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 8, 12, 24, 36, 48, 72, and 96 hours post-dose. Plasma or serum should be separated promptly and immediately frozen until assayed. Following a 2-week washout period, subjects should begin the second phase of the study.

**Subject Monitoring**— Blood pressure and pulse rate should be monitored hourly during the first 4 hours of the study. Subjects with a heart rate less than 45 bpm or greater than 110 bpm should have an electrocardiogram (lead II) performed and have their pulse monitored hourly. Subjects should report any unusual symptoms observed during the study. Subjects should be periodically questioned during each phase of the study for any unusual symptoms experienced after drug administration.



Analytical Methods— Selegiline and its three metabolites desmethylselegiline (DES), methamphetamine (MA), and amphetamine (A) should be assayed using a suitable method fully validated with respect to adequate sensitivity, specificity, linearity, recovery, accuracy, and precision (both within and between days). Stability of the samples under frozen conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be determined. Chromatograms of the analysis of the unknown samples, including all associated standard curve and quality control chromatograms, should be available for regulatory authorities. The sponsor should justify the rejection of any analytical data and provide a rationale for selection of the reported values.

Statistical Analysis of Pharmacokinetic Data— See Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design under General Guidances. Pivotal statistical criteria should be applied to the three selegiline metabolites, desmethylselegiline (DES), methamphetamine (MA), and amphetamine (A). Results should also be reported for selegiline.

#### Limited Food Effects Study—

Objective— The objective is to compare the rate and extent of absorption of selegiline from a generic formulation with that from a reference formulation under nonfasting conditions and to compare the rate and extent of absorption of the drug from a generic formulation under fasting and nonfasting conditions when given as equal labeled doses.

Design— The study design is a single-dose, randomized three-treatment, three-period, six-sequence crossover with a 2-week washout period between Phase I and Phase II, and Phase II and Phase III. An equal number of subjects should be assigned to each of the six dosing sequences.

The limited food effects study should be performed in the same manner as directed under Recommended Protocol for Conducting a Single-Dose Bioequivalence Study under Fasting Conditions, with the following exceptions:

Procedure— An equal number of subjects should be assigned to each of the six dosing sequences possible in a three-treatment, three-period study design (minimum 18 subjects).

Each subject will receive the following treatments:

TREATMENT 1: Generic selegiline hydrochloride product, (two 5-mg tablets, total dose 10 mg) administered after a high-fat breakfast.

TREATMENT 2: Reference product (two 5-mg Eldepryl® tablets, total dose 10 mg) administered after a high-fat breakfast.

TREATMENT 3: Generic selegiline hydrochloride product (two 5-mg tablets, total dose 10 mg) administered under fasting conditions.

Following a 10-hour fast, the subjects receiving the treatments under nonfasting conditions should be served the indicated high-fat breakfast. The subjects should have 30 minutes to finish the entire breakfast, then should immediately receive Treatment 1 or Treatment 2, taken with 240 mL of water. Subjects receiving the treatment under fasting conditions should receive Treatment 3, taken with 240 mL of water only. The same lots of the test and reference products should be used as in the fasting and nonfasting studies. No food should be allowed for at least 4 hours post-dose, with water allowed after the first hour. Subjects should be served scheduled standardized meals throughout the study, and the same meals should be served during all phases of the study.

Statistical Analysis— In general, a comparable food effect will be assumed if the AUC<sub>0-t</sub>, AUC<sub>0-∞</sub>, and C<sub>max</sub> mean values for the test product administered under nonfasting conditions are within 20% of the respective mean values obtained for the reference product administered under nonfasting conditions.

Clinical Report, Side Effects, and Adverse Reactions— Subject medical histories, physical examinations and laboratory reports, and all incidents of possible adverse reactions and side effects to the study formulations should be reported.

Retention of Samples— The laboratory conducting the bioequivalence testing should retain appropriately identified reserve samples of both the test and reference products used to perform the in vivo bioequivalence study for approval of the application. Each reserve sample should consist of at least 200 dosage units or 5 times the amount needed for complete release testing and should be retained for a minimum of 5 years following approval of the application. For more information on retention of bioequivalence samples, refer to 21 CFR 320.63.

#### in vitro testing requirements

Dissolution Testing— Conduct dissolution testing on 12 dosage units of the test and reference products employing the biostudy lots. The current official USP dissolution method should be followed, and should be referenced by the sponsor. The following times should be used to determine the dissolution profile:

Times: 5, 10, 20, and 30 minutes.

The percentage of label claim dissolved at each specified testing interval should be reported for each individual dosage unit. The mean percentage dissolved, the range (highest, lowest) of dissolution, and the coefficient of variation (relative standard deviation) should be reported.

Content Uniformity Test (905)— Content uniformity testing on the test and reference product lots should be performed as described in USP.

#### Tolmetin Sodium Capsules and Tablets—In Vivo Bioequivalence and In Vitro Dissolution Testing<sup>4</sup>

Clinical Pharmacology/Usage— Tolmetin sodium is a nonsteroidal anti-inflammatory agent freely soluble in water. Its mode of action is unknown. The drug is indicated for the relief of signs and symptoms of rheumatoid arthritis and osteoarthritis, both in the treatment of acute episodes and the long-term management of the chronic disease. In adults with rheumatoid arthritis, the recommended starting dose is 400 mg three times daily (1200 mg daily), preferably including a dose on arising and a dose at bedtime.

Pharmacokinetics— The drug is rapidly and almost completely absorbed following oral dosing, with peak plasma levels reached in 30 to 60 minutes. Tolmetin displays a biphasic elimination from plasma consisting of a rapid phase (T<sub>1/2</sub>, about 1 to 2 hours) followed by a slower phase (T<sub>1/2</sub>, about 5 hours). Essentially all of the dose is recovered in the urine within 24 hours either as an active metabolite or as conjugates of tolmetin. Peak plasma levels of about 40 to 50 µg per mL were obtained with a 400-mg oral dose. When taken immediately after a meal, peak plasma drug levels decreased by 50%, while total bioavailability was decreased by 16%. Administration with milk alone had no effect on peak plasma drug concentrations but decreased the total tolmetin bioavailability by 16%.

#### in vivo bioequivalence studies<sup>5</sup>

##### Product Information—

FDA Designated Reference Product: Tolectin® (McNeil Pharmaceutical) 400-mg capsules and 600-mg tablets.

Batch Size— The test batch or lot must be manufactured under production conditions and must be of a size at least 10% that of the largest lot planned for full production or a minimum of 100,000 units, whichever is larger.

Potency— The assayed potency of the reference product should not differ from that of the test product by more than 5%.

#### Types of Studies Required—

1. A single-dose, randomized, two-period, two-treatment, two-sequence crossover study under fasting conditions comparing equal doses of the test and reference products.
2. A single-dose, randomized, three-treatment, three-period, six-sequence crossover, limited food effects study comparing equal doses of the test and reference products when administered immediately following a standard high-fat breakfast.<sup>6</sup>

#### Recommended Protocols for Conducting a Single-Dose, Fasted Bioequivalence Study—

Objective— The objective is to compare the rate and extent of absorption of tolmetin sodium of a generic formulation with that of a reference formulation when given as equal labeled doses.

Design— The study design is a single-dose, two-treatment, two-period, two-sequence crossover with a 1-week washout period between Phase I and Phase II dosing. An equal number of subjects should be randomly assigned to each of the two possible dosing sequences. Before the study begins, the proposed protocol should be approved by an institutional review board.

Facilities— The clinical facilities and analytical laboratory used for the study should be identified along with the names, titles, and curriculum vitae of the medical and scientific or analytical directors.



**Selection of Subjects**—The sponsor should enroll a number of subjects sufficient to ensure statistical validity of the study. It is recommended that a minimum of 24 subjects be used in this study. Subjects should be healthy, preferably nonsmoking, volunteers aged 18 to 55 and within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). Subjects should be selected on the basis of acceptable medical history, physical examination, and clinical laboratory test results. Female subjects must be given a pregnancy test prior to beginning the study. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. If smokers are included, they should be identified as such. Written, informed consent must be obtained from all study participants before they are accepted into the study.

**Procedure**—Following an overnight fast of at least 10 hours, subjects should be administered a single 400-mg dose (for capsules) or a single 600-mg dose (for tablets) of the test or reference product with 240 mL of water.

**Restrictions**—Study volunteers should observe the following restrictions:

- a. Water may be taken except for 1 hour before and after drug administration when no liquid is allowed other than that needed for drug dosing.
- b. Subjects should fast for at least 4 hours after administration of the test or reference treatment. All meals should be standardized during the study, and the same meals should be served during both phases of the study.
- c. No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
- d. Subjects should take no Rx medications, including oral contraceptives, beginning 2 weeks and no OTC medications beginning 1 week before drug administration and until after the study is completed.

**Blood Sampling**—Venous blood samples should be collected pre-dose (0 hours) and at 0.17, 0.33, 0.50, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12, 16, and 20 hours post-dose. Plasma should be separated promptly and immediately frozen until assayed. Following a minimum 1-week washout period, subjects should begin the second phase of the study.

**Subject Monitoring**—Blood pressure and pulse rate should be monitored hourly during the first 4 hours of the study. Subjects with a heart rate less than 45 bpm or greater than 110 bpm should have an electrocardiogram (lead II) performed and have their pulse monitored hourly. Subjects should report any unusual symptoms observed during the study. Subjects should be periodically questioned during each phase of the study for any unusual symptoms experienced after drug administration.

**Analytical Methods**—Tolmetin should be assayed using a suitable method fully validated with respect to adequate sensitivity, specificity, linearity, recovery, accuracy, and precision (both within and between days). Stability of the samples under frozen conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be determined.

Chromatograms of the analysis of the unknown samples, including all associated standard curve and quality control chromatograms, should be available for regulatory authorities. The sponsor should justify the rejection of any analytical data and provide a rationale for selection of the reported values.

**Statistical Analysis of Pharmacokinetic Data**—See Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design under General Guidances.

**Limited Food Effects Study**—

**Objective**—The objective is to compare the rate and extent of absorption of tolmetin sodium from a generic formulation with that from a reference formulation under nonfasting conditions and to compare the rate and extent of absorption of the drug from a generic formulation under fasting and nonfasting conditions when given as equal labeled doses.

**Design**—The study design is a single-dose, three-treatment, three-period, six-sequence crossover with a 1-week washout period between Phase I and Phase II, and Phase II and Phase III.

The limited food effects study (minimum 12 subjects) should be performed in the same manner as the single-dose study under fasting conditions, with the following exceptions:

**Procedure**—An equal number of subjects should be assigned to each of the 6 dosing sequences possible in a three-treatment, three-period study design. Each subject will receive the following treatments:

TREATMENT 1: Generic product, a 400-mg capsule or 600-mg tablet administered after a standard high-fat breakfast.

TREATMENT 2: Reference product, a 400-mg Tolectin® capsule or 600-mg Tolectin® tablet administered after a standard high-fat breakfast.

TREATMENT 3: Generic product, a 400-mg capsule or 600-mg tablet administered under fasting conditions.

Following a 10-hour fast, the subjects receiving treatments under fed conditions should be served a standard high-fat breakfast. The subjects should have 30 minutes to finish the entire breakfast and then should immediately receive Treatment 1 or Treatment 2, taken with 240 mL of water. Subjects receiving the treatment under fasting conditions should receive Treatment 3, taken with 240 mL of water only. The same lots of the test and reference products should be used in the fasted and fed studies. No food should be allowed for at least 4 hours post-dose with water allowed after the first hour. Subjects should be served scheduled standardized meals throughout the study, and the same meals should be served during all phases of the study.

**Statistical Analysis**—In general, a comparable food effect will be assumed if the AUC<sub>0-t</sub>, AUC<sub>0-∞</sub>, and C<sub>max</sub> mean values for the test product administered under fed conditions are within 20% of the respective mean values obtained for the reference product administered under fed conditions.

**Clinical Report, Side Effects, and Adverse Reactions**—Subject medical histories, physical examinations and laboratory reports, and all incidents of possible adverse reactions and side effects to the study formulations should be reported.

**Retention of Samples**—The laboratory conducting the bioequivalence testing should retain appropriately identified reserve samples of both the test and reference products used to perform the *in vivo* bioequivalence studies for approval of the application. Each reserve sample should consist of at least 200 dosage units or 5 times the amount needed for complete release testing, whichever is greater, and should be retained for a minimum of 5 years following approval of the application. For more information on retention of bioequivalence samples refer to 21 CFR 320.63.

**in vitro testing requirements**

**Dissolution Testing**—Conduct dissolution testing on 12 dosage units of the test product and the reference product, employing the biostudy lots. The current official USP dissolution method (see [Dissolution](#) (711)) should be followed and should be referenced by the sponsor. The following times should be used to determine the dissolution profile: Times: 10, 20, 30, and 45 minutes.

The percentage of label claim dissolved at each specified testing interval should be reported for each individual dosage unit. The mean percentage dissolved, the range (highest, lowest) of dissolution, and the coefficient of variation (relative standard deviation) should be reported.

**Content Uniformity Test**—Content uniformity testing on the test and reference product lots should be performed as described in USP.

**waiver requirements**

Waiver of *in vivo* bioequivalence study requirements for the 200-mg strength of the generic tablet product may be granted pursuant to 21 CFR 320.22(d)(2) provided the following conditions are met:

1. The 200-mg tablet is proportionally similar in both active and inactive ingredients to the firm's 600-mg tablet which has been demonstrated to be bioequivalent to the reference product *in vivo*.
2. The 200-mg tablet of the generic product meets dissolution test requirements.

**Auxiliary Information**—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	William E. Brown Senior Scientist 1-301-816-8380	(BPC05) Biopharmaceutics05

## DRUG PROTOCOL CONTENTS

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[Gemfibrozil Capsules and Tablets](#)  
[Glipizide Tablets](#)  
[Glyburide Tablets](#)  
[Guanabenz Acetate](#)  
[Hydroxychloroquine Sulfate Tablets](#)  
[Indapamide Tablets](#)  
[Ketoprofen Capsules](#)  
[Nadolol Tablets](#)  
[Naproxen Tablets](#)  
[Pentoxifylline Extended-Release Tablets](#)  
[Pindolol Tablets](#)  
[Piroxicam Capsules](#)  
[Ranitidine Hydrochloride Tablets](#)  
[Selegiline Hydrochloride Tablets](#)  
[Tolmetin Sodium Capsules and Tablets](#)

## GENERAL GUIDANCES

Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design<sup>2</sup>

## introduction

The FDA Division of Bioequivalence in the Office of Generic Drugs usually evaluates bioequivalence by comparing the in vivo rate and extent of drug absorption of a test and reference formulation in healthy subjects. In a standard in vivo bioequivalence study design, study participants receive test and reference products on separate occasions, in either single or multiple doses, with random assignment to the two possible sequences of product administration. Samples of an accessible biologic fluid such as blood or urine are analyzed for drug and metabolite concentrations, and pharmacokinetic parameters (AUC, Cmax and Tmax) are obtained from the resulting concentration-time curves. These pharmacokinetic parameters are then analyzed statistically to determine if the test and reference products yield comparable values.

Standard statistical methodology based on the null hypothesis is not appropriate to assess bioequivalence. The Division of Bioequivalence has therefore employed a testing procedure termed the two one-sided tests procedure to determine whether average values for pharmacokinetic parameters measured after administration of the test and reference products are comparable. This procedure involves the calculation of a confidence interval for the ratio (or difference) between the test and reference product pharmacokinetic variable averages. The limits of the observed confidence interval must fall within a predetermined range for the ratio (or difference) of the product averages. The determination of the confidence interval range and the statistical level of significance are judgments made by the Division of Bioequivalence.

This guidance provides information about general pharmacokinetic and statistical analyses of bioequivalence data to be conducted by sponsors of abbreviated new drug and antibiotic applications and addresses three specific aspects of the statistical analysis as follows:

1. Logarithmic transformation of pharmacokinetic data
2. Sequence effect
3. Outlier consideration.

This guidance became effective July 1, 1992. Any investigations initiated after this date should generally conform to the recommendations of the guidance. Sponsors following a different approach are encouraged to discuss the matter in advance with the FDA to prevent the expenditure of money and effort on preparing a submission that may later be determined to be unacceptable.

## general methodology

## Pharmacokinetic Analysis—

Single-Dose Studies— At a minimum, the following pharmacokinetic parameters for the substances of interest should be measured in a single-dose bioequivalence study:

- a. Area under the plasma/blood concentration-time curve from time zero to time t (AUC<sub>0-t</sub>), calculated by the trapezoidal rule, where t is the last measurable time point.
- b. Area under the plasma/blood concentration-time curve from time zero to time infinity (AUC<sub>0-∞</sub>), where AUC<sub>0-∞</sub> = AUC<sub>0-t</sub> + Ct /  $\lambda_Z$ , Ct is the last measurable drug concentration, and  $\lambda_Z$  is the terminal elimination rate constant calculated according to an appropriate method. The terminal or elimination half-life of the drug (t<sub>1/2</sub>) should also be reported.
- c. Peak drug concentration (Cmax) and the time to peak drug concentration (Tmax), obtained directly from the data without interpolation.

Multiple-Dose Studies— At a minimum, the following pharmacokinetic parameters for the substances of interest should be measured in a multiple-dose bioequivalence study:



- a. Area under the plasma/blood concentration-time curve from time zero to time  $\tau$  over a dosing interval at steady state ( $AUC_{0-\tau}$ ), where  $\tau$  is the dosing interval.
- b. Peak drug concentration ( $C_{max}$ ) and the time to peak drug concentration ( $T_{max}$ ), obtained directly from the data without interpolation, after the last dose is administered.
- c. Drug concentrations at the end of each dosing interval during steady state ( $C_{min}$ ).
- d. Average drug concentration at steady state ( $C_{av}$ ), where  $C_{av} = AUC_{0-\tau}/\tau$ .
- e. Degree of fluctuation (DF) at steady state, where  $DF = 100\% \times (C_{max} - C_{min})/C_{av}$ .

Evidence of attainment of steady state for the test and reference products should be submitted in the bioequivalence study report.

Statistical Analysis— Parametric (normal-theory) general linear model procedures are recommended for the analysis of pharmacokinetic data derived from in vivo bioequivalence studies. An analysis of variance (ANOVA) should be performed on the pharmacokinetic parameters AUC and  $C_{max}$  using appropriate statistical programs and models. For example, for a conventional two-treatment, two-period, two-sequence ( $2 \times 2$ ) randomized crossover study design, the statistical model often includes factors accounting for the following sources of variation:

1. Sequence (sometimes called Group or Order)
2. Subjects, nested in sequences
3. Period (or Phase)
4. Treatment (sometimes called Drug or Formulation).

The sequence effect should be tested using the [subject (sequence)] mean square from the ANOVA as an error term. All other main effects should be tested against the residual error (error mean square) from the ANOVA. The least square means (LSMEANS) statement should be used to calculate least square means for treatments. Estimates should be obtained for the adjusted differences between treatment means and the standard error associated with these differences.

The two one-sided hypotheses at the  $\alpha = 0.05$  level of significance should be tested for AUC and  $C_{max}$  by constructing the 90% confidence interval for the ratio between the test and reference averages.

logarithmic transformation of pharmacokinetic data

Statistical Assumptions— The assumptions underlying the ANOVA are:

1. Randomization of samples
2. Homogeneity of variances
3. Additivity (linearity) of the statistical model
4. Independence and normality of residuals.

In bioequivalence studies, these assumptions can be interpreted as follows:

1. The subjects chosen for the study should be randomly assigned to the sequences of the study.
2. The variances associated with the two treatments, as well as between the sequence groups, should be equal or at least comparable.
3. The main effects of the statistical model, such as subject, sequence, period, and treatment effect for a standard  $2 \times 2$  crossover study, should be additive. There should be no interactions between these effects.
4. The residuals of the model should be independently and normally distributed. In other words, data from bioequivalence studies should have a normal distribution.

If these assumptions are not met, additional steps should be taken prior to the ANOVA including data transformation to improve the fit of the assumptions or use of a nonparametric statistical test in place of ANOVA. However, the normality and constant variance assumptions in the ANOVA model are known to be relatively robust [i.e., a small or moderate departure from each (or both) of these assumptions will not have a significant effect on the final result].

Rationale for Log Transformation— It is acceptable to use logarithms to the base 10 or natural logarithms ( $\ln$ ). The report must state unambiguously which logarithms were used, and the use must be consistent throughout.

Clinical Rationale— The primary comparison of interest in a bioequivalence study is the ratio of average parameter data from the test and reference formulations rather than the difference between them. Using log transformation, the general linear statistical model employed in the analysis of bioequivalence data allows inferences about the difference between the two means on the log scale, which can then be retransformed into inferences about the ratio of the two averages (means or medians) on the original scale. Log transformation thus achieves the general comparison based on the ratio rather than the difference.

Pharmacokinetic Rationale— Westlake observed that a multiplicative model is postulated for pharmacokinetic parameters in bioavailability/bioequivalence studies, i.e., AUC and  $C_{max}$  (but not  $T_{max}$ ). Assuming that elimination of the drug is first order and only occurs from the central compartment, the following equation holds after an extravascular route of administration:

$$\begin{aligned} AUC_{0-\infty} &= FD/CL \\ &= FD/(V\text{Ke}) \end{aligned}$$

where  $F$  is the fraction absorbed,  $D$  is the administered dose, and  $FD$  is the amount of drug absorbed.  $CL$  is the clearance of a given subject, which is the product of the apparent volume of distribution ( $V$ ) and the elimination rate constant ( $\text{Ke}$ ).<sup>3</sup>

The use of AUC as a measure of the amount of drug absorbed thus involves a multiplicative term ( $CL$ ), which might be regarded as a function of the subject. For this reason, Westlake contends that the subject effect is not additive if the data is analyzed on the original scale of measurement.

Logarithmic transformation of the AUC data will bring the  $CL(V\text{Ke})$  term into the equation in an additive fashion:

$$\ln AUC_{0-\infty} = \ln F + \ln D - \ln V - \ln \text{Ke}$$

Similar arguments were given for  $C_{max}$ . The following equation applies for a drug exhibiting one compartmental characteristic:

$$C_{max} = (FD/V) \times e^{(-\text{Ke} \times T_{max})}$$

where again  $F$ ,  $D$ , and  $V$  are introduced into the model in a multiplicative manner. However, after logarithmic transformation the equation becomes

$$\ln C_{max} = \ln F + \ln D - \ln V - \text{Ke}T_{max}$$



Log transformation of the Cmax data also results in the additive treatment of the V term.

**Statistical Rationale**— Logarithmic transformation of the data from bioequivalence studies can be used to circumvent the use of estimates of the reference product average for computation of the confidence interval for the ratio of product averages. This is an advantage for the cases where a least square estimate for the reference product mean is not well defined. Standard parametric methods are ill-suited to making inferences about the ratio of two averages, though some valid methods do exist. Log transformation changes the problem to one of making inferences about the difference (on the log scale) of two averages, for which the standard methods are well suited.

Many biological data correspond more closely to a log-normal distribution than to a normal distribution. The plasma concentration data, including the derived parameters AUC and Cmax, tend to be skewed, and their variances tend to increase with the means. Log transformation is likely to remedy this situation and make the variances independent of the mean. In addition, frequency distributions skewed to the left (with a long tail to the right) are often made more symmetrical by log transformation.

This argument is actually less persuasive than the argument based on the additivity of the statistical model because it is based largely on the between-subject distribution of AUC and Cmax values. For crossover studies, it is largely the within-subject distribution of values that determines the validity and efficiency of the standard parametric methods of analysis. Despite the arguments regarding the effect of log transformation on normality of bioequivalence data, it is recognized that the limited sample size (20–30 subjects) in a bioequivalence study precludes a reliable determination of the underlying normal distribution of the data set either with or without log transformation.

**General Procedures**— Pharmacokinetic parameters AUC and Cmax should be log transformed. Firms are not encouraged to test for normality of data distribution after log transformation, nor should they employ normality of data distribution as a justification for carrying out the statistical analysis on the original scale. Robustness of a balanced study to non-normality of the data plus use of log transformation will be adequate in most cases.

If a firm believes that the data of a particular bioequivalence study should be statistically analyzed on the untransformed basis rather than the log scale, justification based upon a sound scientific rationale, as well as the statistical methods to be used, ought to be submitted to and reviewed by the FDA Division of Bioequivalence or a comparable regulatory authority.

**Presentation of Data**— The drug concentration in biological fluid at each sampling time point should be furnished untransformed for all the subjects who participated in the study. The derived pharmacokinetic parameters should also be furnished untransformed. The mean, the standard deviation, and the coefficient of variation for each variable should be computed and tabulated in the final report.

To facilitate bioequivalence comparisons, pharmacokinetic parameters for each individual should be displayed in parallel for the formulations tested. In particular, for AUC and Cmax, the difference ( $T - R$ ), ratio ( $T/R$ ), and log of ratio ( $\log T/R$  or  $\ln T/R$ ) between the test and reference values should be tabulated side by side for all the subjects. For each subject, the summary tables should indicate in which sequence (test, reference or reference, test) the subject received the product. Histograms showing the frequency distribution of the difference and  $\ln$  ratio (or log ratio) for the major pharmacokinetic parameters (AUC and Cmax) are useful in the submission.

In addition to the arithmetic mean for the test and reference products, the geometric means (antilog of the means of the logs), means of the logs, and standard deviations of the logs should be calculated for AUC and Cmax. All means, including arithmetic mean, geometric mean, and means of the logs, as well as standard deviations and coefficients of variation, are to be included in the report.

**Equivalence Criteria**— For a broad range of drugs, the FDA Division of Bioequivalence used a range of 80% to 120% for the ratio of the product averages as the standard equivalence criterion when the study data are analyzed on the untransformed basis. This corresponds to a range of  $\pm 20\%$  for the relative difference between the product averages.

When log-transformed data are used in the analysis of AUC and Cmax, using a range of 80% to 125% for the ratio of averages has an advantage over the 80% to 120% criterion in that for the analysis of log-transformed data, the probability of concluding equivalence is at a maximum if the ratio of averages is in fact 1.0 (i.e., exact equality). For the analysis of log-transformed data with a criterion of 80% to 120%, the maximum probability of concluding equivalence occurs when the ratio of product averages equals approximately 0.98. Thus, an equivalence criterion of 80% to 125% is used for the ratio of the product averages.

The 90% confidence interval for the difference in the means of the log-transformed data should be calculated using methods appropriate to the experimental design. The antilogs of the confidence limits constitute the 90% confidence interval for the ratio of the test and reference product averages.

#### sequence effect

A major limitation of a conventional two-treatment, two-period, two-sequence crossover design is the confounding between (i) a true sequence or group effect, (ii) unequal residual or carryover effects, and (iii) treatment-by-period interactions. A true sequence effect (i.e., a difference between the average response for sequence group one and sequence group two) would not bias the determination of bioequivalence. Unequal residual effects, however, would bias this estimate. A treatment-by-period interaction based on an underlying physical basis (i.e., if there were actually something about the periods that caused the difference between the product averages to differ from one period to another), would lead to difficulties in interpreting the estimate of the ratio (difference) in the pharmacokinetic parameters between the test and reference formulations.

Even if there were no true sequence effect, no unequal residual effects, and no treatment-by-period statistical interaction, approximately 10 out of every 100 two-treatment crossover studies would be likely to show an apparent sequence effect, if the testing is carried out at the 10% level of significance.

If the ANOVA test for the presence of a sequence effect results in statistical significance, the actual cause cannot be determined from the data alone. In some cases, plausible causes might be evaluated by examining demographic or physiological subject data, but this examination is seldom conclusive.

On the basis of these considerations, an in vivo two-treatment, two-period, two-sequence crossover bioequivalence study showing a statistically significant sequence effect may be acceptable provided:

1. It is a single-dose study;
2. It includes only healthy, normal subjects;
3. The drug is not an endogenous entity;
4. More than adequate washout period has been allowed between the two phases of the study, and in the second phase, the predose biological matrix samples do not exhibit any detectable drug level in all subjects; and
5. The study meets all scientific and statistical criteria such as:
  - a. It is based upon an acceptable study protocol;
  - b. It contains an acceptable validated assay methodology;
  - c. The study data are acceptable;
  - d. Appropriate statistical analyses of the data are performed; and
  - e. Acceptable confidence intervals for the pharmacokinetic parameters are achieved.

Under all other circumstances, the sponsor may be asked to conduct another study. After regulatory review, multiple-dose studies or studies in patients demonstrating a statistically significant sequence effect may be acceptable provided they meet all other criteria listed above.

#### outlier consideration

Outliers are defined in bioequivalence studies as subjects having discordant values of one or more pharmacokinetic parameters when compared with other values in a study. Because bioequivalence studies are usually carried out as crossover studies, the most important type of outlier is where one or a few subjects differ notably from the rest of the subjects for the test product response versus the reference product response (e.g., test minus reference difference, test/reference ratio, or the  $\log$  of the test/reference ratio).

The existence of an outlier could be indicative of the following problems with interchangeability of two formulations:

**Product Failure**— A subject obtained an unusually high or low response to one or the other of the products because of a problem with the specific dosage unit(s) administered. Examples include a modified- or extended-release dosage form exhibiting dose dumping or a dosage unit whose coating inhibited dissolution.



Subpopulation— A subject may be representative of a type of subject, present in the general population in low numbers, for whom the relative bioavailability of the two products is markedly different than it is for the majority of the population, and for whom the two products are not bioequivalent, even though they might be bioequivalent in the majority of the population.

In the case of product failure, it may make a difference whether the unusual response is observed on the test product or the reference product. In the case of a subpopulation, however, even if the unusual response is observed on the reference product, there may still be concern for lack of interchangeability of the two products.

Statistical tests exist for outlier identification. For detection of a single outlier, one important test is based on the absolute value of the "Studentized Residual." Out of all the data in the study, the test focuses on the most extreme. Approximate critical values for this test have been published by Lund.<sup>9</sup> In principle, however, outliers cannot be dropped from the analysis of the data solely on the basis of a statistical test. When one or more outliers are identified, one should provide scientific evidence or explanations to justify the exclusion of the subject data from statistical analysis.

#### Oral Extended-Release Dosage Forms<sup>4</sup>

This guidance describes *in vivo* bioequivalence studies and *in vitro* drug release testing recommended to applicants intending to submit Abbreviated New Drug Applications (ANDAs) for extended-release products administered orally.

##### nomenclature

Modified-Release Dosage Forms— A modified-release dosage form is one for which the drug release characteristics of time course and/or location are chosen to accomplish therapeutic or convenience objectives not offered by conventional dosage forms such as solutions, ointments, or promptly dissolving dosage forms. Delayed-release and extended-release dosage forms are two types of modified-release dosage forms. This guidance does not consider bioequivalence studies for delayed-release formulations.

Delayed-Release Dosage Forms— A delayed-release dosage form is one that releases a drug(s) at a time other than promptly after administration.

Extended-Release Dosage Forms— An extended-release dosage form is one that allows at least a twofold reduction in dosing frequency or significant increase in patient compliance or therapeutic performance as compared to that presented as a conventional dosage form (e.g., as a solution or a prompt drug-releasing, conventional solid dosage form).

The terms controlled release, prolonged action, and sustained release are used synonymously with extended release. This document uses the term extended release to describe a formulation that does not release active drug substance immediately after oral dosing and that also allows a reduction in dosage frequency. This nomenclature accords generally with the USP definition of extended release but does not specify an impact on dosing frequency. The terms controlled release and extended release are considered interchangeable in this guidance.

##### regulatory background and general requirements

The Drug Price Competition and Patent Term Restoration Act amendments of 1984 to the Food, Drug, and Cosmetic Act gave the Food and Drug Administration statutory authority to accept and approve for marketing ANDAs for generic substitutes of innovator products, including those approved after 1962. To gain approval, ANDAs for a generic extended-release formulation must demonstrate, among other things, that the formulation is both pharmaceutically equivalent and bioequivalent to the innovator extended-release product, which is also termed the reference listed product as identified in FDA's Approved Drug Products with Therapeutic Equivalence Rating, "The Orange Book" (USP DI, Volume III).

Pharmaceutical Equivalence— To be pharmaceutically equivalent, the generic and innovator formulations must (1) contain the same active ingredient; (2) contain the same strength of the active ingredient in the same dosage form; (3) be intended for the same route of administration, and (4) generally be labeled for the same conditions of use. The FDA does not require that the generic and reference listed extended-release products contain the same excipients or that the mechanism by which the active drug substance is released from the formulation be the same.

Bioequivalence Studies— Current regulations require that bioequivalence be demonstrated between a generic extended-release formulation and the reference listed product. The reference listed product is generally an extended-release product subject to an approved full New Drug Application (NDA). For approval, documentation of bioequivalence must be established through performance of a series of *in vivo* bioequivalence studies that are defined under the section *In Vivo Bioequivalence Studies for Approval*. Approval of an ANDA will rely on data derived from evaluation of a biobatch, which is to be manufactured in accordance with the Office of Generic Drugs Procedure and Policy Guide 22-90.

Quality control of the manufacture of an extended-release formulation after approval may be assessed, in part, through performance of *in vitro* dissolution tests. Preapproval submission of these data is required. Recommendations for the conditions under which this test may be performed are described under the section *In Vitro Dissolution for Quality Control Preapproval*. This section also describes how specifications for this test are developed by the applicant and approved by the Division of Bioequivalence. These data are required in the application for approval.

##### *in vivo* bioequivalence studies for approval

*In vivo* bioequivalence studies recommended for approval for extended-release generic formulations are designed to document the following:

- The drug product meets the extended-release claim made for it.
- The drug product does not release the active drug substance at too rapid a rate (dose dumping).
- Performance is equivalent between the generic and the reference listed product following single doses and dosing to steady state.
- The influence of food on the *vivo* performance is comparable for the generic formulation relative to the innovator formulation.

The above objectives are generally met by three *in vivo* studies: (1) a single-dose, randomized, two-period, two-treatment, two-sequence crossover study under fasting conditions, comparing equal doses of the test and reference products; (2) a single-dose, randomized, three-treatment, three-period, six-sequence, crossover, limited food effects study, comparing equal doses of the test product administered under fasting conditions with those of the test and reference products administered immediately after a standard high-fat breakfast<sup>8</sup>; and (3) a multiple-dose, steady state, randomized, two-treatment, two-period, two-sequence crossover study under fasting conditions comparing equal doses of the test and reference formulations. [note—For safety reasons, this study may be performed in the nonfasting state. Applicants are encouraged to submit a study protocol describing the safety considerations requiring deviation from the fasting state to the Division of Bioequivalence for review prior to execution of the study.]

These studies are described in detail below. Under certain circumstances, the Division of Bioequivalence in the Office of Generic Drugs may require additional single-dose or multiple-dose steady state studies. The following general information relative to the three *in vivo* studies is provided:

- FDA-designated reference product is identified by the symbol "+" in The Orange Book.
- The assayed potency of the test product should not differ from that of the reference product by more than 5%.
- The clinical laboratory conducting any *in vivo* study should retain an appropriately identified reserve sample of the test and reference products for a period of 5 years. Each reserve sample should consist of at least 200 dosage units. For more information on retention of bioequivalence samples, refer to 21 CFR 320.63.
- A single-dose two-way crossover study under fasting conditions is required for each strength of a generic extended-release tablet formulation with multiple strengths. The multiple-dose steady state study and the fasting/nonfasting single-dose three-way crossover study are to be conducted with the highest strength only.

For an extended-release bead type capsule formulation to be marketed in multiple strengths, a single-dose bioequivalence study under fasting conditions is required only on the highest strength, provided that the compositions of the lower strengths are proportional to that of the highest strength, and the capsules contain identical beads or pellets. Single-dose *in vivo* bioequivalence studies may be waived for the lower strengths on the basis of acceptable drug release profiles. Multiple-dose steady state and single-dose fasting/nonfasting studies are to be conducted on the highest strength of the capsule formulation.

Batch Size— The test batch or lot must be manufactured under production conditions and must be of a size that is at least 10% of the largest lot planned for full production or a minimum of 100,000 units, whichever is larger.

##### *single-dose* fasting two-way crossover bioequivalence study

Objective— The objective is to compare the rate and extent of absorption of a generic extended-release product with that of the reference-listed product when administered in equal labeled doses.

Design— The study design is a single-dose, two-treatment, two-period, two-sequence crossover with an adequate washout period (usually equal to at least 10 elimination half-lives of



the drug) between the two phases of the study. An equal number of subjects should be randomly assigned to the two possible dosing sequences. Before the study begins, the proposed protocol should be approved by an institutional review board.

**Facilities**— The clinical facilities and analytical laboratory used for the study should be identified along with the names, titles, and curriculum vitae of the medical and scientific or analytical directors.

**Selection of Subjects**— The sponsor should enroll a number of subjects sufficient to ensure statistical validity of the study. It is recommended that a minimum of 24 subjects be used in this study. More subjects may be required for a drug that exhibits high intra-subject variability in metrics of rate and extent of absorption. Subjects should be healthy, preferably nonsmoking, volunteers 18 to 50 years of age, and within 10% of ideal body weight for height and build, although within 15% of ideal body weight is acceptable (Metropolitan Life Insurance Company Statistical Bulletin, 1983). Subjects should be accepted on the basis of acceptable medical history, physical examination, and clinical laboratory tests. Female subjects must be given a pregnancy test prior to beginning the study. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to the administered drug should be excluded from the study. If smokers are included, they should be identified as such. Written, informed consent must be obtained from all subjects before they are accepted into the study.

**Procedure**— Following an overnight fast of at least 10 hours, subjects should be administered a single dose of the test or reference product with 240 mL of water. They should continue fasting for 4 hours after administration of the test or reference treatment.

**Restrictions**— Study volunteers should observe the following restrictions:

- a. No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
- b. Subjects should take no Rx medications, including oral contraceptives, beginning two weeks prior to and no OTC medications beginning one week prior to initiation of the study and until after the study is completed.
- c. Water may be taken except for 1 hour before and after administration, when no liquid is allowed other than that needed for drug dosing.
- d. All meals during the study should be standardized, and the same meals should be served during both phases of the study.

**Blood Sampling**— In addition to the pre-dose (0 hour) sample, venous blood samples should be collected post-dose so that there are at least four sampling time points on the ascending part and six or more on the descending part of the concentration-time curve. The biological matrix (plasma, serum, or whole blood) should be immediately frozen after collection and, as appropriate, centrifugation, and kept frozen until assayed.

**Subject Monitoring**— Blood pressure and pulse rate should be monitored hourly during the first 4 hours of the study. Subjects with a heart rate less than 45 bpm or greater than 110 bpm should have an electrocardiogram (lead II) performed and have their pulse monitored hourly. Subjects should report any unusual symptoms observed during the study. Subjects should be periodically questioned during each phase of the study for any unusual symptoms experienced after drug administration.

**Analysis of Blood Samples**— The active ingredient should be assayed using a suitable analytical method validated with regard to specificity, accuracy, precision (both within and between days), limit of quantitation, linearity, and recovery. Stability of the samples under frozen conditions, at room temperature, and during freeze-thaw cycles, if appropriate, should be determined. If the analytical method is a chromatographic method, chromatograms of unknown samples, including all associated standard curve and quality control chromatograms, should be available for regulatory authorities.

**Pharmacokinetic Analysis of Data**— Calculation of area under the plasma concentration-time curve to the last quantifiable concentration (AUC<sub>0-t</sub>) and to infinity (AUC<sub>0-∞</sub>), C<sub>max</sub>, and T<sub>max</sub> should be performed according to standard techniques.

**Statistical Analysis of Pharmacokinetic Data** (see Statistical Procedures for Bioequivalence Studies Using a Standard Two-treatment Crossover Design)— The log transformed AUC and C<sub>max</sub> data should be analyzed statistically using ANOVA. These two parameters for the test product should be shown to be within 80% to 125% of the reference product using the 90% confidence interval.

**Clinical Report, Side Effects, and Adverse Reactions**— Subject medical histories, physical examination and laboratory reports, and all incidents of possible adverse reactions to the study formulations should be reported.

multiple-dose steady state, two-way crossover bioequivalence study under fasting conditions

**Objective**— The objective is to compare the steady-state rate and extent of absorption of a generic extended-release formulation with that of the reference formulation when given as equal labeled doses.

**Design**— The study design is a multiple-dose, steady-state two-treatment, two-period, two-sequence crossover with an adequate washout period between the two phases of the study. An equal number of subjects should be randomly assigned to the two possible dosing sequences. Before beginning the study, the study protocol should be approved by an institutional review board.

**Facilities and Selection of Subjects**— See the appropriate section under Single-Dose Fasting Two-Way Crossover Bioequivalence Study.

**Procedure**— Extended-release products that are administered once a day should be dosed following an overnight fast of at least 10 hours; subjects should continue fasting for 4 hours post-dose. For extended-release products that are dosed every 12 hours (b.i.d.), the morning dose should be given following an overnight fast of about 10 hours, and subjects should continue fasting for 4 hours post-dose; the evening dose should be administered 12 hours after the morning dose and after a fast of at least 2 hours and subjects should continue fasting for 2 hours post-dose. Each dose should be administered with 240 mL of water.

**Restrictions**— Study volunteers should observe the following restrictions:

- a. No alcohol or xanthine-containing foods or beverages should be consumed for 48 hours prior to dosing and until after the last blood sample is collected.
- b. Subjects should take no Rx medications, including oral contraceptives, beginning two weeks prior to and no OTC medications beginning one week prior to initiation of the study and until after the study is completed.
- c. Water may be taken except for 1 hour before and after administration, when no liquid is allowed other than that needed for drug dosing.

**Blood Sampling**— At least three trough concentrations (C<sub>min</sub>) on three consecutive days should be determined to ascertain that the subjects are at steady state prior to measurement of rate and extent of absorption after a single-dose administration in a dosing interval at steady state. The three consecutive trough samples should be collected at the same time of the day and should be comparable. For extended-release drug products administered more often than every 24 hours, assessment of trough levels just prior to 2 consecutive doses is not recommended because a difference in the consecutive trough values may occur due to circadian rhythm irrespective of whether or not steady state has been attained. Adequate blood samples should be collected at appropriate times during a dosing interval at steady state to permit estimation of the total area under the concentration-time curve, peak concentration (C<sub>max</sub>), and time to peak concentration (T<sub>max</sub>).

**Subject Monitoring and Analysis of Blood Samples**— See under Single-Dose Fasting Two-Way Crossover Bioequivalence Study.

**Pharmacokinetic Analysis of Data**— The following pharmacokinetic data are to be reported for the evaluation of bioequivalence of the generic extended-release product with the reference listed product:

- a. Individual and mean blood drug concentration levels
- b. Individual and mean trough levels (C<sub>min</sub>)
- c. Individual and mean peak levels (C<sub>max</sub>)
- d. Calculation of individual and mean steady state AUC<sub>interdose</sub> is recommended (AUC<sub>interdose</sub> is AUC during a dosing interval at steady state)
- e. Individual and mean percent fluctuation [= 100 × (C<sub>max</sub> – C<sub>min</sub>)/C<sub>min</sub>]
- f. Individual and mean time to peak concentration (T<sub>max</sub>)

**Statistical Analysis of Pharmacokinetic Data**— The log transformed AUC and C<sub>max</sub> data should be analyzed statistically using ANOVA. These two parameters for the test product should be shown to be within 80% to 125% of the reference product using the 90% confidence interval. Fluctuation for the test product should be evaluated for comparability with that for the reference product. For further information on statistical analysis, see Statistical Procedures for Bioequivalence Studies Using a Standard Two-Treatment Crossover Design.

**Clinical Report, Side Effects, and Adverse Reactions**— See under Single-Dose Fasting Two-Way Crossover Bioequivalence Study.



## single-dose, three-way crossover fasting/nonfasting bioequivalence study

Objective— The objective is to compare the rate and extent of absorption of a generic formulation with that of the reference listed formulation under nonfasting conditions and to compare the rate and extent of absorption of the drug from a generic formulation under fasting and nonfasting conditions when given as equal labeled doses.

Design— The study design is a single-dose, three-treatment, three-period, six-sequence crossover with adequate washout period between the three phases of the study. An equal number of subjects should be randomly assigned to each dosing sequence. Before beginning the study, the study protocol should be approved by an institutional review board.

Facilities— See this section under Single-Dose Fasting Two-Way Crossover Bioequivalence Study.

Selection of Subjects— A minimum of 18 subjects should be enrolled in this study. For other information on selection of subjects, see Single-Dose Fasting Two-Way Crossover Bioequivalence Study.

Procedure— Each subject should receive the following three treatments:

treatment 1: Generic extended-release product administered after a high fat content breakfast

treatment 2: Innovator extended-release product (reference listed drug) administered after a high fat content breakfast.

treatment 3: Generic extended-release product administered after fasting.

Following an overnight fast of at least 10 hours, subjects receiving treatments under nonfasting conditions should be served a high-fat breakfast then immediately dosed with Treatment 1 or Treatment 2 with 240 mL of water. Subjects receiving Treatment 3 should be dosed at the same time as Treatments 1 and 2 with 240 mL of water only. No food should be allowed for at least 4 hours post-dose, with water allowed after the first hour. Subjects should be served scheduled standardized meals throughout the study, and the same meals should be served during all phases of the study.

Restrictions, Blood Sampling, Subject Monitoring, and Analysis of Blood Samples— See the appropriate section under Single-Dose Fasting Two-Way Crossover Bioequivalence Study.

Statistical Analysis of Pharmacokinetic Data— In general a comparable food effect will be assumed if the mean values of  $AUC_{0-t}$ ,  $AUC_{0-\infty}$ , and  $C_{max}$  for the generic product administered with food differ by no more than 20% from the respective mean values for the reference listed product administered with food in the study.

Clinical Report, Side Effects, and Adverse Reactions— See under Single-dose Fasting Two-way Crossover Bioequivalence Study.

in vitro drug release for quality control preapproval

Drug Release Testing (724)— Drug release testing should be conducted on 12 individual dosage units of the batches of the test and reference products used in the bioequivalence studies. The potential for pH dependence of drug release from an extended-release product is well recognized. Drug release profiles should therefore be generated in aqueous media at the following pH ranges: 1-1.5, 4-4.5, and 6.0-6.8. Early sampling times of 1, 2, and 4 hours should be included in the sampling schedule to provide assurance against premature release of the drug (dose dumping) from the formulation. Typical drug release conditions are shown below:

Apparatus 1 (for capsules): 100 rpm.

Apparatus 2 (for tablets): 50 and 75 rpm.

Temperature:  $37 \pm 0.5^\circ\text{C}$ .

Units Tested: 12.

Medium: 900 mL of aqueous media at various pH values.

Times: 1, 2, and 4 hours, and every 2 hours thereafter, until 80% of the drug is released.

Tolerances: to be established based on data generated.

Content Uniformity Test (905)— Content uniformity testing on the test and reference product lots should be performed as described in USP.

Specifications— Specifications for the drug release procedure to ensure quality control will be determined on a case-by-case basis. In general, further validation will be required to expand dissolution specifications beyond those established for the biobatch.

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1 Further information about guidances can be obtained by contacting the United States Food and Drug Administration, Division of Bioequivalence, Office of Generic Drugs, 7500 Standish Place, Metro Park North, Rockville, MD 20855 [Phone: (301) 594-2290; FAX: (301) 594-0181]. Copies of the guidances can be obtained from the United States Food and Drug Administration, Center for Drug Evaluation and Research, Consumer Affairs Branch HFD-210 5600 Fishers Lane, Rockville, MD 20857 [Phone: (301) 827-4573, FAX: (301) 827-4577.]

2 This statement, prepared by the Division of Bioequivalence, Office of Generic Drugs (OGD), in consultation with the Division of Biometrics, Office of Epidemiology and Biostatistics, is an informal communication under 21 CFR 10.90 (b)(9) that represents the best judgment of the Division of Bioequivalence and the Office at this time. This statement does not necessarily represent the formal position of the Center for Drug Evaluation and Research (CDER), the Food and Drug Administration (FDA), and does not bind or obligate CDER or FDA to the views expressed.

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3 Note that a more general equation can be written for any multi-compartmental model as  $AUC_{0-\infty} = FD / (Vd \cdot \lambda Z)$ , where  $Vd$  is the volume of distribution relating drug concentration in plasma or blood to the amount of drug in the body during the terminal exponential phase, and  $\lambda Z$  is the terminal slope of the concentration-time curve.

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9 Lund RE. Tables for an approximate test for outliers in linear models. *Technometrics* 1975; 17:473-476.

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4 This statement, prepared by the FDA Division of Bioequivalence in the Office of Generic Drugs, is an informal communication under 21 CFR 10.90(b)(9) that represents the best judgment of the Division at this time. This statement does not necessarily represent the formal position of the Center for Drug Evaluation and Research, Food and Drug Administration, and does not bind or otherwise obligate the Center for Drug Evaluation and Research, Food and Drug Administration, to the views expressed.

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6 Each subject should consume a standardized, high-fat breakfast consisting of the following:

1 buttered English muffin

1 fried egg

1 slice of American cheese

1 slice of Canadian bacon

1 serving of hash brown potatoes

8 fluid oz. (240 mL) of whole milk

6 fluid oz. (180 mL) of orange juice

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5 The sponsoring firm is advised that an Investigational New Drug (IND) application may be required if dosing levels exceed those recommended in the official labeling. See Policy and Procedure Guide 36-92, "Submission of an 'Investigational New Drug Application' to the FDA Office of Generic Drugs (OGD)" and 21 CFR 312.2 and 320.31(b)(1).

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7 Glipizide (5 and 10 mg) and glyburide (1.25, 2.5, and 5 mg), the second generation sulfonylurea antidiabetic agents, are comparatively more potent than tolbutamide (250 and 500 mg) and tolazamide (100, 250, and 500 mg). Therefore, in a fasting bioequivalence study involving normal subjects, hypoglycemic events occur more frequently with glipizide and glyburide than with tolbutamide and tolazamide. In the case of glipizide, the hypoglycemic episodes in normal subjects participating in a fasting bioequivalence study were fewer when the glucose was given to subjects every 15 minutes than when it was given every 30 minutes. In a study with such a design, measurement of plasma glucose is not necessary because it will not reflect the pharmacodynamic endpoint. However, such design is preferable to the usual fasting study design to ensure the welfare of the subjects and to avoid excessive drop out rate.

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8 Each subject should consume the following high-fat breakfast:



2 eggs fried in butter  
2 slices of toasted white bread spread with butter  
2 slices of bacon  
2 oz. of hash brown potatoes  
8 fluid oz. (240 mL) of whole milk

#### 1091 LABELING OF INACTIVE INGREDIENTS

This informational chapter provides guidelines for labeling of inactive ingredients present in dosage forms.

Within the past few years a number of trade associations representing pharmaceutical manufacturers have adopted voluntary guidelines for the disclosure and labeling of inactive ingredients. This is helpful to individuals who are sensitive to particular substances and who wish to identify the presence or confirm the absence of such substances in drug products.

Because of the actions of these associations, the labeling of therapeutically inactive ingredients currently is deemed to constitute good pharmaceutical practice.

Although the manufacturers represented by these associations produce most of the products sold in this country, not all manufacturers, repackagers, or labelers here or abroad are members of these associations. Further, there are some differences in association guidelines. The guidelines presented here are designed to help promote consistency in labeling.

In accordance with good pharmaceutical practice, all dosage forms [note—for requirements on parenteral and topical preparations, see the General Notices] should be labeled to state the identity of all added substances (therapeutically inactive ingredients) present therein, including colors, except that flavors and fragrances may be listed by the general term "flavor" or "fragrance." Such listing should be in alphabetical order by name and be distinguished from the identification statement of the active ingredient(s).

The name of an inactive ingredient should be taken from the current edition of one of the following reference works (in the following order of precedence): (1) the United States Pharmacopeia or the National Formulary; (2) USAN and the USP Dictionary of Drug Names; (3) CTFA Cosmetic Ingredient Dictionary; (4) Food Chemicals Codex. An ingredient not listed in any of the aforementioned reference works should be identified by its common or usual name (the name generally recognized by consumers or health-care professionals) or, if no common or usual name is available, by its chemical or other technical name.

An ingredient that may be, but not always is, present in a product should be qualified by words such as "or" or "may also contain."

The name of an ingredient whose identity is a trade secret may be omitted from the list if the list states "and other ingredients." For the purposes of this guideline, an ingredient is considered to be a trade secret only if its presence confers a significant competitive advantage upon its manufacturer and if its identity cannot be ascertained by the use of modern analytical technology.

An incidental trace ingredient having no functional or technical effect on the product need not be listed unless it has been demonstrated to cause sensitivity reactions or allergic responses.

Inactive ingredients should be listed on the label of a container of a product intended for sale without prescription, except that in the case of a container that is too small, such information may be contained in other labeling on or within the package.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Andrzej Wilk, Ph.D.</a> Scientist 1-301-816-8305	(NOM05) Nomenclature 05

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#### 1092 THE DISSOLUTION PROCEDURE: DEVELOPMENT AND VALIDATION

The USP dissolution procedure is a performance test applicable to many dosage forms. It is one test in a series of tests that constitute the dosage form's public specification (tests, procedures for the tests, acceptance criteria). To satisfy the performance test, USP provides the general test chapters [Disintegration](#) 701, [Dissolution](#) 711, and [Drug Release](#) 724. These chapters provide information about conditions of the procedure. For dissolution, these include information about (1) medium, (2) apparatus/agitation rate, (3) study design, (4) assay, and (5) acceptance criteria. Overall the dissolution procedure yields data to allow an accept/reject decision relative to the acceptance criteria, which are frequently based on a regulatory decision. This chapter provides recommendations on how to develop and validate a dissolution procedure.

##### GENERAL COMMENTS

The dissolution procedure requires an apparatus, a dissolution medium, and test conditions that provide a method that is discriminating yet sufficiently rugged and reproducible for day-to-day operation and capable of being transferred between laboratories.

The acceptance criteria should be representative of multiple batches with the same nominal composition and manufacturing process, typically including key batches used in pivotal studies, and representative of performance in stability studies.

The procedure should be appropriately discriminating, capable of distinguishing significant changes in a composition or manufacturing process that might be expected to affect in vivo performance. It is also possible for the procedure to show differences between batches when no significant difference is observed in vivo. This situation requires careful evaluation of whether the procedure is too sensitive or appropriately discriminating. Assessing the results from multiple batches that represent typical variability in composition and manufacturing parameters may assist in this evaluation. It is sometimes valuable to intentionally vary manufacturing parameters, such as lubrication, blend time, compression force, or drying parameters, to further characterize the discriminatory power of the procedure.

With regard to stability, the dissolution test should appropriately reflect relevant changes in the drug product over time that are caused by temperature, humidity, photosensitivity, and other stresses.

A properly designed test should result in data that are not highly variable and should not be associated with significant analytical solution stability problems. High variability in results can make it difficult to identify trends or effects of formulation changes. Dissolution results may be considered highly variable if the relative standard deviation (RSD) is greater than 20% at time points of 10 minutes or less and greater than 10% RSD at later time points.<sup>4</sup> However, most dissolution results exhibit less variability than this. The source of the variability should be investigated when practical, and attempts should be made to reduce variability whenever possible. The two most likely causes are the formulation itself (e.g., drug substance, excipients, or manufacturing process) or artifacts associated with the test procedure (e.g., coning, tablets sticking to the vessel wall or basket screen). Visual observations are often helpful for understanding the source of the variability and whether the dissolution test itself is contributing to the variability. Any time the dosage contents do not disperse freely throughout the vessel in a uniform fashion, aberrant results can occur. Depending on the problem, the usual remedies include changing the apparatus type, speed of agitation, or deaeration; consideration and/or examination of sinker type; and changing the composition of the medium. Modifications to the apparatus may also be useful, with proper justification and validation.

Many causes of variability can be found in the formulation and manufacturing process. For example, poor content uniformity, process inconsistencies, a reaction taking place at different rates during dissolution, excipient interactions or interference, film coating, capsule shell aging, and hardening or softening of the dosage form on stability may be sources of variability and interferences. During routine testing of the product, variability outside the expected range should be investigated from analytical, formulation, and processing perspectives.

##### MEDIUM

Physical and chemical data for the drug substance and dosage unit need to be determined before selecting the dissolution medium. Two key properties of the drug are the solubility



... solution state stability of the drug as a function of the pH value. When selecting the composition of the medium, the influence of buffers, pH value, and surfactants on the solubility and stability of the drug need to be evaluated. Key properties of the dosage unit that may affect dissolution include release mechanism (immediate, delayed, or modified) and disintegration rate as affected by hardness, friability, presence of solubility enhancers, and presence of other excipients.

Generally, when developing a dissolution procedure, one goal is to have sink conditions, defined as the volume of medium at least three times that required in order to form a saturated solution of drug substance. When sink conditions are present, it is more likely that dissolution results will reflect the properties of the dosage form. A medium that fails to provide sink conditions may be acceptable if it is shown to be more discriminating or otherwise appropriately justified.

Using an aqueous-organic solvent mixture as a dissolution medium is discouraged; however, with proper justification this type of medium may be acceptable.

Purified water is often used as the dissolution medium, but is not ideal for several reasons. First, the quality of the water can vary depending on the source of the water, and the pH value of the water is not controlled. Second, the pH value can vary from day to day and can also change during the run, depending on the active substance and excipients. Despite these limitations, water is inexpensive, readily available, easily disposed of, ecologically acceptable, and suitable for products with a release rate independent of the pH value of the medium.

The dissolution characteristics of an oral formulation should be evaluated in the physiologic pH range of 1.2 to 6.8 (1.2 to 7.5 for modified-release formulations). During method development, it may be useful to measure the pH before and after a run to discover whether the pH changes during the test. Selection of the most appropriate conditions for routine testing is then based on discriminatory capability, ruggedness, stability of the analyte in the test medium, and relevance to in vivo performance, where possible.

Typical media for dissolution may include the following (not listed in order of preference): dilute hydrochloric acid, buffers in the physiologic pH range of 1.2 to 7.5, simulated gastric or intestinal fluid (with or without enzymes), water, and surfactants (with or without acids or buffers) such as polysorbate 80, sodium lauryl sulfate, and bile salts.

The molarity of the buffers and acids used can influence the solubilizing effect, and this factor may be evaluated.

For compounds with high solubility and high permeability (as defined by the Biopharmaceutics Classification System), the choice of medium and apparatus may be influenced by the referenced FDA Guidance<sup>4</sup>.

For very poorly soluble compounds, aqueous solutions may contain a percentage of a surfactant (e.g., sodium lauryl sulfate, polysorbate, or lauryldimethylamine oxide) that is used to enhance drug solubility. The need for surfactants and the concentrations used can be justified by showing profiles at several different concentrations. Surfactants can be used either as wetting agents or to solubilize the drug substance.

#### Volume

Normally, for basket and paddle apparatus, the volume of the dissolution medium is 500 mL to 1000 mL, with 900 mL as the most common volume. The volume can be raised to between 2 and 4 L, using larger vessels and depending on the concentration and sink conditions of the drug; justification for this procedure is expected.

#### Deareration

The significance of deareration of the medium should be determined, because air bubbles can interfere with the test results, acting as a barrier to dissolution if present on the dosage unit or basket mesh. Further, bubbles can cause particles to cling to the apparatus and vessel walls. On the other hand, bubbles on the dosage unit may increase buoyancy, leading to an increase in the dissolution rate, or may decrease the available surface area, leading to a decrease in the dissolution rate. A deareration method is described as a footnote in the Procedure section under [Dissolution](#) (711). Typical steps include heating the medium, filtering, and drawing a vacuum for a short period of time. Other methods of deareration are available and in routine use throughout the industry. Media containing surfactants are not usually dearerated because the process results in excessive foaming. To determine whether deareration of the medium is necessary, results from dissolution samples run in nondearated medium and dearerated medium should be compared.

#### Enzymes

The use of enzymes in the dissolution medium is permitted in accordance with [Dissolution](#) (711) when dissolution failures occur as a result of cross-linking with gelatin capsules or gelatin-coated products.

#### In Vitro-In Vivo Correlation (IVIVC)

An in-depth discussion on IVIVC can be found in [In Vitro and In Vivo Evaluation of Dosage Forms](#) (1088). A brief discussion follows.

Biorelevant medium is a medium that has some relevance to the in vivo performance of the dosage unit. Choice of a biorelevant medium is based on (1) a mechanistic approach that considers the absorption site, if known, and (2) whether the rate-limiting step to absorption is the dissolution or permeability of the compound. In some cases, the biorelevant medium will be different from the test conditions chosen for the regulatory test, and the time points are also likely to be different. If the compound dissolves quickly in the stomach and is highly permeable, gastric emptying time may be the rate-limiting step to absorption. In this case, the dissolution test should demonstrate that the drug is released quickly under typical gastric (acidic) conditions. On the other hand, if dissolution occurs primarily in the intestinal tract (e.g., for a poorly soluble, weak acid), a higher pH range (e.g., simulated intestinal fluid with a pH of 6.8) may be more appropriate. The fed and fasted states may also have significant effects on the absorption or solubility of a compound. Compositions of media that simulate the fed and fasted states can be found in the literature. These media reflect changes in pH, bile concentrations, and osmolarity after meal intake and therefore have a composition different from that of typical compendial media. They are primarily used to establish in vitro-in vivo correlations during formulation development and to assess potential food effects and are not intended for quality control purposes. For quality control purposes, the substitution of natural surfactants (bile components) with appropriate synthetic surfactants is permitted and encouraged because of the expense of the natural substances and the labor-intensive preparation of the biorelevant media.

#### APPARATUS/AGITATION

##### Apparatus

The choice of apparatus is based on knowledge of the formulation design and the practical aspects of dosage form performance in the in vitro test system. For solid oral dosage forms, Apparatus 1 and Apparatus 2 are used most frequently.

When Apparatus 1 or 2 is not appropriate, another official apparatus may be used. Apparatus 3 (Reciprocating Cylinder) has been found to be especially useful for bead-type modified-release dosage forms. Apparatus 4 (Flow-Through Cell) may offer advantages for modified-release dosage forms that contain active ingredients with limited solubility. In addition, Apparatus 3 or Apparatus 4 may have utility for soft gelatin capsules, bead products, suppositories, or poorly soluble drugs. Apparatus 5 (Paddle over Disk) and Apparatus 6 (Rotating Cylinder) have been shown to be useful for evaluating and testing transdermal dosage forms. Apparatus 7 (Reciprocating Holder) has been shown to have application to nondisintegrating oral modified-release dosage forms, as well as to transdermal dosage forms.

Some changes can be made to the apparatus; for example, a basket mesh size other than the typical 40-mesh basket (e.g., 10, 20, 80 mesh) may be used when the need is clearly documented by supporting data. In countries where available mesh sizes vary from the USP-specified mesh value, basket material with the nearest metric dimension should be used.

Care must be taken that baskets are uniform and meet the dimensional requirements specified under [Dissolution](#) (711). If the basket screens become clogged during dissolution of capsule or tablet formulations, it may be advisable to switch to the paddle method. The volume can be increased from the typical 900 to 1000 mL by using 2- and 4-L vessels to assist in meeting sink conditions for poorly soluble drugs.

A noncompendial apparatus may have some utility with proper justification, qualification, and documentation of superiority over the standard equipment. For example, a small-volume apparatus with mini paddles and baskets may be considered for low-dosage strength products. The rotating bottle or static tubes (jacketed stationary tubes enclosed with a water jacket and equipped with a magnetic stirrer) may also have utility for microspheres and implants, peak vessels for eliminating coning, and modified flow-through cells for special dosage forms, including powders and stents.

#### Sinkers

When sinkers are used, a detailed description of the sinker must be stated in the written procedure. It may be useful to evaluate different sinkers, recognizing that sinkers can



significantly influence the dissolution profile of a dosage unit. When transferring the procedure, the sinkers should be duplicated as closely as possible in the next facility. There are several types of commercially available sinkers. A method for making sinkers by hand, sinkers that are similar to "a few turns of wire helix" as described in Apparatus 2 (Paddle Apparatus) under [Dissolution](#) (711), is described below.

Materials— Use 316 stainless steel wire or other inert material, typically 0.032 inch/20 gauge; and cylinders of appropriate diameter (e.g., cork borers). Sizes are shown in the accompanying table.

Capsule Shell Type	Length of Wire (cm)	Diameter (cm)	Cork Bore Number
#0, elongated	12	0.8	4
#1 and #2	10	0.7	3
#3 and #4	8	0.55	2

Procedure— Cut the specified length of wire, coil around a cylinder of the appropriate size, and use small pliers to curve in the ends. Use caution, because wire ends may be rough and may need to be filed.

If the sinker is handmade, the sinker material and construction procedure instructions should be documented; if a commercial sinker is used, the vendor part number should be reported.

#### Agitation

For immediate-release capsule or tablet formulations, Apparatus 1 (baskets) at 100 rpm or Apparatus 2 (paddles) at 50 or 75 rpm are most commonly used. Other agitation speeds and apparatus are acceptable with appropriate justification.

Rates outside 25 to 150 rpm are usually inappropriate because of the inconsistency of hydrodynamics below 25 rpm and because of turbulence above 150 rpm. Agitation rates between 25 and 50 rpm are generally acceptable for suspensions. For dosage forms that exhibit coning (mounding) under the paddle at 50 rpm, the coning can be reduced by increasing the paddle speed to 75 rpm, thus reducing the artifact and improving the data. If justified, 100 rpm may be used, especially for extended-release products. Decreasing or increasing the apparatus rotation speed may be justified if the profiles better reflect *in vivo* performance and/or the method results in better discrimination without adversely affecting method reproducibility.

Selection of the agitation and other study design elements for modified-release dosage forms is similar to that for immediate-release products. These elements should conform to the requirements and specifications given in [Dissolution](#) (711) when the apparatus has been appropriately calibrated.

#### STUDY DESIGN

##### Time Points

For immediate-release dosage forms, the duration of the procedure is typically 30 to 60 minutes; in most cases, a single time point specification is adequate for Pharmacopeial purposes. Industrial and regulatory concepts of product comparability and performance may require additional time points, which may also be required for product registration or approval. A sufficient number of time points should be selected to adequately characterize the ascending and plateau phases of the dissolution curve. According to the Biopharmaceutics Classification System referred to in several FDA Guidance, highly soluble, highly permeable drugs formulated with rapidly dissolving products need not be subjected to a profile comparison if they can be shown to release 85% or more of the active drug substance within 15 minutes. For these types of products a one-point test will suffice. However, most products do not fall into this category. Dissolution profiles of immediate-release products typically show a gradual increase reaching 85% to 100% at about 30 to 45 minutes. Thus, dissolution time points in the range of 15, 20, 30, 45, and 60 minutes are usual for most immediate-release products. For rapidly dissolving products, including suspensions, useful information may be obtained from earlier points, e.g., 5 to 10 minutes. For slower-dissolving products, time points later than 60 minutes may be useful. Dissolution test times for compendial tests are usually established on the basis of an evaluation of the dissolution profile data.

So-called infinity points can be useful during development studies. To obtain an infinity point, the paddle or basket speed is increased at the end of the run for a sustained period (typically 15 to 60 minutes), after which time an additional sample is taken. Although there is no requirement for 100% dissolution in the profile, the infinity point can provide data that may supplement content uniformity data and may provide useful information about formulation characteristics during initial development or about method bias.

For an extended-release dosage form, at least three test time points are chosen to characterize the *in vitro* drug release profile for Pharmacopeial purposes. Additional sampling times may be required for drug approval purposes. An early time point, usually 1 to 2 hours, is chosen to show that there is little probability of dose dumping. An intermediate time point is chosen to define the *in vitro* release profile of the dosage form, and a final time point is chosen to show the essentially complete release of the drug. Test times and specifications are usually established on the basis of an evaluation of drug release profile data. For products containing more than a single active ingredient, drug release is to be determined for each active ingredient.

##### Observations

Visual observations and recordings of product dissolution and disintegration behavior are very useful because dissolution and disintegration patterns can be indicative of variables in the formulation or manufacturing process. To accomplish visual observation, proper lighting (with appropriate consideration of photodegradation) of the vessel contents and clear visibility in the bath are essential. Documenting observations by drawing sketches and taking photographs or videos can be instructive and helpful for those who are not able to observe the real time dissolution test. Observations are especially useful during method development and formulation optimization. Examples of typical observations include, but are not limited to, the following:

1. Uneven distribution of particles throughout the vessel. This can occur when particles cling to the sides of the vessel, when there is coning or mounding directly under the apparatus, when particles float at the surface of the medium, when film-coated tablets stick to the vessel, and/or when off-center mounds are formed.
2. Air bubbles on the inside of the vessel or on the apparatus or dosage unit. Sheen on the apparatus is also a sign of air bubbles. This observation would typically be made when assessing the need to deaerate the medium.
3. Dancing or spinning of the dosage unit, or the dosage unit being hit by the paddle.
4. Adhesion of particles to the paddle or the inside of the basket, which may be observed upon removal of the stirring device at the end of the run.
5. Pellicles or analogous formations, such as transparent sacs or rubbery, swollen masses surrounding the capsule contents.
6. Presence of large floating particles or chunks of the dosage unit.
7. Observation of the disintegration rate (e.g., percentage reduction in size of the dosage unit within a certain time frame).
8. Complex disintegration of the coating of modified or enteric-coated products—for example, the partial opening and splitting apart (like a clamshell) or incomplete opening of the shell accompanied by the release of air bubbles and excipients.

##### Sampling

Manual— Manual sampling uses plastic or glass syringes, a stainless steel cannula that is usually curved to allow for vessel sampling, a filter, and/or a filter holder. The sampling site must conform to specifications under [Dissolution](#) (711).

Autosampling— Autosampling is a useful alternative to manual sampling, especially if the test includes several time points. However, because regulatory labs may perform the dissolution test using manual sampling, autosampling requires validation with manual sampling.

There are many brands of autosamplers, including semiautomated and fully automated systems. Routine performance checks, cleaning, and maintenance as described in the pertinent standard operating procedures or metrology documents are useful for reliable operation of these devices.



Some instruments are equipped with sampling through the basket or paddle shaft. Proper validation (e.g., demonstrated equivalence to results with the usual sampling procedure) may be required.

The disturbance of the hydrodynamics of the vessel by sampling probes should be considered and adequate validation performed to ensure that the probes are not introducing a significant change in the dissolution rate.

Comparison of manual and automated procedures should be performed to evaluate the interchangeability of the procedures. This can be accomplished by comparing data from separate runs or, in some cases, by sampling both ways from the same vessel. Results should be consistent with the requirements for intermediate precision (described in this chapter in Validation) if the procedures are to be considered interchangeable.

Other aspects of automation validation may include carryover of residual drug, effect of an in-residence probe (simultaneous sampling as mentioned above may not be suitable in this case), adsorption of drug, and cleaning and/or rinse cycles.

#### Filters

Filtration of the dissolution samples is usually necessary to prevent undissolved drug particles from entering the analytical sample and further dissolving. Also, filtration removes insoluble excipients that may otherwise cause high background or turbidity. Prewetting of the filter with the medium may be necessary.

Filters can be in-line or at the end of the sampling probe or both. The pore size can range from 0.45 to 70  $\mu\text{m}$ . The usual types of filters are depth, disk, and flow-through. However, if the excipient interference is high, if the filtrate has a cloudy appearance, or if the filter becomes clogged, an alternative type of filter or pore size should be evaluated.

Adsorption of the drug(s) onto the filter needs to be evaluated. If drug adsorption occurs, the amount of initial filtrate discarded may need to be increased. If results are still unsuitable, an alternative filter material may be sought.

Filter validation may be accomplished by preparing a suitable standard solution or a completely dissolved sample solution (e.g., prepared as a typical sample in a vessel or a sample put in a beaker and stirred with a magnetic stirrer for 1 hour). For standard solutions, compare the results for filtered solutions (after discarding the appropriate volume) to those for the unfiltered solutions. For sample solutions, compare the results for filtered solutions (after discarding the appropriate volume) to those for centrifuged, unfiltered solutions.

#### Centrifugation

Centrifugation of samples is not preferred, because dissolution can continue to occur and because there may be a concentration gradient in the supernatant. A possible exception might be for compounds that adsorb onto all common filters.

#### ASSAY

The usual assay for a dissolution sample is either spectrophotometric determination or HPLC. The preferred method of analysis is spectrophotometric determination because results can be obtained faster, the analysis is simpler, and fewer solvents are used. HPLC methods are used when there is significant interference from excipients or among drugs in the formulation to improve analytical sensitivity and/or when the analysis can be automated. It may be useful to obtain data for the drug with a stability-indicating assay (e.g., HPLC chromatograms) in the medium of choice, even if the primary assay is based on a spectrophotometric method.

#### VALIDATION

The validation topics described in this section are typical but not all-inclusive. The validation elements addressed may vary, depending on the phase of development or the intended use for the data.<sup>2</sup> The acceptance criteria are presented as guidelines only and may differ for some products. Firms should document the appropriate acceptance criteria for their products in pertinent SOPs. Other considerations may be important for special dosage forms. The extent of validation depends on the phase of the product development. Full validation takes place by the time of Phase III clinical studies. Validation studies should address the variations associated with different profile time points. For products containing more than a single active ingredient, the dissolution method needs to be validated for each active ingredient.

##### Specificity/Placebo Interference

It is necessary to demonstrate that the results are not unduly affected by placebo constituents, other active drugs, or degradates.

The placebo consists of all the excipients and coatings (inks, sinker, and capsule shell are also included when appropriate) without the active ingredient. Placebo interference may be determined by weighing samples of the placebo blend and dissolving or dispersing them in dissolution medium at concentrations that would be encountered during testing. It may be desirable to perform this experiment at 37° by comparing it to the 100% standard by the formula:

$$100C(AP / AS)(V/L)$$

in which C is the concentration, in mg per mL, of the standard; AP and AS are the absorbances of the placebo and the standard, respectively; V is the volume, in mL, of the medium; and L is the label claim, in mg. The interference should not exceed 2%.

note—For extended-release products, a placebo version of the finished dosage form may be more appropriate to use than blends, because this placebo formulation will release the various excipients in a manner more nearly reflecting the product than will a simple blend of the excipients. In this case, it may be appropriate to evaluate potential interference at multiple sampling points in the release profile.

If the placebo interference exceeds 2%, then method modification—such as (1) choosing another wavelength, (2) baseline subtraction using a longer wavelength, or (3) using HPLC—may be necessary in order to avoid the interference. When other active drugs or significant levels of degradates are present, it is necessary to demonstrate that these do not significantly affect the results. One procedure for doing this is to measure the matrix in the presence and absence of the other active drug or degradate: any interference should not exceed 2%.

##### Linearity and Range

Linearity and range are typically established by preparing solutions of the drug, ranging in concentration from below the lowest expected concentration to above the highest concentration during release. This may be done in conjunction with accuracy/recovery determination. The scheme may be altered if different flow-cell sizes or injection volumes are used.

Typically, solutions are made from a common stock if possible. For the highest concentration, the determination may not exceed the linearity limits of the instrument.

Organic solvents may be used to enhance drug solubility for the preparation of the standard solutions; however, no more than 5% (v/v) of organic solvent in the final solution should be used, unless validated.

Linearity is typically calculated by using an appropriate least-squares regression program. Typically, a square of the correlation coefficient ( $r^2 \geq 0.98$ ) demonstrates linearity. In addition, the y-intercept must not be significantly different from zero.

##### Accuracy/Recovery

Accuracy/recovery are typically established by preparing multiple samples containing the drug and any other constituents present in the dosage form (e.g., excipients, coating materials, capsule shell) ranging in concentration from below the lowest expected concentration to above the highest concentration during release.

In cases of poor drug solubility, it may be appropriate to prepare a stock solution by dissolving the drug substance in a small amount of organic solvent (typically not exceeding 5%) and diluting to the final concentration with dissolution medium. An amount of stock solution equivalent to the targeted label claim may be added to the vessel instead of the drug powder. Similarly, for very low strengths, it may be more appropriate to prepare a stock solution than to attempt to weigh very small amounts. The measured recovery is typically 95% to 105% of the amount added. Bracketing or matrixing of multiple strengths may be useful.



A special case for validation is the Acid Stage procedure described in Delayed-Release Dosage Forms under [Dissolution](#) 711. The limit of not more than 10% needs to be validated. If the compound degrades in acid, the validation experiment must address this fact.

#### Precision

Repeatability— Repeatability is determined by replicate measurements of standard and/or sample solutions. It can be measured by calculating the RSD of the multiple injections or spectrophotometric readings for each standard solution, or from the accuracy or linearity data.

Intermediate Precision— Intermediate precision may be evaluated to determine the effects of random events on the precision of the analytical procedure. This evaluation is typically done later in the development of the drug product. The precision can be across the range of product strengths. Typical variations to study include days, analysts, and equipment. The use of an experimental matrix design is encouraged for evaluation of intermediate precision. If possible, intermediate precision can be evaluated using a well-characterized lot of drug product of tight content uniformity. In cases where a well-characterized product is not available, placebo and active ingredient may be used to identify intermediate precision.

The dissolution profiles on the same sample may be run by at least two different analysts, each analyst preparing the standard solutions and the medium. Typically, the analysts use different dissolution baths, spectrophotometers or HPLC equipment (including columns), and autosamplers; and they perform the test on different days. This procedure may not need to be performed for each strength; instead, bracketing with high and low strengths may be acceptable.

A typical acceptance criterion is that the difference in the mean value between the dissolution results at any two conditions using the same strength does not exceed an absolute 10% at time points with less than 85% dissolved and does not exceed 5% for time points above 85%. Acceptance criteria may be product-specific, and other statistical tests and limits may be used.

#### Robustness

The evaluation of robustness, which assesses the effect of making small, deliberate changes to the dissolution conditions, typically is done later in the development of the drug product. The number of replicates (typically 3 or 6) is dependent on the intermediate precision.

Parameters to be varied are dependent on the dissolution procedure and analysis type. They may include medium composition (e.g., buffer or surfactant concentration), pH, volume, agitation rate, and temperature. For HPLC analysis, parameters may include mobile phase composition (percentage organic, buffer concentration, pH), flow rate, wavelength, column temperature, and multiple columns (of the same type). For spectrophotometric analysis, the wavelength may be varied.

#### Standard and Sample Solution Stability

The standard solution is stored under conditions that ensure stability. The stability of the standard is analyzed over a specified period of time, using a freshly prepared standard solution at each time interval for comparison. The acceptable range for standard solution stability is typically between 98% and 102%.

The sample solution is typically stored at room temperature. The sample is analyzed over a specified period of time using the original sample solution response for comparison. The typical acceptable range for sample solution stability may be between 98% and 102% compared with the initial analysis of the sample solutions. If the solution is not stable, aspects to consider could be temperature (refrigeration may be needed), light protection, and container material (plastic or glass).

The procedure may state that the standards and samples need to be analyzed within a time period demonstrating acceptable standard and sample solution stability.

#### Spectrophotometric Analysis

Samples may be automatically introduced into the spectrophotometer using autosippers and flow cells. Routine performance checks, cleaning, and maintenance as described in the standard operating procedures or metrology documents are useful for reliable operation of these instruments. Cells with path lengths ranging from 0.02 to 1 cm are typically used. Cell alignment and air bubbles could be sources of error. The smaller path length cells are used to avoid diluting the sample; however, acceptable linearity and standard error need to be demonstrated.

During analysis, standard solutions are typically prepared and analyzed at just one concentration at 100% (or the selected Q value) of the dosage strength. During profile analysis, other concentrations may be useful. A typical blank, standard, and sample may be analyzed in a sequence that brackets the sample with standards and blanks, especially at the beginning and end of the analysis.

In most cases, the mean absorbance of the dissolution medium blank may not exceed 1% of the standard. Values higher than 1% must be evaluated on a case-by-case basis. The typical RSD for UV analysis is usually not more than 2%.

The absorptivity is calculated by dividing the mean standard absorbance by the concentration, in mg per mL, divided by the flow-cell path length in cm. After enough historical data are accumulated, an acceptable absorptivity range for the analyte (using the appropriate flow cell) may be determined. This value may be useful in troubleshooting aberrant data.

Fiber optics as a sampling and determinative method, with proper validation, is an option.

It may be useful to examine the UV spectrum of the drug in solution to select the optimum wavelength.

#### HPLC

For HPLC analysis, the compatibility of dissolution media and mobile phase may be examined, especially if large injector volumes (over 100  $\mu$ L) are needed. Samples are normally analyzed with HPLC using a spectrophotometric detector and an auto-injector. Single injections of each vessel time point with standards throughout the run constitute a typical run design. System suitability tests include, at a minimum, the retention window and injection precision. Typically, the repeatability of an HPLC analysis should be less than or equal to 2% RSD for five or six standard determinations. The standard level is typically at the 100% label claim level, especially for a single-point analysis.

Preparation of the placebo samples for the HPLC analysis is to be performed in the same way as in the spectrophotometric analysis. Examine the chromatogram for peaks eluting at the same retention time as the drug. If there are extraneous peaks, inject the standard solution, and compare retention times. If the retention times are too close, spike the placebo solution with the drug. Chromatograms may also be obtained over an extended run time using the blank (dissolution medium), standard, and sample solution to identify late eluters that may interfere with subsequent analyses.

The validation documentation may include overlaid representative chromatograms or spectra of blank dissolution medium, a filtered placebo solution, a standard solution, and a filtered dissolution sample. Absence of interfering peaks in the placebo chromatogram or lack of absorbance by the placebo at the analytical wavelength demonstrates specificity.

#### ACCEPTANCE CRITERIA

Typical acceptance criteria for the amount of active ingredient dissolved, expressed as a percentage of the labeled content (Q), are in the range of 75% to 80% dissolved. A Q value in excess of 80% is not generally used, because allowance needs to be made for assay and content uniformity ranges.<sup>3</sup> Acceptance criteria including test times are usually established on the basis of an evaluation of the dissolution profile data. Acceptance criteria should be consistent with historical data, and there is an expectation that acceptable batches (e.g., no significant differences in *in vivo* performance, composition, or manufacturing procedure) will have results that fall within the acceptance criteria.

1 The Biopharmaceutics Classification System is outlined in the FDA Guidance for Industry: Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System, August 2000; <http://www.fda.gov/cder/guidance/3618fnl.htm>, accessed 6/22/2005.

2 Boudreau, S.P.; McElvain, J.S.; Martin, L.D.; Dowling, T.; Fields, S.M. Method Validation by Phase of Development, an Acceptable Analytical Practice. *Pharmaceutical Technology* 2004; 28(11):54-66.

3 See the FDA Guidance for Industry: Dissolution Testing of Immediate-Release Solid Oral Dosage Forms, August 1997; <http://www.fda.gov/cder/guidance/1713bp1.pdf>, accessed 6/22/2005.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">William E. Brown</a> Senior Scientist 1-301-816-8380	(BPC05) Biopharmaceutics05

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Pharmacopeial Forum: Volume No. 31(5) Page 1463

**1101 MEDICINE DROPPER**

The Pharmacopeial medicine dropper consists of a tube made of glass or other suitable transparent material that generally is fitted with a collapsible bulb and, while varying in capacity, is constricted at the delivery end to a round opening having an external diameter of about 3 mm. The dropper, when held vertically, delivers water in drops each of which weighs between 45 mg and 55 mg.

In using a medicine dropper, one should keep in mind that most medicinal liquids do not have the same surface tension and viscosity characteristics as water, and therefore the size of drops will vary from one preparation to another.

Where accuracy of dosage is important, a dropper that has been calibrated specifically for the preparation with which it is supplied should be employed. The volume error incurred in measuring any liquid by means of a calibrated dropper should not exceed 15% under normal use conditions.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">William E. Brown</a> Senior Scientist 1-301-816-8380	(PDF05) Pharmaceutical Dosage Forms 05

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Pharmacopeial Forum: Volume No. 30(6) Page 2137

**1111 MICROBIOLOGICAL ATTRIBUTES OF NONSTERILE PHARMACEUTICAL PRODUCTS**

Few raw materials used in making pharmaceutical products are sterile as received, and special treatment may be required to render them microbiologically acceptable for use. Strict adherence to effective environmental control and sanitation, equipment cleaning practices, and good personal hygiene practices in pharmaceutical manufacture is vital in minimizing both the type and the number of microorganisms.

Monitoring, in the form of regular surveillance, should include an examination of the microbiological attributes of Pharmacopeial articles and a determination of compliance with such microbiological standards as are set forth in the individual monographs. It may be necessary also to monitor the early and intermediate stages of production, with emphasis being placed on raw materials, especially those of animal or botanical origin, or from natural mineral sources, which may harbor objectionable microorganisms not destroyed during subsequent processing. It is essential that ingredients and components be stored under conditions designed to deter microbial proliferation.

The nature and frequency of testing vary according to the product. Monographs for some articles require freedom from one or more species of selected indicator microorganisms such as *Salmonella* species, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. For some articles, a specific limit on the total aerobic count of viable microorganisms and/or the total combined molds and yeasts count is set forth in the individual monograph; in these cases a requirement for freedom from specified indicator microorganisms may also be included. The significance of microorganisms in nonsterile pharmaceutical products should be evaluated in terms of the use of the product, the nature of the product, and the potential hazard to the user. Also taken into account is the processing of the product in relation to an acceptable quality for pharmaceutical purposes.

It is suggested that certain categories of products should be tested routinely for total microbial count and for specified indicator microbial contaminants, e.g., natural plant, animal, and some mineral products for *Salmonella* species; oral solutions and suspensions for *E. coli*; articles applied topically for *P. aeruginosa* and *S. aureus*; and articles intended for rectal, urethral, or vaginal administration for yeasts and molds.

Definitive microbial limits (stipulated microorganisms and/or counts) are incorporated into specific monographs on the basis of a major criterion, i.e., the potential of the stipulated microorganisms and/or counts, and of any others that they may reflect, to constitute a hazard in the end product. Such considerations also take into account the processing to which the product components are subjected, the current technology for testing, and the availability of desired quality material. Any of these may preclude the items from specific requirements under [Microbial Enumeration Tests \(61\)](#) and [Tests for Specified Microorganisms \(62\)](#). Regardless of such preclusion, it remains essential to apply strict good manufacturing practices to assure a lowest possible load of microorganisms.

The relevant tests for determining the total count of viable aerobic microorganisms and the total combined molds and yeasts count, and for detection and identification of designated species are given under [Microbial Enumeration Tests \(61\)](#) and [Tests for Specified Microorganisms \(62\)](#). For reliable results, the personnel responsible for the conduct of the test should have specialized training in microbiology and in the interpretation of microbiological data.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Radhakrishna S Tirumalai, Ph.D.</a> Senior Scientist 1-301-816-8339	(MSA05) Microbiology and Sterility Assurance

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Pharmacopeial Forum: Volume No. 29(5) Page 1733

**1111 MICROBIOLOGICAL EXAMINATION OF NONSTERILE PRODUCTS: ACCEPTANCE CRITERIA FOR PHARMACEUTICAL PREPARATIONS AND SUBSTANCES FOR PHARMACEUTICAL USE**

The presence of certain microorganisms in nonsterile preparations may have the potential to reduce or even inactivate the therapeutic activity of the product and has a potential to adversely affect the health of the patient. Manufacturers have therefore to ensure a low bioburden of finished dosage forms by implementing current guidelines on Good Manufacturing Practice during the manufacture, storage, and distribution of pharmaceutical preparations.

Microbial examination of nonsterile products is performed according to the methods given in the texts on [Microbial Enumeration Tests \(61\)](#) and [Tests for Specified Microorganisms \(62\)](#). Acceptance criteria for nonsterile pharmaceutical products based upon the total aerobic microbial count (TAMC) and the total combined yeasts and molds count (TYMC) are given in [Tables 1](#) and [2](#). Acceptance criteria are based on individual results or on the average of replicate counts when replicate counts are performed (e.g., direct plating methods).

When an acceptance criterion for microbiological quality is prescribed, it is interpreted as follows:

- 101 cfu: maximum acceptable count = 20;
- 102 cfu: maximum acceptable count = 200;
- 103 cfu: maximum acceptable count = 2000; and so forth.

Table 1. Acceptance Criteria for Microbiological Quality of Nonsterile Dosage Forms



Route of Administration	Total Aerobic Microbial Count (cfu/g or cfu/mL)	Total Combined Yeasts/Molds Count (cfu/g or cfu/mL)	Specified Microorganism(s)
Nonaqueous preparations for oral use	103	102	Absence of Escherichia coli (1 g or 1 mL)
Aqueous preparations for oral use	102	101	Absence of Escherichia coli (1 g or 1 mL)
Rectal use	103	102	—
Oromucosal use	102	101	Absence of Staphylococcus aureus (1 g or 1 mL) Absence of Pseudomonas aeruginosa (1 g or 1 mL)
Gingival use	102	101	Absence of Staphylococcus aureus (1 g or 1 mL) Absence of Pseudomonas aeruginosa (1 g or 1 mL)
Cutaneous use	102	101	Absence of Staphylococcus aureus (1 g or 1 mL) Absence of Pseudomonas aeruginosa (1 g or 1 mL)
Nasal use	102	101	Absence of Staphylococcus aureus (1 g or 1 mL) Absence of Pseudomonas aeruginosa (1 g or 1 mL)
Auricular use	102	101	Absence of Staphylococcus aureus (1 g or 1 mL) Absence of Pseudomonas aeruginosa (1 g or 1 mL)
Vaginal use	102	101	Absence of Pseudomonas aeruginosa (1 g or 1 mL) Absence of Staphylococcus aureus (1 g or 1 mL) Absence of Candida albicans (1 g or 1 mL)
Transdermal patches (limits for one patch including adhesive layer and backing)	102	101	Absence of Staphylococcus aureus (1 patch) Absence of Pseudomonas aeruginosa (1 patch)
Inhalation use (special requirements apply to liquid preparations for nebulization)	102	101	Absence of Staphylococcus aureus (1 g or 1 mL) Absence of Pseudomonas aeruginosa (1 g or 1 mL) Absence of bile-tolerant Gram-negative bacteria (1 g or 1 mL)

[Table 1](#) includes a list of specified microorganisms for which acceptance criteria are set. The list is not necessarily exhaustive, and for a given preparation it may be necessary to test for other microorganisms depending on the nature of the starting materials and the manufacturing process.

If it has been shown that none of the prescribed tests will allow valid enumeration of microorganisms at the level prescribed, a validated method with a limit of detection as close as possible to the indicated acceptance criterion is used.

Table 2. Acceptance Criteria for Microbiological Quality of Nonsterile Substances for Pharmaceutical Use

	Total Aerobic Microbial Count (cfu/g or cfu/mL)	Total Combined Yeasts/Molds Count (cfu/g or cfu/mL)
Substances for pharmaceutical use	103	102

In addition to the microorganisms listed in [Table 1](#), the significance of other microorganisms recovered should be evaluated in terms of the following:

- The use of the product: hazard varies according to the route of administration (eye, nose, respiratory tract).
- The nature of the product: does the product support growth? does it have adequate antimicrobial preservation?
  - The method of application.
  - The intended recipient: risk may differ for neonates, infants, the debilitated.
    - Use of immunosuppressive agents, corticosteroids.
    - The presence of disease, wounds, organ damage.

Where warranted, a risk-based assessment of the relevant factors is conducted by personnel with specialized training in microbiology and in the interpretation of microbiological data. For raw materials, the assessment takes account of the processing to which the product is subjected, the current technology of testing, and the availability of materials of the desired quality.

(Official May 1, 2009)

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Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Radhakrishna S Tirumalai, Ph.D.</a> Senior Scientist 1-301-816-8339	(MSA05) Microbiology and Sterility Assurance

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#### 1112 APPLICATION OF WATER ACTIVITY DETERMINATION TO NONSTERILE PHARMACEUTICAL PRODUCTS

The determination of the water activity of nonsterile pharmaceutical dosage forms aids in the decisions relating to the following:

- optimizing product formulations to improve antimicrobial effectiveness of preservative systems,
- reducing the degradation of active pharmaceutical ingredients within product formulations susceptible to chemical hydrolysis,
- reducing the susceptibility of formulations (especially liquids, ointments, lotions, and creams) to microbial contamination, and



- d. providing a tool for the rationale for reducing the frequency of microbial limit testing and screening for objectionable microorganisms for product release and stability testing, using methods contained in the general test chapter [Microbial Enumeration Tests](#) [61](#) and [Tests for Specified Microorganisms](#) [62](#).

Reduced water activity ( $a_W$ ) will greatly assist in the prevention of microbial proliferation in pharmaceutical products; and the formulation, manufacturing steps, and testing of nonsterile dosage forms should reflect this parameter.

Low water activity has traditionally been used to control microbial deterioration of foodstuffs. Examples where the available moisture is reduced are dried fruit, syrups, and pickled meats and vegetables. Low water activities make these materials self-preserved. Low water activity will also prevent microbial growth within pharmaceutical drug products. Other product attributes, for example, low or high pH, absence of nutrients, presence of surfactants, and addition of antimicrobial agents, as well as low water activity, help to prevent microbial growth. However, it should be noted that more resistant microorganisms, including spore-forming *Clostridium* spp., *Bacillus* spp., *Salmonella* spp. and filamentous fungi, although they may not proliferate in a drug product with a low water activity, may persist within the product.

When formulating an aqueous oral or topical dosage form, candidate formulations should be evaluated for water activity so that the drug product may be self-preserving, if possible. For example, small changes in the concentration of sodium chloride, sucrose, alcohol, propylene glycol, or glycerin in a formulation may result in the creation of a drug product with a lower water activity that can discourage the proliferation of microorganisms in the product. This is particularly valuable with a multiple-use product that may be contaminated by the user. Packaging studies should be conducted to test product stability and to determine that the container–closure system protects the product from moisture gains that would increase the water activity during storage.

Reduced microbial limits testing may be justified through risk assessment. This reduction in testing, when justified, may entail forgoing full microbial limits testing, implementing skip-lot testing, or eliminating routine testing.

Nonaqueous liquids or dry solid dosage forms will not support spore germination or microbial growth due to their low water activity. The frequency of their microbial monitoring can be determined by a review of the historic testing database of the product and the demonstrated effectiveness of microbial contamination control of the raw materials, ingredient water, manufacturing process, formulation, and packaging system. The testing history would include microbial monitoring during product development, scale-up, process validation, and routine testing of sufficient marketed product lots (e.g., up to 20 lots) to ensure that the product has little or no potential for microbial contamination. Because the water activity requirements for different Gram-reactive bacteria, bacterial spores, yeasts, and molds are well described in the literature,<sup>1</sup> the appropriate microbial limit testing program for products of differing water activities can be established. For example, Gram-negative bacteria including the specific objectionable microorganisms, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella* species will not proliferate or survive in preserved products with water activities below 0.91, while Gram-positive bacteria such as *Staphylococcus aureus* will not proliferate below 0.86, and *Aspergillus niger* will not proliferate below 0.77. Furthermore, even the most osmophilic yeast and xerophilic fungi will not proliferate below 0.60, and they cannot be isolated on compendial microbiological media.<sup>1</sup> The water activity requirements measured at 25° for the growth of a range of representative microorganisms are presented in [Table 1](#).

Table 1. Water Activities ( $a_W$ ) Required to Support the Growth of Representative Microorganisms

Bacteria	Water Activity ( $a_W$ )	Molds and Yeast	Water Activity ( $a_W$ )
<i>Pseudomonas aeruginosa</i>	0.97	<i>Rhizopus nigricans</i>	0.93
<i>Bacillus cereus</i>	0.95	<i>Mucor plumbeus</i>	0.92
<i>Clostridium botulinum</i> , Type A	0.95	<i>Rhodotorula mucilaginosa</i>	0.92
<i>Escherichia coli</i>	0.95	<i>Saccharomyces cerevisiae</i>	0.90
<i>Clostridium perfringens</i>	0.95	<i>Paecilomyces variotti</i>	0.84
<i>Lactobacillus viridescens</i>	0.95	<i>Penicillium chrysogenum</i>	0.83
<i>Salmonella</i> spp.	0.95	<i>Aspergillus fumigatus</i>	0.82
<i>Enterobacter aerogenes</i>	0.94	<i>Penicillium glabrum</i>	0.81
<i>Bacillus subtilis</i>	0.90	<i>Aspergillus flavus</i>	0.78
<i>Micrococcus lysodekticus</i>	0.93	<i>Aspergillus niger</i>	0.77
<i>Staphylococcus aureus</i>	0.86	<i>Zygosachcharomyces rouxii</i> (osmophilic yeast)	0.62
<i>Halobacterium halobium</i> (halophilic bacterium)	0.75	<i>Xeromyces bisporus</i> (xerophilic fungi)	0.61

Pharmaceutical drug products with water activities well below 0.75 (e.g., direct compression tablets, powder and liquid-filled capsules, nonaqueous liquid products, ointments, and rectal suppositories) would be excellent candidates for reduced microbial limit testing for product release and stability evaluation. This is especially true when pharmaceutical products are made from ingredients of good microbial quality, when manufacturing environments do not foster microbial contamination, when there are processes that inherently reduce the microbial content, when the formulation of the drug product has antimicrobial activity, and when manufacturing sites have an established testing history of low bioburden associated with their products. [Table 2](#) contains suggested microbial limit testing strategies for typical pharmaceutical and over-the-counter (OTC) drug products based on water activity. Other considerations, as listed above, would be applied when setting up the microbial limits testing program for individual drug products because water activity measurements cannot solely be used to justify the elimination of microbial content testing for product release.

Similar arguments could be made for the microbial limits testing of pharmaceutical ingredients. However, this would require pharmaceutical manufacturers to have a comprehensive knowledge of the pharmaceutical ingredient manufacturer's manufacturing processes, quality programs, and testing record. This could be obtained through a supplier audit program.

Table 2. Microbial Limit Testing Strategy for Representative Pharmaceutical and OTC Drug Products Based on Water Activity

Products	Water Activity ( $a_W$ )	Greatest Potential Contaminants	Testing Recommended
Nasal inhalant	0.99	Gram-negative bacteria	TAMC,* TCYMC, absence of <i>S. aureus</i> and <i>P. aeruginosa</i>
Hair shampoo	0.99	Gram-negative bacteria	TAMC, TCYMC, absence of <i>S. aureus</i> and <i>P. aeruginosa</i>
Antacid	0.99	Gram-negative bacteria	TAMC, TCYMC, absence of <i>E. coli</i> and <i>Salmonella</i> spp.
Topical cream	0.97	Gram-positive bacteria	TAMC, TCYMC, absence of <i>S. aureus</i> and <i>P. aeruginosa</i>
Oral liquid	0.90	Gram-positive bacteria and fungi	TAMC and TCYMC
Oral suspension	0.87	Fungi	TAMC and TCYMC
Topical ointment	0.55	None	Reduced testing
Lip balm	0.36	None	Reduced testing
Vaginal and rectal suppositories	0.30	None	Reduced testing
Compressed tablets	0.36	None	Reduced testing
Liquid-filled capsule	0.30	None	Reduced testing

\* TAMC = Total aerobic microbial count; TCYMC = Total combined yeast and mold count.

note—The water activities cited in [Table 2](#) for the different dosage forms are representative, and companies are urged to test their individual products before developing a testing



Water activity,  $aW$ , is the ratio of vapor pressure of  $H_2O$  in product (P) to vapor pressure of pure  $H_2O$  ( $P_0$ ) at the same temperature. It is numerically equal to 1/100 of the relative humidity (RH) generated by the product in a closed system. RH can be calculated from direct measurements of partial vapor pressure or dew point or indirect measurement by sensors whose physical or electric characteristics are altered by the RH to which they are exposed.

The relationship between  $aW$  and equilibrium relative humidity (ERH) is represented by the following equations:

$$aW = P/P_0 \text{ and } ERH(\%) = aW \times 100$$

The  $aW$  measurement may be conducted using the dew point/chilled mirror method.<sup>2</sup> A polished, chilled mirror is used as the condensing surface. The cooling system is electronically linked to a photoelectric cell into which light is reflected from the condensing mirror. An air stream, in equilibrium with the test sample, is directed at the mirror which cools until condensation occurs on the mirror. The temperature at which this condensation begins is the dew point from which the ERH is determined. Sample preparation should be considered as it may affect the water activity level of the material tested. Commercially available instruments using the dew point/chilled mirror method or other technologies need to be evaluated for suitability, validated, and calibrated when used to make water activity determinations. These instruments are typically calibrated using saturated salt solutions at 25°, as listed in [Table 3](#).

Table 3. Standard Saturated Salt Solutions Used to Calibrate Water Activity Determination Instruments

Saturated Salt Solutions	ERH (%)	$aW$
Potassium sulfate ( $K_2SO_4$ )	97.3	0.973
Barium chloride ( $BaCl_2$ )	90.2	0.902
Sodium chloride ( $NaCl$ )	75.3	0.753
Magnesium nitrate ( $Mg(NO_3)_2$ )	52.9	0.529
Magnesium chloride ( $MgCl_2$ )	32.8	0.328

1 J. A. Troller, D. T. Bernard, and V. W. Scott. Measurement of Water Activity. In: Compendium of Methods for the Microbiological Examination of Foods. American Public Health Association, Washington, DC, 1984 pp.124–134.

2 AOAC International Official Method 978.18. In: Official Methods of Analysis of AOAC International, 17th edition, AOAC International, Gaithersburg, Maryland.

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Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Radhakrishna S Tirumalai, Ph.D.</a> Senior Scientist 1-301-816-8339	(MSA05) Microbiology and Sterility Assurance

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Pharmacopeial Forum: Volume No. 30(5) Page 1709

#### 1116 MICROBIOLOGICAL EVALUATION OF CLEAN ROOMS AND OTHER CONTROLLED ENVIRONMENTS

The purpose of this informational chapter is to review the various issues that relate to aseptic processing of bulk drug substances, dosage forms, and in certain cases, medical devices; and to the establishment, maintenance, and control of the microbiological quality of controlled environments.

This chapter includes discussions on (1) the classification of a clean room based on particulate count limits; (2) microbiological evaluation programs for controlled environments; (3) training of personnel; (4) critical factors in design and implementation of a microbiological evaluation program; (5) development of a sampling plan; (6) establishment of microbiological Alert and Action levels; (7) methodologies and instrumentation used for microbiological sampling; (8) media and diluents used; (9) identification of microbial isolates; (10) operational evaluation via media fills; and (11) a glossary of terms. Excluded from this chapter is a discussion of controlled environments for use by licensed pharmacies in the preparation of sterile products for home use, which is covered under [Pharmaceutical Compounding—Sterile Preparations](#) (797).

There are alternative methods to assess and control the microbiological status of controlled environments for aseptic processing. Numerical values included in this chapter are not intended to represent absolute values or specifications, but are informational. Given the variety of microbiological sampling equipment and methods, one cannot reasonably suggest that the attainment of these values guarantees the needed level of microbial control or that excursions beyond values in this chapter indicate a loss of control. The improper application of microbiological sampling and analysis may cause significant variability and the potential for inadvertent contamination. Sampling media and devices, and methods indicated in this chapter, are not specifications but only informational.

A large proportion of sterile products are manufactured by aseptic processing. Because aseptic processing relies on the exclusion of microorganisms from the process stream and the prevention of microorganisms from entering open containers during filling, product bioburden as well as microbial bioburden of the manufacturing environment are important factors relating to the level of sterility assurance of these products.

##### Establishment of Clean Room Classifications

The design and construction of clean rooms and controlled environments are covered in Federal Standard 209E. This standard of air cleanliness is defined by the absolute concentration of airborne particles. Methods used for the assignment of air classification of controlled environments and for monitoring of airborne particulates are included. This federal document only applies to airborne particulates within a controlled environment and is not intended to characterize the viable or nonviable nature of the particles.

The application of Federal Standard 209E to clean rooms and other controlled environments in the pharmaceutical industry has been used by manufacturers of clean rooms to provide a specification for building, commissioning, and maintaining these facilities. However, data available in the pharmaceutical industry provide no scientific agreement on a relationship between the number of nonviable particulates and the concentration of viable microorganisms.

The criticality of the number of nonviable particulates in the electronic industry makes the application of Federal Standard 209E a necessity, while the pharmaceutical industry has a greater concern for viable particulates (i.e., microorganisms) rather than total particulates as specified in Federal Standard 209E. A definite concern for counts of total particulates in injectable products exists in the pharmaceutical industry (see [Particulate Matter in Injections](#) (788)).

The rationale that the fewer particulates present in a clean room, the less likely it is that airborne microorganisms will be present is accepted and can provide pharmaceutical manufacturers and builders of clean rooms and other controlled environments with engineering standards in establishing a properly functioning facility.

Federal Standard 209E, as applied in the pharmaceutical industry is based on limits of all particles with sizes equal to or larger than 0.5  $\mu m$ . [Table 1](#) describes Airborne Particulate Cleanliness Classes in Federal Standard 209E as adapted to the pharmaceutical industry. The pharmaceutical industry deals with Class M3.5 and above. Class M1 and M3 relate to the electronic industry and are shown in [Table 1](#) for comparison purposes. It is generally accepted that if fewer particulates are present in an operational clean room or other controlled environment, the microbial count under operational conditions will be less, provided that there are no changes in airflow, temperature, and humidity. Clean rooms are maintained under a state of operational control on the basis of dynamic (operational) data.

Table 1. Airborne Particulate Cleanliness Classes\*

Class Name	Particles equal to and larger than 0.5 $\mu m$
U.S.	

SI	Customary	(m <sup>3</sup> )	(ft <sup>3</sup> )
M1	—	10.0	0.283
M1.5	1	35.3	1.00
M2	—	100	2.8
M2.5	10	353	10.0
M3	—	1,000	28.3
M3.5	100	3,530	100
M4	—	10,000	283
M4.5	1,000	35,300	1,000
M5	—	100,000	2,830
M5.5	10,000	353,000	10,000
M6	—	1,000,000	28,300
M6.5	100,000	3,530,000	100,000
M7	—	10,000,000	283,000

\* Adapted from U.S. Federal Standard 209E, September 11, 1992—"Airborne Particulate Cleanliness Classes in Clean Rooms and Clean Zones."

#### Importance of a Microbiological Evaluation Program for Controlled Environments

Monitoring of total particulate count in controlled environments, even with the use of electronic instrumentation on a continuous basis, does not provide information on the microbiological content of the environment. The basic limitation of particulate counters is that they measure particles of 0.5  $\mu\text{m}$  or larger. While airborne microorganisms are not free-floating or single cells, they frequently associate with particles of 10 to 20  $\mu\text{m}$ . Particulate counts as well as microbial counts within controlled environments vary with the sampling location and the activities being conducted during sampling. Monitoring the environment for nonviable particulates and microorganisms is an important control function because they both are important in achieving product compendial requirements for Particulate Matter and Sterility under [Injections](#) 1.

Microbial monitoring programs for controlled environments should assess the effectiveness of cleaning and sanitization practices by and of personnel that could have an impact on the bioburden of the controlled environment. Microbial monitoring, regardless of how sophisticated the system may be, will not and need not identify and quantitate all microbial contaminants present in these controlled environments. However, routine microbial monitoring should provide sufficient information to ascertain that the controlled environment is operating within an adequate state of control.

Environmental microbial monitoring and analysis of data by qualified personnel will permit the status of control to be maintained in clean rooms and other controlled environments. The environment should be sampled during normal operations to allow for the collection of meaningful data. Microbial sampling should occur when materials are in the area, processing activities are ongoing, and a full complement of operating personnel is on site.

Microbial monitoring of clean rooms and some other controlled environments, when appropriate, should include quantitation of the microbial content of room air, compressor air that enters the critical area, surfaces, equipment, sanitization containers, floors, walls, and personnel garments (e.g., gowns and gloves). The objective of the microbial monitoring program is to obtain representative estimates of bioburden of the environment. When data are compiled and analyzed, any trends should be evaluated by trained personnel. While it is important to review environmental results on the basis of recommended and specified frequency, it is also critical to review results over extended periods to determine whether trends are present. Trends can be visualized through the construction of statistical control charts that include alert and action levels. The microbial control of controlled environments can be assessed, in part, on the basis of these trend data. Periodic reports or summaries should be issued to alert the responsible manager.

When the specified microbial level of a controlled environment is exceeded, a documentation review and investigation should occur. There may be differences in the details of the investigation, depending on the type and processing of the product manufactured in the room. Investigation should include a review of area maintenance documentation; sanitization documentation; the inherent physical or operational parameters, such as changes in environmental temperature and relative humidity; and the training status of personnel involved. Following the investigation, actions taken may include reinforcement of training of personnel to emphasize the microbial control of the environment; additional sampling at increased frequency; additional sanitization; additional product testing; identification of the microbial contaminant and its possible source; and an evaluation of the need to reassess the current standard operating procedures and to revalidate them, if necessary.

Based on the review of the investigation and testing results, the significance of the microbial level being exceeded and the acceptability of the operations or products processed under that condition may be ascertained. Any investigation and the rationale for the course of action should be documented and included as part of the overall quality management system.

A controlled environment such as a clean zone or clean room is defined by certification according to a relevant clean room operational standard. Parameters that are evaluated include filter integrity, air velocity, air patterns, air changes, and pressure differentials. These parameters can affect the microbiological bioburden of the clean room operation. The design, construction, and operation of clean rooms varies greatly, making it difficult to generalize requirements for these parameters. An example of a method for conducting a particulate challenge test to the system by increasing the ambient particle concentration in the vicinity of critical work areas and equipment has been developed by Ljungquist and Reinmuller.<sup>1</sup> First, smoke generation allows the air movements to be visualized throughout a clean room or a controlled environment. The presence of vortices or turbulent zones can be visualized, and the airflow pattern may be fine-tuned to eliminate or minimize undesirable effects. Then, particulate matter is generated close to the critical zone and sterile field. This evaluation is done under simulated production conditions, but with equipment and personnel in place.

Proper testing and optimization of the physical characteristics of the clean room or controlled environment is essential prior to completion of the validation of the microbiological monitoring program. Assurance that the controlled environment is operating adequately and according to its engineering specifications will give a higher assurance that the bioburden of the environment will be appropriate for aseptic processing. These tests should be repeated during routine certification of the clean room or controlled environment and whenever changes made to the operation, such as personnel flow, processing, operation, material flow, air-handling systems, or equipment layout, are determined to be significant.

#### Training of Personnel

Aseptically processed products require manufacturers to pay close attention to detail and to maintain rigorous discipline and strict supervision of personnel in order to maintain the level of environmental quality appropriate for the sterility assurance of the final product.

Training of all personnel working in controlled environments is critical. This training is equally important for personnel responsible for the microbial monitoring program, where contamination of the clean working area could inadvertently occur during microbial sampling. In highly automated operations, the monitoring personnel may be the employees who have the most direct contact with the critical zones within the processing area. Monitoring of personnel should be conducted before or after working in the processing area.

Microbiological sampling has the potential to contribute to microbial contamination due to inappropriate sampling techniques. A formal personnel training program is required to minimize this risk. This formal training should be documented for all personnel entering controlled environments.

Management of the facility must assure that all personnel involved in operations in clean rooms and controlled environments are well versed in relevant microbiological principles. The training should include instruction on the basic principles of aseptic processing and the relationship of manufacturing and handling procedures to potential sources of product contamination. This training should include instruction on the basic principles of microbiology, microbial physiology, disinfection and sanitation, media selection and preparation, taxonomy, and sterilization as required by the nature of personnel involvement in aseptic processing. Personnel involved in microbial identification will require specialized training on required laboratory methods. Additional training on the management of the environmental data collected must be provided to personnel. Knowledge and understanding of applicable standard operating procedures is critical, especially those standard operating procedures relating to corrective measures that are taken when environmental conditions so dictate.



Understanding of regulatory compliance policies and each individual's responsibilities with respect to good manufacturing practices (GMPs) should be an integral part of the training program as well as training in conducting investigations and in analyzing data.

The major source of microbial contamination of controlled environments is the personnel. Contamination can occur from the spreading of microorganisms by individuals, particularly those with active infections. Only healthy individuals should be permitted access to controlled environments.

These facts underscore the importance of good personal hygiene and a careful attention to detail in the aseptic gowning procedure used by personnel entering the controlled environment. Once these employees are properly gowned—including complete facial coverage—they must be careful to maintain the integrity of their gloves and suits at all times. Since the major threat of contamination of product being aseptically processed comes from the operating personnel, the control of microbial contamination associated with these personnel is one of the most important elements of the environmental control program.

The importance of thorough training of personnel working in controlled environments, including aseptic techniques, cannot be overemphasized. The environmental monitoring program, by itself, will not be able to detect all events in aseptic processing that could compromise the microbiological quality of the environment. Therefore, periodic media-fill or process simulation studies to revalidate the process are necessary to assure that the appropriate operating controls and training are effectively maintained.

#### Critical Factors Involved in the Design and Implementation of a Microbiological Environmental Control Program

An environmental control program should be capable of detecting an adverse drift in microbiological conditions in a timely manner that would allow for meaningful and effective corrective actions. It is the responsibility of the manufacturer to develop, initiate, implement, and document such a microbial environmental monitoring program.

Although general recommendations for an environmental control program will be discussed, it is imperative that such a program be tailored to specific facilities and conditions. A general microbiological growth medium such as Soybean Casein Digest Medium should be suitable in most cases. This medium may be supplemented with additives to overcome or to minimize the effects of sanitizing agents, or of antibiotics if used or processed in these environments. The detection and quantitation of yeasts and molds should be considered.

General mycological media, such as Sabouraud's, Modified Sabouraud's, or Inhibitory Mold Agar are acceptable. Other media that have been validated for promoting the growth of fungi, such as Soybean-Casein Digest Agar, can be used. In general, testing for obligatory anaerobes is not performed routinely. However, should conditions or investigations warrant, such as the identification of these organisms in sterility testing facilities, more frequent testing is indicated. The ability of the selected media to detect and quantitate these anaerobes or microaerophilic microorganisms should be evaluated.

The selection of time and incubation temperatures is made once the appropriate media have been selected. Typically, incubation temperatures in the  $22.5 \pm 2.5^{\circ}\text{C}$  and  $32.5 \pm 2.5^{\circ}\text{C}$  ranges have been used with an incubation time of 72 and 48 hours, respectively. Sterilization processes used to prepare growth media for the environmental program should be

validated and, in addition, media should be examined for sterility and for growth promotion as indicated under [Sterility Tests](#) (71). In addition, for the Growth Promotion test, representative microflora isolated from the controlled environment or ATCC strain preparations of these isolates may also be used to test media. Media must be able to support growth when inoculated with less than 100 colony-forming units (cfu) of the challenge organisms.

An appropriate environmental control program should include identification and evaluation of sampling sites and validation of methods for microbiological sampling of the environment.

The methods used for identification of isolates should be verified using indicator microorganisms (see [Microbial Enumeration Tests](#) (61) and [Tests for Specified Microorganisms](#) (62)).

#### Establishment of Sampling Plan and Sites

During initial start-up or commissioning of a clean room or other controlled environment, specific locations for air and surface sampling should be determined. Consideration should be given to the proximity to the product and whether air and surfaces might be in contact with a product or sensitive surfaces of container-closure systems. Such areas should be considered critical areas requiring more monitoring than non-product-contact areas. In a parenteral vial filling operation, areas of operation would typically include the container-closure supply, paths of opened containers, and other inanimate objects (e.g., fomites) that personnel routinely handle.

The frequency of sampling will depend on the criticality of specified sites and the subsequent treatment received by the product after it has been aseptically processed. [Table 2](#) shows suggested frequencies of sampling in decreasing order of frequency of sampling and in relation to the criticality of the area of the controlled environment being sampled.

Table 2. Suggested Frequency of Sampling on the Basis of Criticality of Controlled Environment

Sampling Area	Frequency of Sampling
Class 100 or better room designations	Each operating shift
Supporting areas immediately adjacent to Class 100 (e.g., Class 10,000)	Each operating shift
Other support areas (Class 100,000)	Twice/week
Potential product/container contact areas	Twice/week
Other support areas to aseptic processing areas but non-product contact (Class 100,000 or lower)	Once/week

As manual interventions during operation increase, and as the potential for personnel contact with the product increases, the relative importance of an environmental monitoring program increases. Environmental monitoring is more critical for products that are aseptically processed than for products that are processed and then terminally sterilized. The determination and quantitation of microorganisms resistant to the subsequent sterilization treatment is more critical than the microbiological environmental monitoring of the surrounding manufacturing environments. If the terminal sterilization cycle is not based on the overkill cycle concept but on the bioburden prior to sterilization, the value of the bioburden program is critical.

The sampling plans should be dynamic with monitoring frequencies and sample plan locations adjusted based on trending performance. It is appropriate to increase or decrease sampling based on this performance.

#### Establishment of Microbiological Alert and Action Levels in Controlled Environments

The principles and concepts of statistical process control are useful in establishing Alert and Action levels and in reacting to trends.

An Alert level in microbiological environmental monitoring is that level of microorganisms that shows a potential drift from normal operating conditions. Exceeding the Alert level is not necessarily grounds for definitive corrective action, but it should at least prompt a documented follow-up investigation that could include sampling plan modifications.

An Action level in microbiological environmental monitoring is that level of microorganisms that when exceeded requires immediate follow-up and, if necessary, corrective action.

Alert levels are usually based upon historical information gained from the routine operation of the process in a specific controlled environment.

In a new facility, these levels are generally based on prior experience from similar facilities and processes; and at least several weeks of data on microbial environmental levels should be evaluated to establish a baseline.

These levels are usually re-examined for appropriateness at an established frequency. When the historical data demonstrate improved conditions, these levels can be re-examined and changed to reflect the conditions. Trends that show a deterioration of the environmental quality require attention in determining the assignable cause and in instituting a corrective action plan to bring the conditions back to the expected ranges. However, an investigation should be implemented and an evaluation of the potential impact this has on a product should be made.



## Microbial Considerations and Action Levels for Controlled Environments

Classification of clean rooms and other controlled environments is based on Federal Standard 209E based on total particulate counts for these environments. The pharmaceutical and medical devices industries have generally adopted the classification of Class 100, Class 10,000, and Class 100,000, especially in terms of construction specifications for the facilities.

Although there is no direct relationship established between the 209E controlled environment classes and microbial levels, the pharmaceutical industry has been using microbial levels corresponding to these classes for a number of years; and these levels have been those used for evaluation of current GMP compliance.<sup>2</sup> These levels have been shown to be readily achievable with the current technology for controlled environments. There have been reports and concerns about differences in these values obtained using different sampling

systems, media variability, and incubation temperatures. It should be recognized that, although no system is absolute, it can help in detecting changes, and thus trends, in environmental quality. The values shown in [Tables 3, 4, and 5](#) represent individual test results and are suggested only as guides. Each manufacturer's data must be evaluated as part of an overall monitoring program.

Table 3. Air Cleanliness Guidelines in Colony-Forming Units (cfu) in Controlled Environments (Using a Slit-to-Agar Sampler or Equivalent)

Class <sup>*</sup>		cfu per cubic meter of air <sup>**</sup>	cfu per cubic feet of air
SI	U.S. Customary		
M3.5	100	Less than 3	Less than 0.1
M5.5	10,000	Less than 20	Less than 0.5
M6.5	100,000	Less than 100	Less than 2.5

\* As defined in Federal Standard 209E, September 1992.

\*\* A sufficient volume of air should be sampled to detect excursions above the limits specified.

Table 4. Surface Cleanliness Guidelines of Equipment and Facilities in cfu in Controlled Environments

Class		cfu per Contact Plate <sup>*</sup>	
SI	U.S. Customary		
M3.5	100	3 (including floor)	
M5.5	10,000	5	
		10 (floor)	

\* Contact plate areas vary from 24 to 30 cm<sup>2</sup>. When swabbing is used in sampling, the area covered should be greater than or equal to 24 cm<sup>2</sup> but no larger than 30 cm<sup>2</sup>.

Table 5. Surface Cleanliness Guidelines in Controlled Environments of Operating Personnel Gear in cfu

Class		cfu per Contact Plate <sup>*</sup>	
SI	U.S. Customary	Gloves	Personnel Clothing & Garb
M3.5	100	3	5
M5.5	10,000	10	20

\* See in [Table 4](#) under (\*).

## Methodology and Instrumentation for Quantitation of Viable Airborne Microorganisms

It is generally accepted by scientists that airborne microorganisms in controlled environments can influence the microbiological quality of the intermediate or final products manufactured in these areas. Also, it generally is accepted that estimation of the airborne microorganisms can be affected by instruments and procedures used to perform these assays. Therefore, where alternative methods or equipment is used, the general equivalence of the results obtained should be ascertained. Advances in technology in the future are expected to bring innovations that would offer greater precision and sensitivity than the current available methodology and may justify a change in the absolute numbers of organisms that are detected.

Today, the most commonly used samplers in the U.S. pharmaceutical and medical device industry are the impaction and centrifugal samplers. A number of commercially available samplers are listed for informational purposes. The selection, appropriateness, and adequacy of using any particular sampler is the responsibility of the user.

**Slit-to-Agar Air Sampler (STA)**— This sampler is the instrument upon which the microbial guidelines given in [Table 3](#) for the various controlled environments are based. The unit is powered by an attached source of controllable vacuum. The air intake is obtained through a standardized slit below which is placed a slowly revolving Petri dish containing a nutrient agar. Particles in the air that have sufficient mass impact on the agar surface and viable organisms are allowed to grow out. A remote air intake is often used to minimize disturbance of the laminar flow field.

**Sieve Impactor**— The apparatus consists of a container designed to accommodate a Petri dish containing a nutrient agar. The cover of the unit is perforated, with the perforations of a predetermined size. A vacuum pump draws a known volume of air through the cover, and the particles in the air containing microorganisms impact on the agar medium in the Petri dish. Some samplers are available with a cascaded series of containers containing perforations of decreasing size. These units allow for the determination of the distribution of the size ranges of particulates containing viable microorganisms, based on which size perforations admit the particles onto the agar plates.

**Centrifugal Sampler**— The unit consists of a propeller or turbine that pulls a known volume of air into the unit and then propels the air outward to impact on a tangentially placed nutrient agar strip set on a flexible plastic base.

**Sterilizable Microbiological Atrium**— The unit is a variant of the single-stage sieve impactor. The unit's cover contains uniformly spaced orifices approximately 0.25 inch in size. The base of the unit accommodates one Petri dish containing a nutrient agar. A vacuum pump controls the movement of air through the unit, and a multiple-unit control center as well as a remote sampling probe are available.

**Surface Air System Sampler**— This integrated unit consists of an entry section that accommodates an agar contact plate. Immediately behind the contact plate is a motor and turbine that pulls air through the unit's perforated cover over the agar contact plate and beyond the motor, where it is exhausted. Multiple mounted assemblies are also available.

**Gelatin Filter Sampler**— The unit consists of a vacuum pump with an extension hose terminating in a filter holder that can be located remotely in the critical space. The filter consists of random fibers of gelatin capable of retaining airborne microorganisms. After a specified exposure time, the filter is aseptically removed and dissolved in an appropriate diluent and then plated on an appropriate agar medium to estimate its microbial content.

**Settling Plates**— This method is still widely used as a simple and inexpensive way to qualitatively assess the environments over prolonged exposure times. The exposure of open agar-filled Petri dishes, or settling plates, is not to be used for quantitative estimations of the microbial contamination levels of critical environments.

One of the major limitations of mechanical air samplers is the limitation in sample size of air being sampled. Where the microbial level in the air of a controlled environment is expected to contain not more than three cfu per cubic meter, several cubic meters of air should be tested if results are to be assigned a reasonable level of precision and accuracy. Often this is not practical. To show that microbial counts present in the environment are not increasing over time, it might be necessary to extend the time of sampling to determine if the time of sampling is a limiting factor or not. Typically, slit-to-agar samplers have an 80-liter-per-minute sampling capacity (the capacity of the surface air system is somewhat higher). If one cubic meter of air is tested, then it would require an exposure time of 15 minutes. It may be necessary to use sampling times in excess of 15 minutes to obtain a representative environmental sample. Although there are samplers reported to be capable of very high sampling volume rates, consideration in these situations should be given to the potential for disruption of the airflow patterns in any critical area or to the creation of a turbulence that could increase the probability of contamination.



For centrifugal air samplers, a number of earlier studies showed that the samples demonstrated a selectivity for larger particles. The use of this type of sampler may have resulted in higher airborne counts than the other types of air samplers because of that inherent selectivity.

When selecting a centrifugal sampler, the effect of the sampler on the linearity of the airflow in the controlled zone where it is placed for sampling should be taken into consideration. Regardless of the type of sampler used, the use of a remote probe requires determining that the extra tubing does not have an adverse effect on the viable airborne count. This effect should either be eliminated or, if this is not possible, a correction factor should be introduced in the reporting of results.

#### Methodology and Equipment for Sampling of Surfaces for Quantitation of Viable Microbial Contaminants in Controlled Environments

Another component of the microbial environmental control program in controlled environments is surface sampling of equipment, facilities, and personnel gear used in these environments. The standardization of surface sampling methods and procedures has not been as widely addressed in the pharmaceutical industry as the standardization of air sampling procedures.<sup>3</sup> To minimize disruptions to critical operations, surface sampling is performed at the conclusion of operations. Surface sampling may be accomplished by the use of contact plates or by the swabbing method. Surface monitoring is generally performed on areas that come in contact with the product and on areas adjacent to those contact areas.

Contact plates filled with nutrient agar are used when sampling regular or flat surfaces and are directly incubated at the appropriate time for a given incubation temperature for quantitation of viable counts. Specialized agar can be used for specific quantitation of fungi, spores, etc.

The swabbing method may be used for sampling of irregular surfaces, especially for equipment. Swabbing is used to supplement contact plates for regular surfaces. The swab is then placed in an appropriate diluent and the estimate of microbial count is done by plating of an appropriate aliquot on or in specified nutrient agar. The area to be swabbed is defined using a sterile template of appropriate size. In general, it is in the range of 24 to 30 cm<sup>2</sup>. The microbial estimates are reported per contact plate or per swab.

#### Culture Media and Diluents Used for Sampling or Quantitation of Microorganisms

The type of medium, liquid or solid, that is used for sampling or quantitation of microorganisms in controlled environments will depend on the procedure and equipment used. A commonly used all-purpose medium is Soybean-Casein Digest Agar when a solid medium is needed. Other media, liquid or solid, are listed below.

Liquid Media <sup>a</sup>	Solid Media <sup>a</sup>
Tryptone saline	Soybean-casein digest agar
Peptone water	Nutrient agar
Buffered saline	Tryptone glucose extract agar
Buffered gelatin	Lecithin agar
Enriched buffered gelatin	Brain heart infusion agar
Brain heart infusion	Contact plate agar
Soybean-casein medium	

<sup>a</sup> Liquid and solid media are sterilized using a validated process.

These media are commercially available in dehydrated form. They are also available in ready-to-use form. When disinfectants or antibiotics are used in the controlled area, consideration should be given to using media with appropriate inactivating agents.

Alternative media to those listed can be used provided that they are validated for the purpose intended.

#### Identification of Microbial Isolates from the Environmental Control Program

The environmental control program includes an appropriate level of identification of the flora obtained from sampling. A knowledge of the normal flora in controlled environments aids in determining the usual microbial flora anticipated for the facility being monitored; evaluating the effectiveness of the cleaning and sanitization procedures, methods, and agents; and recovery methods. The information gathered by an identification program can also be useful in the investigation of the source of contamination, especially when the Action levels are exceeded.

Identification of isolates from critical areas and areas immediate to these critical areas should take precedence over identification of microorganisms from noncritical areas. Identification methods should be verified, and ready-to-use kits should be qualified for their intended purpose (see Critical Factors Involved in the Design and Implementation of Environmental Control Program).

#### Operational Evaluation of the Microbiological Status of Aseptically Filled Products in Clean Rooms and Other Controlled Environments

The controlled environment is monitored through an appropriate environmental monitoring program. To assure that minimal bioburden is achieved, additional information on the evaluation of the microbiological status of the controlled environment can be obtained by the use of media fills. An acceptable media fill shows that a successful simulated product run can be conducted on the manufacturing line at that point in time. However, other factors are important, such as appropriate construction of facilities, environmental monitoring and training of personnel.

When an aseptic process is developed and installed, it is generally necessary to qualify the microbiological status of the process by running at least three successful consecutive media fills. A media fill utilizes growth medium in lieu of products to detect the growth of microorganisms. Issues in the development of a media fill program that should be considered are the following: media-fill procedures, media selection, fill volume, incubation, time and temperature, inspection of filled units, documentation, interpretation of results, and possible corrective actions required.

Since a media fill is designed to simulate aseptic processing of a specified product, it is important that conditions during a normal product run are in effect during the media fill. This includes the full complement of personnel and all the processing steps and materials that constitute a normal production run. During the conduct of media fill, various predocumented interventions that are known to occur during actual product runs should be planned (e.g., changing filling needles, fixing component jams).

Alternatively, in order to add a safety margin, a combination of possible conditions can be used. Examples may include frequent start and stop sequences, unexpected repair of processing system, replacement of filters, etc. The qualification of an aseptic process need not be done for every product, but should be done for each processing line. Since the geometry of the container (size as well as opening of the container) and the speed of the line are factors that are variable in the use of an aseptic processing line, appropriate combination of these factors, preferably at the extremes, should be used in the qualification of the line. A rationale for products used should be documented.

The 1987 FDA Guideline on Sterile Drug Products Produced by Aseptic Processing indicates that media-fill runs be done to cover all production shifts for line/product/container combinations. This guideline should be considered not only for qualification media-fill runs, but also for periodic reevaluation or revalidation. Media fill programs should also simulate production practices over extended runs. This can be accomplished by doing media-fill runs at the end of production runs.

In general, an all-purpose, rich medium such as Soybean Casein Broth that has been checked for growth promotion with a battery of indicator organisms (see [Sterility Tests](#) (71)) at a level of below 100 cfu/unit, can be used. Isolates from the controlled environment where aseptic processing is to be conducted may also be used. Following the aseptic processing of the medium, the filled containers are incubated at  $22.5 \pm 2.5^{\circ}\text{C}$  or at  $32.5 \pm 2.5^{\circ}\text{C}$ . All media filled containers should be incubated for a minimum of 14 days. If two temperatures are used for incubation of media filled samples, then these filled containers should be incubated for at least 7 days at each temperature. Following incubation, the medium-filled containers should be inspected for growth. Media filled isolates are identified by genus and, when possible, by species in order to investigate the sources of contamination.

Critical issues in performing media fills are the number of fills to qualify an aseptic process, the number of units filled per media fill, the interpretation of results, and implementation of



Corrective actions. Historically, three media-fill runs during initial qualification or start-up of a facility are conducted to demonstrate consistency of the aseptic processing line. The minimum number of units to demonstrate a contamination rate of not more than 0.1%, which is the criterion for acceptance of a successful media-fill run, is at least 3,000. It should be emphasized that many firms in the United States and other countries are filling more than 3,000 units in a single media-fill run.<sup>4</sup> Pilot plant facilities used for preparing small clinical lots may use smaller media fills.

A number of international documents (i.e., ISO and EU-GMP) have also cited an expectation of zero positives out of 3,000 media filled units at the 95% confidence level. However, it is recognized that repeated media runs are required in order to confirm the statistical validity of the observed contamination rate for the process.

PDA Technical Monograph Number 17,<sup>4</sup> "A Survey of Current Sterile Manufacturing Practices," indicated that many manufacturers believe that their aseptic processes are capable of contamination rates below 0.1%.

Since the most critical source of contamination in the clean room is the personnel, visual documentation that can be helpful in correlating production activities to contamination events during media fills is encouraged. The widespread use of isolator systems for sterility testing has demonstrated that elimination of personnel does reduce contamination in aseptic handling.

#### An Overview of the Emerging Technologies for Advanced Aseptic Processing

Because of the strong correlation between human involvement and intervention and the potential for product contamination in aseptic processing, production systems in which personnel are removed from critical zones have been designed and implemented. Methods developed to reduce the likelihood of contamination include equipment automation, barriers, and isolator systems. Facilities that employ these advanced aseptic processing strategies are already in operation. In facilities where personnel have been completely excluded from the critical zone, the necessity for room classification based on particulate and environmental microbiological monitoring requirements may be significantly reduced.

The following are definitions of some of the systems currently in place to reduce the contamination rate in aseptic processing:

**Barriers**—In the context of aseptic processing systems, a barrier is a device that restricts contact between operators and the aseptic field enclosed within the barrier. These systems are used in hospital pharmacies, laboratories, and animal care facilities, as well as in aseptic filling. Barriers may not be sterilized and do not always have transfer systems that allow passage of materials into or out of the system without exposure to the surrounding environment. Barriers range from plastic curtains around the critical production zones to rigid enclosures found on modern aseptic-filling equipment. Barriers may also incorporate such elements as glove ports, half-suits, and rapid-transfer ports.

**Blow/Fill/Seal**—This type of system combines the blow-molding of container with the filling of product and a sealing operation in one piece of equipment. From a microbiological point of view, the sequence of forming the container, filling with sterile product, and formation and application of the seal are achieved aseptically in an uninterrupted operation with minimal exposure to the environment. These systems have been in existence for about 30 years and have demonstrated the capability of achieving contamination rates below 0.1%.

Contamination rates of 0.001% have been cited for blow/fill/seal systems when combined media-fill data are summarized and analyzed.

**Isolator**—This technology is used for a dual purpose. One is to protect the product from contamination from the environment, including personnel, during filling and closing, and the other is to protect personnel from deleterious or toxic products that are being manufactured.

Isolator technology is based on the principle of placing previously sterilized components (containers/products/closures) into a sterile environment. These components remain sterile during the whole processing operation, since no personnel or nonsterile components are brought into the isolator. The isolator barrier is an absolute barrier that does not allow for interchanges between the protected and unprotected environments. Isolators either may be physically sealed against the entry of external contamination or may be effectively sealed by the application of continuous overpressure. Manipulations of materials by personnel are done via use of gloves, half-suits, or full suits. All air entering the isolator passes through either an HEPA or UPLA filter, and exhaust air typically exits through an HEPA-grade filter. Peracetic acid and hydrogen peroxide vapor are commonly used for the surface sterilization of the isolator unit's internal environment. The sterilization of the interior of isolators and all contents are usually validated to a sterility assurance level of 10<sup>-6</sup>.

Equipment, components, and materials are introduced into the isolator through a number of different procedures: use of a double-door autoclave; continuous introduction of components via a conveyor belt passing through a sterilizing tunnel; use of a transfer container system through a docking system in the isolator enclosure. It is also necessary to monitor closely an isolator unit's integrity, calibration, and maintenance.

The requirements for controlled environments surrounding these newer technologies for aseptic processing depend on the type of technology used.

**Blow/Fill/Seal** equipment that restricts employee contact with the product may be placed in a controlled environment, especially if some form of employee intervention is possible during production.

Barrier systems will require some form of controlled environment. Because of the numerous barrier system types and applications, the requirements for the environment surrounding the barrier system will vary. The design and operating strategies for the environment around these systems will have to be developed by the manufacturers in a logical and rational fashion. Regardless of these strategies, the capability of the system to produce sterile products must be validated to operate in accordance with pre-established criteria.

In isolators, the air enters the isolator through integral filters of HEPA quality or better, and their interiors are sterilized typically to a sterility assurance level of 10<sup>-6</sup>; therefore, isolators contain sterile air, do not exchange air with the surrounding environment, and are free of human operators. However, it has been suggested that when the isolator is in a controlled environment, the potential for contaminated product is reduced in the event of a pinhole leak in the suit or glove.

The extent and scope of an environmental microbiological monitoring of these advanced systems for aseptic processing depends on the type of system used. Manufacturers should balance the frequency of environmental sampling systems that require human intervention with the benefit accrued by the results of that monitoring. Since barrier systems are designed to reduce human intervention to a minimum, remote sampling systems should be used in lieu of personnel intervention. In general, once the validation establishes the effectiveness of the barrier system, the frequency of sampling to monitor the microbiological status of the aseptic processing area could be reduced, as compared to the frequency of sampling of classical aseptic processing systems.

Isolator systems require relatively infrequent microbiological monitoring. Continuous total particulate monitoring can provide assurance that the air filtration system within the isolator is working properly. The methods for quantitative microbiological air sampling described in this chapter may not have sufficient sensitivity to test the environment inside an isolator. Experience with isolators indicates that under normal operations pinhole leaks or tears in gloves represent the major potential for microbiological contamination; therefore, frequent testing of the gloves for integrity and surface monitoring of the gloves is essential. Surface monitoring within the isolator may also be beneficial on an infrequent basis.

#### GLOSSARY

**Airborne Particulate Count** (also referred to as Total Particulate Count)—Particles detected are 0.5  $\mu\text{m}$  and larger. When a number of particles is specified, it is the maximum allowable number of particles per cubic meter of air (or per cubic foot of air).

**Airborne Viable Particulate Count** (also referred to as Total Airborne Aerobic Microbial Count)—When a number of microorganisms is specified, it is the maximum number of colony-forming units (cfu) per cubic meter of air (or per cubic foot of air) that is associated with a Cleanliness Class of controlled environment based on the Airborne Particulate Count.

**Aseptic Processing**—A mode of processing pharmaceutical and medical products that involves the separate sterilization of the product and of the package (containers/closures or packaging material for medical devices) and the transfer of the product into the container and its closure under microbiologic critically controlled conditions.

**Air Sampler**—Devices or equipment used to sample a measured amount of air in a specified time to quantitate the particulate or microbiological status of air in the controlled environment.

**Air Changes**—The frequency per unit of time (minutes, hours, etc.) that the air within a controlled environment is replaced. The air can be recirculated partially or totally replaced.

**Action Levels**—Microbiological levels in the controlled environment, specified in the standard operating procedures, which when exceeded should trigger an investigation and a corrective action based on the investigation.



Alert Levels—Microbial levels, specified in the standard operating procedures, which when exceeded should result in an investigation to ensure that the process is still within control. Alert levels are specific for a given facility and are established on the basis of a baseline developed under an environmental monitoring program. These Alert levels can be modified depending on the trend analysis done in the monitoring program. Alert levels are always lower than Action levels.

Bioburden—Total number of microorganisms detected in or on an article.

Clean Room—A room in which the concentration of airborne particles is controlled to meet a specified airborne particulate Cleanliness Class. In addition, the concentration of microorganisms in the environment is monitored; each Cleanliness Class defined is also assigned a microbial level for air, surface, and personnel gear.

Clean Zone—A defined space in which the concentration of airborne particles and microorganisms are controlled to meet specific Cleanliness Class levels.

Controlled Environment—Any area in an aseptic process system for which airborne particulate and microorganism levels are controlled to specific levels, appropriate to the activities conducted within that environment.

Commissioning of a Controlled Environment—Certification by engineering and quality control that the environment has been built according to the specifications of the desired cleanliness class and that, under conditions likely to be encountered under normal operating conditions (or worst-case conditions), it is capable of delivering an aseptic process. Commissioning includes media-fill runs and results of the environmental monitoring program.

Corrective Action—Actions to be performed that are in standard operating procedures and that are triggered when certain conditions are exceeded.

Environmental Isolates—Microorganisms that have been isolated from the environmental monitoring program.

Environmental Monitoring Program—Documented program, implemented through standard operating procedures, that describes in detail the procedures and methods used for monitoring particulates as well as microorganisms in controlled environments (air, surface, personnel gear). The program includes sampling sites, frequency of sampling, and investigative and corrective actions that should be followed if Alert or Action levels are exceeded. The methodology used for trend analysis is also described.

Equipment Layout—Graphical representation of an aseptic processing system that denotes the relationship between and among equipment and personnel. This layout is used in the Risk Assessment Analysis to determine sampling site and frequency of sampling based on potential for microbiological contamination of the product/container/closure system. Changes must be assessed by responsible managers, since unauthorized changes in the layout for equipment or personnel stations could result in increase in the potential for contamination of the product/container/closure system.

Federal Standard 209E—"Airborne Particulate Cleanliness Classes in Clean Rooms and Clean Zones" is a standard approved by the Commissioner, Federal Supply Services, General Service Administration, for the use of "All Federal Agencies." The Standard establishes classes of air cleanliness based on specified concentration of airborne particulates. These classes of air cleanliness have been developed, in general, for the electronic industry "super-clean" controlled environments. In the pharmaceutical industry, the Federal Standard 209E is used to specify the construction of controlled environment. Class 100, Class 10,000, and Class 100,000 are generally represented in an aseptic processing system. If the classification system is applied on the basis of particles equal to or greater than 0.5  $\mu\text{m}$ , these classes are now represented in the SI system by Class M3.5, M5.5, and M6.5, respectively.

Filter Integrity—Testing that ensures that a filter functional performance is satisfactory [e.g., diethyl phthalate (DOP) and bubble point test].

Material Flow—The flow of material and personnel entering controlled environments should follow a specified and documented pathway that has been chosen to reduce or minimize the potential for microbial contamination of the product/closure/container systems. Deviation from the prescribed flow could result in increase in potential for microbial contamination.

Material/personnel flow can be changed, but the consequences of the changes from a microbiological point of view should be assessed by responsible managers and must be authorized and documented.

Media Growth Promotion—Procedure that references Growth Promotion under [Sterility Tests](#) 71 to demonstrate that media used in the microbiological environmental monitoring program, or in media-fill runs, are capable of supporting growth of indicator microorganisms and of environmental isolates from samples obtained through the monitoring program or their corresponding ATCC strains.

Media Fill—Microbiological simulation of an aseptic process by the use of growth media processed in a manner similar to the processing of the product and with the same container/closure system being used.

Out-of-Specification Event—Temporary or continuous event when one or more of the requirements included in standard operating procedures for controlled environments are not fulfilled.

Product Contact Areas—Areas and surfaces in a controlled environment that are in direct contact with either products, containers, or closures and the microbiological status of which can result in potential microbial contamination of the product/container/closure system. Once identified, these areas should be tested more frequently than non-product-contact areas or surfaces.

Risk Assessment Analysis—Analysis of the identification of contamination potentials in controlled environments that establish priorities in terms of severity and frequency and that will develop methods and procedures that will eliminate, reduce, minimize, or mitigate their potential for microbial contamination of the product/container/closure system.

Sampling Plan—A documented plan that describes the procedures and methods for sampling a controlled environment; identifies the sampling sites, the sampling frequency, and number of samples; and describes the method of analysis and how to interpret the results.

Sampling Sites—Documented geographical location, within a controlled environment, where sampling for microbiological evaluation is taken. In general, sampling sites are selected because of their potential for product/container/closure contacts.

Standard Operating Procedures—Written procedures describing operations, testing, sampling, interpretation of results, and corrective actions that relate to the operations that are taking place in a controlled environment and auxiliary environments. Deviations from standard operating procedures should be noted and approved by responsible managers.

Sterile Field—In aseptic processing or in other controlled environments, it is the space at the level of or above open product containers, closures, or product itself, where the potential for microbial contamination is highest.

Sterility—Within the strictest definition of sterility, an article is deemed sterile when there is complete absence of viable microorganisms. Absolute sterility cannot be practically demonstrated without testing every article in a batch. Sterility is defined in probabilistic terms, where the likelihood of a contaminated article is acceptably remote.

Swabs—Devices provided that are used to sample irregular as well as regular surfaces for determination of microbial status. The swab, generally composed of a stick with an absorbent extremity, is moistened before sampling and used to sample a specified unit area of a surface. The swab is then rinsed in sterile saline or other suitable menstruum and the contents plated on nutrient agar plates to obtain an estimate of the viable microbial load on that surface.

Trend Analysis—Data from a routine microbial environmental monitoring program that can be related to time, shift, facility, etc. This information is periodically evaluated to establish the status or pattern of that program to ascertain whether it is under adequate control. A trend analysis is used to facilitate decision-making for requalification of a controlled environment or for maintenance and sanitization schedules.

1 Interaction Between Air Movements and the Dispersion of Contaminants: Clean Zones with Unidirectional Air Flow, *Journal of Parenteral Science and Technology*, 47(2), 1993.

2 NASA, 1967—*Microbiology of Clean Rooms*.

3 The Sixteenth Edition of Standard Methods for the Examination of Dairy Products (the American Health Association) provides a section on surface sampling.

4 A Parenteral Drug Association Survey (Technical Monograph 17) showed that out of 27 respondents, 50% were filling more than 3,000 units per run.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.



Topic/Question	Contact	Expert Committee
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## 1117 MICROBIOLOGICAL BEST LABORATORY PRACTICES

### INTRODUCTION

Good laboratory practices in a microbiology laboratory consist of activities that depend on several principles: aseptic technique, control of media, control of test strains, control of equipment, diligent recording and evaluation of data, and training of the laboratory staff. Because of the known variability in microbiology data, reliability and reproducibility are dependent on the use of accepted methods and adherence to good laboratory practices.

### MEDIA PREPARATION AND QUALITY CONTROL

#### Media Preparation

Culture media are the basis for most microbiological tests. Safeguarding the quality of this media is therefore critical to the success of the microbiology laboratory. Media preparation, proper storage, and quality control testing can assure a consistent supply of high quality media.

It is important to choose the correct media or components in making media based on the use of accepted sources or references for formulas. The manufacturer's formula and instructions for preparation routinely accompany dehydrated media and ready-made media. Because different media types may have different preparation requirements (e.g., heating, additives, and pH adjustment), it is important to follow these instructions to ensure preparation of acceptable media quality. A certificate of analysis describing expiry dating and recommended storage conditions accompanies ready-made media, as well as the quality control organisms used in growth-promotion and selectivity testing of that media.

Water is the universal diluent for microbiological media. Purified Water is most often used for media preparation, but in certain cases the use of deionized or distilled water may be appropriate. The volume of the water used should be recorded.

Consistent preparation of media requires accurate weighing of dehydrated media or media constituents. A calibrated balance with the appropriate weight range for the ingredients should be used. Clean weighing containers and tools (such as spatulas) should be used to prevent foreign substances that may alter the composition of the finished media from entering the formulation. The weight of the components should be recorded.

Dehydrated media should be thoroughly dissolved in water prior to dispensing and sterilization. If heating is necessary to help dissolve media, care should be taken not to overheat media as all culture media, to a greater or lesser extent, are heat-sensitive. Equipment used in the preparation of media should be appropriate to allow for controlled heating, constant agitation, and mixing of the media. Darkening of media (Maillard-type reaction or nonenzymatic browning) is a general indication of overheating. When adding required supplements to media, adequate mixing of the medium after adding the supplement should be performed.

Preparation of media in poorly cleaned glassware can allow inhibitory substances to enter the media. Inhibitory substances can come from detergent residue after cleaning glassware or from prior materials used in the glassware. Be sure that the cleaning process removes debris and foreign matter, and that the detergent is thoroughly rinsed out with Purified Water.

See [Cleaning Glass Apparatus](#) (1051) for additional guidance.

Sterilization of media should be performed within the parameters provided by the manufacturer or validated by the user. Commercially prepared media should provide documentation of the sterilization method that was used. Ideally the manufacturer should provide the sterility assurance level (SAL) of the media against a recognized biological indicator. Autoclaving by moist heat is the preferred sterilization technique, except in instances when boiling is required in order to avoid deterioration of heat-labile components of the media. Sterilization by filtration may also be appropriate for some formulations.

The effects of the sterilization method and conditions on the media should be validated by sterility and growth-promotion testing of the media. In addition, if sterilized by moist heat, the autoclave cycle should be validated to ensure proper heat distribution for selected loads and volumes. Typically, manufacturers recommend using an autoclave cycle of 121° for 15 minutes using a validated autoclave. These conditions apply to time at temperature of the media. As the load configuration of the autoclave will influence the rate of heating, longer cycles may be required for larger loads. However, the sterilization time will be dependent on the media volume and autoclave load. Sterilization cycles in which the autoclave is slow to come up to temperature may result in overheating of the media. Therefore, care must be taken to validate a sterilization cycle to deliver the minimum SAL required, balancing the need for a sterile media against the tendency of the media to degrade under excessive heating. Storage of the media in the autoclave after the liquid cycle is completed is not recommended after cooling, as it may damage the media. Improper heating or sterilizing conditions—for commercially prepared or internally prepared media—may result in a difference in color change, loss of clarity, altered gel strength, or pH drift from the manufacturer's recommended range.

The pH of each batch of medium should be confirmed after it has cooled to room temperature (25°) by aseptically withdrawing a sample for testing. A flat pH probe is recommended for agar surfaces, and an immersion probe is recommended for liquids. The pH of media should be in a range of  $\pm 0.2$  of the value indicated by the manufacturer, unless a wider range is acceptable by the validated method.

Prepared media should be checked by appropriate inspection of plates and tubes for:

- Cracked containers or lids
  - Unequal filling of containers
  - Dehydration resulting in cracks or dimpled surfaces on solid medium
    - Hemolysis
    - Excessive darkening or color change
    - Crystal formation from possible freezing
      - Excessive number of bubbles
      - Microbial contamination
    - Status of redox indicators (if appropriate)
  - Lot number and expiry date checked and recorded
    - Sterility of the media
- Media Storage

It is prudent to consider how the manufacturer or supplier transports and stores media prior to distribution to the end user. Manufacturers of media should use transport and storage conditions that minimize the loss of moisture, control the temperature, and provide mechanical protection to the prepared media.

Media should be labeled properly with batch or lot numbers, preparation and expiration dates, and media identification. Media should be stored according to the manufacturer's instructions. Media prepared in-house should be stored under validated conditions. Do not store agar at or below 0°, as freezing could damage the gel structure. Protect stored media from exposure to light and excessive temperature. Before prolonged storage, agar plates should be placed into a sealed package or container to retard moisture loss.

Remelting of an original container of solid media should be performed only once to avoid media whose quality is compromised by overheating or potential contamination. It is recommended that remelting be performed in a heated water bath or by using free-flowing steam. The use of microwave ovens and heating plates is common, but care should be taken to avoid damaging media by overheating and to avoid the potential injury to laboratory personnel from glass breakage and burns. The molten agar medium should be held in a monitored water bath at a temperature of 45° to 50° for not more than 8 hours. Caution should be taken when pouring the media from a container immersed in a water bath to prevent



water from the bath commingling with the poured sterile media. Wiping the exterior of the container dry prior to pouring may be advisable.

Disposal of used cultured media (as well as expired media) should follow local biological hazard safety procedures.

#### Quality Control Testing

While growth media can be prepared in a laboratory from individual components, many laboratories, for their ease-of-use, use dehydrated media or purchase commercially prepared media in plates or glass containers. Manufacturers of media attempt to standardize raw materials from biological sources, but must constantly deal with unavoidable differences in raw materials obtained from natural sources, and therefore, lot-to-lot variability of media must be considered. The performance of media prepared in a laboratory or by a manufacturer is highly dependent on preparation. Improper media preparation can cause unsatisfactory conditions for microbial growth or recovery and unreliable results.

Quality control tests should be performed on all prepared media. Tests routinely performed on in-house prepared media are pH, growth promotion, and periodic stability checks to confirm the expiry dating.

When in-house prepared microbiological media are properly prepared and sterilized using a validated method, the growth-promotion testing may be limited to each incoming lot of dehydrated media, unless otherwise instructed by the relevant compendial method. If the media preparation was not validated, then every batch of media would be subjected to growth-promotion testing. Test organisms may be selected from the appropriate compendial test chapter, based on the manufacturer's recommendation for a particular medium, or may include representative environmental isolates.

Expiration dates on media should have supporting growth-promotion testing to indicate that the performance of the media still meets acceptance criteria up to and including the expiration date. The length of shelf life of a batch of media will depend on the stability of the ingredients and formulation under specified conditions, as well as the type of container and closure.

When a batch of media does not meet the requirements of growth-promotion testing, an investigation should be initiated to identify the cause. This investigation should include a corrective action plan to prevent the recurrence of the problem. Any nonconforming lot should not be used if an assignable cause or corrective resolution relative to nongrowth support is undetermined.

Some reagents are used for diagnostic purposes to help support identification of microbial organisms, e.g., Gram stain and oxidase test reagents. These may have attributes that can be quality control tested similar to microbiological media. Select the correct quality control standard microorganisms, following the manufacturer's instructions, and perform the testing prior to unknown sample diagnostic testing.

Special care should be taken with media that is used in environmental monitoring studies. Media used for environmental monitoring of critical areas should preferably be double-wrapped and terminally sterilized. If terminal sterilization is not performed, media should be subjected to pre-incubation and 100% inspection prior to use within a critical area. This will prevent extraneous contamination from being carried into controlled environments and will prevent false-positive results. A raised agar level for surface contact plates should be verified.

#### MAINTENANCE OF MICROBIOLOGICAL CULTURES

Biological specimens can be the most delicate standards to handle because their viability and characteristics are dependent on adequate handling and storage. Standardizing the handling and storage of cultures by the user laboratory should be done in a way that will minimize the opportunity for contamination or alteration of growth characteristics. The careful and consistent treatment of stock cultures is critically important to the consistency of microbiological test results. Cultures for use in compendial tests should be acquired from a national culture collection. They can be acquired frozen, freeze-dried, on slants, or in ready-to-use forms. Confirmation of the purity of the culture and the identity of the culture should be performed prior to its use in quality control testing. Ready-to-use cultures may require confirmation of purity, identity, and inoculum size. This confirmation of identity for commonly used laboratory strains should ideally be done at the level of genotypic analysis (i.e., DNA fingerprinting, 16S rRNA gene sequencing, or PCR analysis using suitably validated probes).

Preparation and resuscitation of cultures should follow the instructions of the supplier or a validated, established method. The "Seed-Lot" technique is recommended for storage of stock cultures.

The original sample from the national culture collection is resuscitated and grown in an appropriate medium. Aliquots of this stock culture (the first transfer or passage) are suspended in a cryoprotective medium, transferred to vials, and frozen at  $-30^{\circ}$  or below, until use. If stored at  $-70^{\circ}$ , or in lyophilized form, strains may be kept indefinitely. These frozen stocks should then be used to inoculate monthly or weekly working cultures. Once opened, do not refreeze unused cell suspensions after culturing a working suspension. The unused portion should be discarded to minimize the risk of loss of viability and contamination of the stock.

The number of transfers of working control cultures should be tracked to prevent excessive subculturing that increases the risk of phenotypic alteration. One passage is defined as the transfer of organisms from a viable culture to a fresh medium with growth of the microorganisms. Any form of subculturing is considered to be a transfer/passage.

#### MAINTENANCE OF LABORATORY EQUIPMENT

Most equipment (incubators, water baths, and autoclaves) is subject to standard validation practices of incoming qualification, operational qualification, and performance qualification. Additionally, periodic calibration (generally annually) is commonly required. New equipment, critical to the operation of the laboratory, should be qualified according to a protocol approved by the quality assurance unit (QAU).

Instruments (pH meters and spectrophotometers) used in a microbiology laboratory should be calibrated on a regular schedule and tested to verify performance on a routine basis. The frequency of calibration and performance verification will vary based on the type of instrument and the importance of that equipment to the generation of data in the laboratory.

#### LABORATORY LAYOUT AND OPERATIONS

Laboratory layout and design should carefully consider the requirements of good microbiological practices and laboratory safety. It is essential that cross-contamination of microbial cultures be minimized to the greatest extent possible, and it is also important that microbiological samples be handled in an environment that makes contamination highly unlikely.

In general, a laboratory should be divided into clean or aseptic areas and live culture areas. Areas in which environmental or sterile product samples are handled and incubated should be maintained completely free of live cultures, if possible. If complete separation of live and clean culture zones cannot be accomplished, then other barriers and aseptic practices should be employed to reduce the likelihood of accidental contamination. These barriers include protective clothing, sanitization and disinfection procedures, and biological safety cabinets designated for clean or aseptic operations only. Procedures for handling spills or mishaps with live cultures should be in place, and all relevant technical personnel should be trained regarding these methods.

Some samples will demonstrate microbial growth and require further laboratory analysis to identify the contaminants. When growth is detected, the sample should be taken from the clean section of the laboratory to the live culture section without undue delay. Subculturing, staining, microbial identification, or other investigational operations should be undertaken in the live culture section of the laboratory. If possible, any sample found to contain growing colonies should not be opened in the clean zone of the laboratory. Careful segregation of contaminated samples and materials will reduce false-positive results.

Staff engaged in sampling activities should not enter or work in the live culture handling section of a laboratory unless special precautions are taken, including wearing protective clothing and gloves, and careful sanitization of hands upon exiting. Ideally, staff assigned to sampling activities, particularly those in support of aseptic processing, should not work in the vicinity of live culture laboratory operations. Also, all microbiological samples should be taken using aseptic techniques, including those taken in support of nonsterile products. If possible, all microbiological samples should be taken under full aseptic conditions in specialized sampling areas.

It is important to consider that microbial contamination of samples, which leads to false-positive results, is always possible unless careful aseptic precautions are taken. Facilities should be designed so that raw material and excipient sampling can be done under controlled conditions, including proper gowning and sterilized sampling equipment. It may not always be possible to sample utility systems, such as water systems, under full aseptic conditions; however, it should be noted that when samples are not taken aseptically, their



reliability is inevitably compromised.

Environmental sampling methods should require minimal aseptic handling in loading and unloading sampling instruments. Whenever possible, sampling equipment should be loaded with its microbiological recovery media in the environment that is to be sampled.

All testing in laboratories used for critical testing procedures, such as sterility testing of final dosage forms, bulk product, seed cultures for biological production, or cell cultures used in biological production, should be performed under controlled conditions. Isolator technology is also appropriate for critical, sterile microbiological testing. Isolators have been shown to have lower levels of environmental contamination than manned clean rooms, and therefore, are generally less likely to produce false-positive results. Proper validation of isolators is critical both to ensure environmental integrity and to prevent the possibility of false-negative results as a result of chemical disinfection of materials brought into or used within isolators (see [Sterility Testing—Validation of Isolator Systems \(1208\)](#)).

#### TRAINING OF PERSONNEL

Each person engaged in all phases of pharmaceutical manufacture should have the education, training, and experience to do his or her job. The demands of microbiological testing require that the core educational background of the staff, supervisors, and managers be in microbiology or a closely related biological science. They should be assigned responsibilities in keeping with their level of skill and experience.

A coherent system of operating procedures is necessary to run the microbiology laboratory. These procedures serve two purposes in a training program. Firstly, these SOPs describe the methodology that the microbiologist will follow to obtain accurate and reproducible results, and so serve as the basis for training. Secondly, by tracking the procedures in which a particular microbiologist has demonstrated proficiency, the procedure number or title also serves to identify what training the microbiologist has received specific to his or her job function.

Training curricula should be established for each laboratory staff member specific for his or her job function. They should not independently conduct a microbial test until they are qualified to run the test. Training records should be current, documenting the microbiologist's training in the proper revision to the particular SOP.

Periodic performance assessment is a wise investment in data quality. This performance testing should provide evidence of competency in core activities of the microbiology laboratory such as hygiene, plating, aseptic technique, documentation, and others as suggested by the microbiologist's job function.

Microbiologists with supervisory or managerial responsibilities should have appropriate education and in-house training in supervisory skills, laboratory safety, scheduling, laboratory investigations, technical report writing, relevant SOPs, and other critical aspects of the company's processes as suggested in their role of directing a laboratory function.

#### DOCUMENTATION

Documentation should be sufficient to demonstrate that the testing was performed in a laboratory and by methods that were under control. This includes, but is not limited to, documentation of the following:

- Microbiologist training and verification of proficiency
- Equipment validation, calibration, and maintenance
- Equipment performance during test (e.g., 24-hour/7-day chart recorders)
- Media preparation, sterility checks, and growth-promotion and selectivity capabilities
  - Media inventory and control testing
- Critical components of test conducted as specified by a procedure
  - Data and calculations verified
- Reports reviewed by QAU or a qualified responsible manager
  - Investigation of data deviations (if needed)

#### MAINTENANCE OF LABORATORY RECORDS

Proper recording of data and studies is critical to the success of the microbiology laboratory. The over-riding principle is that the test should be performed as written in the SOP, the SOP should be written to reflect how the test is actually performed, and the laboratory notebook should provide a record of all critical details needed to confirm the integrity of the data. At a minimum, the laboratory write-up should include the following:

- Date
- Material tested
- Microbiologist's name
- Procedure number
- Document test results
- Deviations (if any)
- Documented parameters (equipment used, microbial stock cultures used, media lots used)
- Management/Second review signature

Every critical piece of equipment should be noted in the write-up, and all should be on a calibration schedule documented by SOP and maintenance records. Where appropriate, logbooks or forms should be available and supportive of the laboratory notebook records. Equipment temperatures (waterbaths, incubators, autoclaves) should be recorded and traceable.

The governing SOP and revision should be clearly noted in the write-up. Changes in the data should be crossed off with a single line and initialed. Original data should not be erased or covered over.

Test results should include the original plate counts, allowing a reviewer to recreate the calculations used to derive the final test results. Methods for data analysis should be detailed in cited SOPs.

All laboratory records should be archived and protected against catastrophic loss. A formal record retention and retrieval program should be in place.

#### INTERPRETATION OF ASSAY RESULTS

Analytical microbiological assay results can be difficult to interpret for several important reasons: (1) Microorganisms are both ubiquitous in nature and common environmental contaminants, particularly organisms associated with humans predominate in many types of microbiological analysis; (2) The analyst has the potential to introduce contaminating organisms during sample handling or processing in the laboratory; (3) Microorganisms may not be homogeneously distributed within a sample or an environment; and (4) Microbiological assays are subject to considerable variability of outcome. Therefore, minor differences from an expected outcome may not be significant.

Because of these characteristics of microbiological analysis, laboratory studies should be conducted with the utmost care to avoid exogenous contamination as previously discussed in this chapter. Equally important, results must be interpreted from a broad microbiological perspective considering not only the nature of the putative contaminant, but the likelihood of that organism(s) surviving in the pharmaceutical ingredient, excipient, or environment under test. In addition, the growth characteristics of the microorganism should be considered (especially in questions of the growth of filamentous fungi in liquid media).

When results are observed that do not conform to a compendial monograph or another established quantitative target, an investigation into the finding is required. There are generally two distinct reasons for the observation of microbial contamination that does not comply with a target or requirement: There may be either a laboratory error or laboratory environmental conditions that produced an invalid result, or the product contains a level of contamination or specific types of contaminants outside established levels or limits. In either



case, laboratory management and, in most cases, general management should be notified immediately.

A full and comprehensive evaluation of the situation surrounding the result should be undertaken. All microbiological conditions or factors that could bring about the observed condition should be fully considered, including the magnitude of the excursion compared to established limits or levels. It is critical to know if the finding is statistically significant in light of assay variability.

The laboratory environment, the protective conditions in place for sampling, historical findings concerning the material under test, and the nature of the material, particularly with regard to microbial survival or proliferation in contact with the material, should be considered in the investigation. In addition, interviews with the laboratory analyst(s) may provide information regarding the actual conduct of the assay that can be valuable in determining the reliability of the result and in determining an appropriate course of action. If laboratory operations are identified as the cause of the nonconforming test outcome, then a corrective action plan should be developed to address the problem(s). Following the approval and implementation of the corrective action plan, the situation should be carefully monitored and the adequacy of the corrective action determined.

If assay results are invalidated based upon the discovery of an attributable error, this action must be documented. Laboratories also should have approved procedures for confirmatory testing (retesting), and if necessary, resampling where specific regulatory or compendial guidance do not govern the conduct of an assay investigation.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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#### 1118 MONITORING DEVICES—TIME, TEMPERATURE, AND HUMIDITY

This chapter provides background on the science and technology of temperature and humidity monitoring. It describes the available technologies and their performance characteristics, and it provides recommendations for verification and validation of performance. The shelf life of a drug is a function of the temperature and humidity conditions under which it is stored and transported as well as the chemical and physical properties of the drug substance and preparation. For this reason, the ability to monitor those conditions is important in the shipping and storage of temperature- and humidity sensitive preparations. Historic geographic and seasonal trends may be used as a planning tool in selecting among the types of temperature and humidity monitoring devices. Meteorological forecasts are available for any pertinent location.

#### TEMPERATURE MEASUREMENT TECHNOLOGIES

The devices described in this section are those most commonly used to monitor temperature in the storage and distribution of drugs in North America. The measurement of temperature at extremes, such as close to absolute zero or above those reasonably expected to be experienced by drugs, is not addressed.

**Alcohol or Mercury Thermometers**— These devices are based on the change in volume of a liquid as a function of temperature. Mercury thermometers are typically used in the ranges from 0° to 50° with a precision of about 0.1°. [note—Some local regulations apply to mercury-based thermometers. Alcohol thermometers may have a precision as good as 0.01°, but they must be quite large to measure temperatures in ranges of more than a few degrees. Both types of thermometers may be designed to indicate the maximum and minimum temperatures measured. See [Thermometers \(21\)](#).]

**Chemical Device**— This is a device based on a phase change or chemical reaction that occurs as a function of temperature. Examples include liquid crystals, waxes, and lacquers that change phase, and thereby their appearance, as a function of temperature. Such materials represent the least expensive form of temperature measurement, but they may be difficult to interpret.

Other types of chemical sensors include systems in which a reaction rate or diffusion process is used to deduce a temperature equivalent integrated over time rather than the temperature at a specific moment in time such as a spike or critical threshold, for which a separate device may be preferred. Thus, chemical sensors provide a measure of accumulated heat rather than instantaneous temperature. It should be noted that these devices are generally irreversible; once a color change or diffusion process has taken place, exposure to low temperatures will not restore the device to its original state. Accuracy and precision vary widely among different types, to differentiate often limited by their ability or their ability to visually interpret diffusion distances.

**Infrared Device**— This is a device based on measuring the IR radiation from the article whose temperature is being determined; the IR radiation varies as a function of the object's temperature. The advantage of the device is that the article may be at some distance from the IR sensor. However, IR devices are expensive compared to other temperature sensors.

**Resistance Temperature Detector (RTD)**— This is a device based on the change in electrical resistance of a material as a function of temperature. Precision and accuracy depend on the quality of the electronics used to measure the resistance. Therefore, although RTDs are among the most stable and accurate temperature sensors, their accuracy may change with the age and temperature of the device as its electronic components are affected. A particular type of RTD uses platinum or platinum alloy wire as the sensor. These are referred to as platinum resistance temperature detectors (PRT or PRTD).

**Solid State Device**— This is a device based on the effect of temperature on either an integrated circuit (see Thermistor below) or a micromechanical or microelectrical system. These devices can attain the highest precision available and also have the advantage of producing a digital output. Their accuracy is typically limited by the accuracy of the calibrating system employed.

**Thermistor**— This is a semiconductor device whose resistance varies with temperature. Thermistors are able to detect very small changes in temperature. They are accurate over a broad range of temperatures.

**Thermocouple**— This is a device based on the change in the junction potential of two dissimilar metals as a function of temperature. Many metal pairs may be used, with each pair providing a unique range, accuracy, and precision. Precision and accuracy depend on the quality of the electronics used to measure the voltage and the type of temperature reference used. Accuracy may be a function of temperature reference used. Thermocouples have relatively poor stability and low sensitivity, but are simple and cover a wide temperature range.

**Thermomechanical Device**— This is a device based on the change in volume of a solid material as a function of temperature. For example, a mechanical spring, which expands or contracts as a function of temperature, thus opening and closing an electrical circuit or moving a chart pen, is such a device. Precision may be as good as 0.05°, but in practice it is rarely better than 0.5°. Accuracy is often in the range of ±1.0°, but it may change with the age and temperature of the device.

#### TIME-TEMPERATURE INTEGRATORS

Time-temperature integrators, commonly referred to as TTIs, change color or physical appearance as a result of exposure to a temperature above a specific threshold for a specific time duration, and thus accumulate heat. TTIs are typically single use, disposable devices that react irreversibly. Once the color changes, it will not revert to the original one even if the temperature returns to the acceptable, normal range. The four basic types of chemical-based TTIs are described below.

**Table 1** lists the four types of chemical TTIs presently in use. The closer the activation energy of the TTI's color change to the activation energy of the degradation process of the drug being monitored, the more accurately the TTI will reflect the status of the drug. In actual practice, the activation energy for degradation of a particular drug is not known precisely enough to enable selection of a particular type of TTI. The range of possible activation energies of a TTI is given in the table to provide a sense of the flexibility of that particular technology. A TTI with a range of possible activation energies can be configured to cover a wider range of time and temperature thresholds.

Table 1. Characteristics of TTI Technologies

	Activation		

Type	Storage	Energy (kcal/mol)	Indication	Placement	Activation
Chemical-Physical	Controlled room temperature	13-80	Readable message or image	Primary label or primary package	Placement of activator tape over indicator
Polymerization	-44°	21 or 37	Readable message or image	Primary label or primary package	Removal from frozen environment
Diffusion	Controlled room temperature	9.8	Progressive color diffusion observed through clear window	Primary package	Removal of barrier film
Enzymatic	Controlled room temperature; cold for extended storage	8-30	Color change observed through clear window	Primary package	Breaking seal to mix liquids

An important characteristic of chemical TTIs is the precision with which the endpoint can be determined. It is difficult to quantify an indication such as a gradual color change. Accuracy may also vary widely with the control and quality of the manufacturing process. As discussed below in Validation of Temperature and Humidity Monitoring Devices, it is not possible to calibrate an individual chemical TTI because the test is, by the nature of the device, necessarily destructive. Chemical time-temperature indicators are relatively inexpensive and may be customized for a wide range of applications.

**Chemical-Physical Based TTI**— This type of TTI is based on a temperature-dependent diffusion/chemical reaction process. It consists of a pressure-sensitive tape structure, which is composed of an indicator tape and an activator tape. The indicator tape contains a dye dispersed in a polymer carrier. The activator is incorporated into an adhesive on the activator tape. Laminating the activator tape over the indicator tape causes activation. A color change or readable message occurs as the activator migrates into the indicator as a function of temperature and time. These TTIs can be manufactured to provide a wide array of time-temperature configurations. Also, because they can be made using a printing process, they can be directly integrated into a product label or provided as a stand-alone label if required.

**Chemical Polymerization Based TTI**— This type of TTI uses a polymerization process in which a color change occurs as a function of time and temperature. The color change happens when a small, colorless molecule polymerizes into a larger, colored molecule on exposure to temperatures above a specific threshold for a specified period of time. These TTIs can be applied as print process, permitting direct integration into a product label or stand-alone label. Since this type of TTI does not require activation, it must be shipped from the manufacturer on dry ice and stored at temperatures below freezing prior to use. Chemical polymerization based TTIs have somewhat limited selections of time-temperature threshold configurations.

**Diffusion Based TTI**— This type of TTI is composed of a color-dyed fat, an ester that diffuses along a porous filter paper strip or wick once the temperature exceeds the melting point of the ester. The distance the colored fat migrates is a function of the time the TTI is exposed to temperatures above the melting point of the ester. Removing a barrier film that separates the dyed fat from the wick activates these devices. They can be modified for various applications by selecting esters of different melting points, and by changing the length of the wick. These TTIs are contained within their own packaging and have limited time-temperature threshold configurations.

**Enzyme Based TTI**— This type of TTI uses an enzyme-catalyzed color generating reaction that occurs as a function of time and temperature. The color change is caused by esterase hydrolysis of a fatty substance, accompanied by a decrease in pH. The enzyme and the fatty substrate are in separate solutions in adjacent compartments. Breaking the barrier between the two compartments and mixing the two solutions activates the device. Enzymatic reactions provide a wide variety of time-temperature configurations.

#### ELECTRONIC TIME-TEMPERATURE HISTORY RECORDERS

These devices, which may serve as an alternative to chemical-based TTIs, use one of the electronic temperature measurement technologies described above and create a record of the temperature history experienced by a device. Some are simple electronic devices that record and save temperature values representative of the cumulative temperature history over a period of time. These may be designated as electronic TTIs. They have the advantages of being able to calculate the Mean Kinetic Temperature (MKT) based on the measurements recorded and they can be calibrated.

**Data Loggers**— A more capable device records the temperature at very short intervals and is able to download the temperature history record to a peripheral system, such as a personal computer. Such devices may be termed electronic temperature data loggers. In addition, data loggers may record the humidity using sensors described below. Data loggers may be permanently fixed within a storage facility or they may be portable and travel with a product. Data loggers equipped with transmitting devices (hard-wire or radio transmission) can be used to monitor temperature and humidity of a product while in transit, with the ability to download the recorded data when the data loggers arrive at a destination.

#### RELATIVE HUMIDITY MEASUREMENT TECHNOLOGIES

Relative humidity may be defined as the ratio of the observed partial pressure of water vapor in a volume of air to the saturation pressure at that temperature. In other words, the relative humidity is the amount of water vapor present divided by the theoretical amount of moisture that could be held by that volume of air at a given temperature. Extensive tables of data are available. Devices for measuring relative humidity are called hygrometers. Several different technologies exist for measuring relative humidity.

**Sling Psychrometer**— The simplest type of hygrometer is based on the temperature difference observed between two identical thermometers, one ordinary, and one with a wet cloth wick over its bulb. The two thermometers are whirled at the end of a chain, and the evaporation of water from the wick cools the wet bulb thermometer. The temperature difference between the wet and dry thermometers is then compared to a table, specific to that psychrometer, based on dry bulb temperature, and the relative humidity is determined. The use of a sling psychrometer in a commercial setting is impractical.

**Hair Hygrometer**— This type of device is based on the fact that the length of a synthetic or human hair increases as a function of the relative humidity. This change is used to move an indicator or affect a strain gauge. A hair hygrometer can be accurate to  $\pm 3\%$ , but it is unable to respond to rapid changes in humidity and loses accuracy at very high or very low levels of relative humidity.

**Infrared Hygrometer**— This type of hygrometer determines relative humidity by comparing the absorption of two different wavelengths of IR radiation through air. One wavelength is absorbed by water vapor and the other is not. This type of hygrometer can accurately measure relative humidity in large or small volumes of air. It is sensitive to rapid changes of humidity and can be integrated with an electronic data handling system.

**Dew Point Hygrometer**— This type of device uses a chilled mirror to determine the dew point of an air sample. The dew point is the temperature at which water vapor in the air begins to condense, that is, the temperature at which the relative humidity is 100%. From this measurement and an accurate measurement of the ambient temperature, the relative humidity can be calculated. The dew point hygrometer is the standard against which most commercially available instruments are calibrated.

**Capacitive Thin-Film Hygrometer**— The principle of this type of hygrometer is that the dielectric of a nonconductive polymer changes in direct proportion to the relative humidity. This change is measured as a change in capacitance. This type of hygrometer is accurate to  $\pm 3\%$ .

**Resistive Thin-Film Hygrometer**— This type of hygrometer is similar to the capacitive thin-film type in that it uses the effect of changing relative humidity on an electrical circuit. In the resistive thin-film hygrometer the sensor is an organic polymer whose electrical resistance changes in logarithmic proportion to the relative humidity. This type of hygrometer is accurate to  $\pm 5\%$ .

#### VALIDATION OF TEMPERATURE AND HUMIDITY MONITORING DEVICES

Thermometers and hygrometers, used to provide data about the temperature and humidity exposure of a product, must be suitable for their intended use. Specifically, they must be appropriately validated. Validation is a process that assures the user of the monitoring device that the device has been tested prior to use either by the manufacturer or the user, to assess the measurement accuracy, measurement responsiveness, and time accuracy, where appropriate. Monitors used in manufacturing, storage, and transport of drugs should be



properly qualified by their users to ensure that the monitors have been received and maintained in proper working order. Pharmacies and consumers may accept the validation performed by the manufacturer of the device.

**Measurement Accuracy**— For temperature and humidity monitoring devices, measurement accuracy refers to the closeness of the value obtained with a particular device to the true value being measured. In practice, this is determined by comparison with a device that has been calibrated against a standard that is obtained from or traceable to the National Institute of Standards and Technology (NIST).

**Measurement Responsiveness**— Any monitor takes time to respond to a change in the temperature or humidity. The more rapid the response, the clearer the picture of the environmental history of a monitored product will be. Measurement responsiveness may be defined as the time,  $t_{1/2}$ , required for a device to read a value of  $(x + y)/2$  after an instantaneous change in the property being measured from  $x$  to  $y$ . Measurement responsiveness is typically defined for the operating range of a device.

Different levels of responsiveness are needed for different monitoring applications. For devices used to monitor storage locations, where the temperature and humidity are unlikely to change rapidly, a  $t_{1/2} \leq 15$  minutes may be appropriate. For devices used to monitor transport, where more rapid changes are possible, a  $t_{1/2} \leq 5$  minutes may be needed.

**Time Accuracy**— Most commonly, time accuracy is expressed as a  $\pm$  percentage of total duration of the recording period. For pharmaceutical applications, a  $\pm 0.5\%$  time accuracy is adequate.

**Validation of Chemical-Based TTIs**— This type of device presents a problem for validation because testing the individual device causes its destruction. For this reason, calibration of individual chemical-based TTIs against an NIST traceable standard is not possible. Ideally, chemical-based TTIs would be made using Good Manufacturing Practices, and their use in connection with monitoring the storage and transport environment of drugs would be appropriately regulated. In the absence of those conditions, the performance of a batch of these devices may be assessed statistically by subjecting an appropriately sized sample to elevated temperature conditions for a set period of time and observing the results. Appropriate acceptance criteria should be adopted.

#### THE USE OF HISTORIC TEMPERATURE DATA

It is clear that the type of temperature monitoring needed is a function of the environmental conditions that can be expected. Therefore, climatic data are useful when selecting the most appropriate local storage conditions and monitoring methods. For example, an inexpensive limit detector may be all that is needed when there is a low probability that excessive temperatures will be experienced. Alternatively, a data logger may be preferred when it would be useful to demonstrate that exposure to the highest temperatures was very brief.

It should be noted, however, that outside temperatures are not necessarily reliable indicators of the temperatures experienced by different items in the distribution chain. For example, recent studies reported significant departures from ambient temperatures on summer days for mailboxes, trucks, and warehouses. Detailed historical temperature data are available from the National Oceanic and Atmospheric Administration showing the daily mean maximum and minimum temperature on any given day of the year in a geographical region of interest (e.g., <http://www.cdc.noaa.gov/USclimate/states.fast.html>).

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General Chapter	<a href="#">Desmond G. Hunt, Ph.D.</a> Scientist 1-301-816-8341	(PS05) Packaging and Storage 05

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1119 NEAR-INFRARED SPECTROSCOPY

#### INTRODUCTION

Near-infrared (NIR) spectroscopy is a branch of vibrational spectroscopy that shares many of the principles that apply to other spectroscopic measurements. The NIR spectral region comprises two subranges associated with detectors used in the initial development of NIR instrumentation. The short-wavelength (Herschel or silicon region) extends from approximately 780 to 1100 nm (12,821–9000  $\text{cm}^{-1}$ ); and longer wavelengths, between 1100 and 2500 nm, compose the traditional (lead sulfide) NIR region. Applications of NIR spectroscopy use spectra displayed in either wavelength or wavenumber units. As is the case with other spectroscopy measurements, interactions between NIR radiation and matter provide information that can be for both qualitative and quantitative assessment of the chemical composition of samples. In addition, qualitative and quantitative characterization of a sample's physical properties can be made because of the sample's influence on NIR spectra. Measurements can be made directly on samples *in situ* in addition to applications during standard sampling and testing procedures.

Applications of qualitative analysis include identification of raw material, in-process sample, or finished product. These applications often involve comparing an NIR spectrum from a sample to reference spectra and assessing similarities against acceptance criteria developed and validated for a specific application. In contrast, applications of quantitative analysis involve the development of a predictive relationship between NIR spectral attributes and sample properties. These applications typically use numerical models to quantitatively predict chemical and/or physical properties of the sample on the basis of NIR spectral attributes.

Vibrational spectroscopy in the NIR region is dominated by overtones and combinations that are much weaker than the fundamental mid-IR vibrations from which they originate. Because molar absorptivities in the NIR range are low, radiation can penetrate several millimeters into materials, including solids. Many materials, such as glass, are relatively transparent in this region. Fiber-optic technology is readily implemented in the NIR range, which allows monitoring of processes in environments that might otherwise be inaccessible.

The instrument qualification tests and acceptance criteria provided in this chapter may not be appropriate for all instrument configurations. In such cases, alternative instrument qualification and performance checks should be scientifically justified and documented. In addition, validation parameters discussed in this chapter may not be applicable for all applications of NIR spectroscopy. Validation parameters characterized for a specific NIR application should demonstrate suitability of the NIR application for its intended use.

#### Transmission and Reflection

The most common measurements performed in the NIR spectral range are transmission and reflection spectroscopy. Incident NIR radiation is absorbed or scattered by the sample and is measured as transmittance or reflectance, respectively. Transflection spectrometry is a hybrid of transmission and reflection wherein a reflector is placed behind the sample so that the optical path through the sample and back to the detector is doubled compared to a transmission measurement of a sample of the same thickness. Transflection is used to describe any double-pass transmission technique. The light may be reflected from a diffuse or specular (mirror) reflector placed behind the sample. This configuration can be adapted to share instrument geometry with certain reflection or fiber-optic probe systems in which the source and the detector are on the same side of the sample.

transmittance,  $T$ , is a measure of the decrease in radiation intensity as a function of wavelength when radiation is passed through a sample. The sample is placed in the optical beam between the source and the detector. The results of both transmission and transflection measurements are usually presented directly in terms of absorbance, i.e.,  $\log(1/T)$ .

reflectance,  $R$ , is a measure of the ratio of the intensity of light reflected from the sample,  $I$ , to that reflected from a background or reference reflective surface,  $IR$ . Most reflection measurements in the NIR are made of scattering samples such as powders and slurries. For such materials NIR radiation can penetrate a substantial distance into the sample, where it can be absorbed when the wavelength of the radiation corresponds to a transition between the ground vibrational state of the analyte and either a harmonic of a given vibrational mode (an overtone) or the sum of two or more different modes (a combination band). Nonabsorbed radiation is scattered back from the sample to the detector. NIR reflection spectra are accessed by calculating and plotting  $\log(1/R)$  versus wavelength. This logarithmic form is the pseudo-absorbance of the material and is commonly called absorbance.

#### Factors That Affect NIR Spectra

The following list is not exhaustive, but it includes many of the major factors that affect NIR spectra.



Sample Temperature— Sample temperature influences spectra obtained from aqueous solutions and other hydrogen-bonded liquids, and a difference of a few degrees may result in significant spectral changes. Temperature may also affect spectra obtained from less polar liquids, as well as solids that contain solvents and/or water.

Moisture and Solvent— Moisture and solvent present in the sample material and analytical system may change the spectrum of the sample. Both absorption by moisture and solvent and their influence on hydrogen bonding of the APIs and excipients can change the NIR spectrum.

Sample Thickness— Sample thickness is a known source of spectral variability and must be understood and/or controlled. The sample thickness in transmission mode is typically controlled by using a fixed optical path length for the sample. In diffuse reflection mode, the sample thickness is typically controlled by using samples that are “infinitely thick” relative to the detectable penetration depth of NIR light into a solid material. Here “infinite thickness” implies that the reflection spectrum does not change if the thickness of the sample is increased.

Sample Optical Properties— In solids, both surface and bulk scattering properties of calibration standards and analytical samples must be taken into account. Surface morphology and refractive index properties affect the scattering properties of solid materials. For powder materials, particle size and bulk density influence scattering properties and the NIR spectrum.

Polymorphism— Variation in crystalline structure (polymorphism) from materials with the same chemical composition can influence NIR spectral response. Different polymorphs and amorphous forms of solid material may be distinguished from one another on the basis of their NIR spectral properties. Similarly, different crystalline hydration or solvation states of the same material can display different NIR spectral properties.

Age of Samples— Samples may exhibit changes in their chemical, physical, or optical properties over time. Care must be taken to ensure that both samples and standards used for NIR analysis are suitable for the intended application.

## INSTRUMENTATION

### Apparatus

All NIR measurements are based on exposing material to incident NIR light radiation and measuring the attenuation of the emerging (transmitted, scattered, or reflected) light. Several spectrophotometers are available; they are based on different operating principles—for example: filters, grating-based dispersive, acousto-optical tunable filter (AOTF), Fourier-transform NIR (FT-NIR), and liquid crystal tunable filter (LCTF). Silicon, lead sulfide, indium gallium arsenide, and deuterated triglycine sulphate are common detector materials. Conventional cuvette sample holders, fiber-optic probes, transmission dip cells, and spinning or traversing sample holders are common examples of sample interfaces for introducing the sample to the optical train of a spectrometer.

The selection of specific NIR instrumentation and sampling accessories should be based on the intended application, and particular attention should be paid to the suitability of the sampling interface for the type of sample that will be analyzed.

### Near-Infrared Reference Spectra

NIR references, by providing known stable measurements to which other measurements can be compared, are used to minimize instrumental variations that would affect the measurement.

Transmittance— The measurement of transmittance requires a background reference spectrum for determining the absorption by the sample relative to the background. Suitable transmittance reference materials depend on the specific NIR application and include air, an empty cell, a solvent blank, or a reference sample.

Reflectance— The measurement of reflectance requires the measurement of a reference reflection spectrum to determine the attenuation of reflected light relative to the unattenuated incident beam. The reflectance spectrum is calculated as the ratio of the single-beam spectrum of the sample to that of the reference material. Suitable reflectance reference materials depend on the specific NIR application and include ceramic, perfluorinated polymers, gold, and other suitable materials.

### Qualification of NIR Instruments

Qualification— Qualification of an NIR instrument can be divided into three elements: Installation Qualification (IQ); Operational Qualification (OQ); and Performance Qualification (PQ). For further discussion, see the proposed general information chapter Analytical Instrument Qualification (1058).

Installation Qualification— The IQ requirements help ensure that the hardware and software are installed to accommodate safe and effective use of the instrument at the desired location.

Operational Qualification— In operational qualification, an instrument's performance is characterized using standards to verify that the system operates within target specifications. The purpose of operational qualification is to demonstrate that instrument performance is suitable. Because there are so many different approaches for measuring NIR spectra, operational qualification using standards with known spectral properties is recommended. Using external traceable reference standard materials does not justify omitting the instrument's internal quality control procedures. As is the case with any spectroscopic device, wavelength uncertainty, photometric linearity, and noise characteristics of NIR instruments should be qualified against target specifications for the intended application.

Performance Qualification— Performance qualification demonstrates that the NIR measurement consistently operates within target specifications defined by the user for a specific application; it is often referred to as system suitability. Performance qualification for NIR measurements can include comparing a sample or standard spectrum to previously recorded spectra. Comparisons of spectra taken over time from identical and stable samples or reference standard materials can form the basis for evaluating the long-term stability of an NIR measurement system. The objective is to demonstrate that no abnormal wavelength shift or change in detector sensitivity has occurred during ongoing analysis.

Characterizing Instrument Performance— Specific procedures, acceptance criteria, and time intervals for characterizing NIR instrument performance depend on the instrument and intended application. Many NIR applications use previously validated models that relate NIR spectral response to a physical or chemical property of interest. Demonstrating stable instrument performance over extended periods of time provides some assurance that reliable measurements can be taken from sample spectra using previously validated NIR models.

Wavelength Uncertainty— NIR spectra from sample and/or reference standard materials can be used to demonstrate an instrument's suitable wavelength dispersion performance against target specifications. The USP Near IR System Suitability Reference Standard or the National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 2036 for reflectance measurement and NIST SRM 2035 for transmittance measurement can be used for wavelength verification. Suitable materials for demonstrating wavelength dispersion performance include polystyrene, mixtures of rare earth oxides, and absorption by water vapor for instruments that use an interferometer for wavelength dispersion. With appropriate justification, alternative standards may be used. Wavelength uncertainty typically is characterized from a single spectrum (collected with the same spectral resolution to obtain the standard value) using a minimum of three peaks that cover a suitable spectral range of the instrument. Typical tolerances for agreement with standard values are  $\pm 1.0$  nm from approximately 700 to 2000 nm and  $\pm 1.5$  nm above 2000 nm to approximately 2500 nm ( $\pm 8$  cm $^{-1}$  below 5000 cm $^{-1}$  and  $\pm 4$  cm $^{-1}$  from 5000 cm $^{-1}$  to approximately 14,000 cm $^{-1}$ ). Alternative tolerances may be used when justified for specific applications.

Photometric Linearity and Response Stability— NIR spectra from samples and/or reference standard materials with known relative transmittance or reflectance can be used to demonstrate a suitable relationship between NIR light attenuation (due to absorption) and instrument response. For reflectance measurements, commercially-available reflectance standards with known reflectance properties are often used. Spectra obtained from reflection standards are subject to variability as a result of the difference between the experimental conditions under which they were factory calibrated and those under which they are subsequently put to use. Hence, the reflectance values supplied with a set of calibration standards may not be useful in the attempt to establish an “absolute” calibration for a given instrument. Provided that (1) the standards do not change chemically or physically, (2) the same reference background is also used to obtain the standard values, and (3) the instrument measures each standard under identical conditions (including precise sample positioning), the reproducibility of the photometric scale will be established over the range of standards. Subsequent measurements on the identical set of standards give information on long-term stability. Photometric linearity is typically characterized using a minimum of four reference standards in the range from 10% to 90% reflection (or transmission). NIR applications based on measuring an absorbance larger than 1.0 may require standards with reflectivity properties between 2% and 5% reflection (or transmission) for characterizing instrument performance at low reflectance. The purpose is to demonstrate a linear relationship between NIR reflectance and/or transmittance and instrument response over the scanning range of the instrument. Typical tolerances for a linear relationship are  $1.00 \pm 0.05$  for the slope and  $0.00 \pm 0.05$  for the intercept of a plot of the measured photometric response versus standard photometric response. Alternative tolerances may occur when justified for specific applications.

Spectroscopic Noise— NIR instrument software may include built-in procedures to automatically determine system noise and to provide a statistical report of noise or S/N over the



...instrument's operating range. In addition, it may be desirable to supplement such checks with measurements that do not rely directly on manufacturer-supplied procedures. Typical procedures involve measuring spectra of traceable reference materials with high and low reflectance. Tolerances for these procedures should demonstrate suitable S/N for the intended application.

high-flux noise—Instrument noise is evaluated at high-light flux by measuring reflectance or transmittance of the reference standard, with the reference material (e.g., 99% reflection standard) acting as both the sample and the background reference.

low-flux noise—The same procedure may be used with a lower-reflectivity reference material (e.g., 10% reflectance standard) to determine system noise at reduced light flux. The source, optics, detector, and electronics make significant contributions to the noise under these conditions.

## METHOD VALIDATION

### Introduction

The objective of NIR method validation, as is the case with the validation of any analytical procedure, is to demonstrate that the measurement is suitable for its intended purpose. NIR spectroscopy is somewhat different from conventional analytical techniques because validation of the former generally is achieved by the assessment of chemometric parameters, but these parameters can still be related to the fundamental validation characteristics required for any analytical method.

Data pretreatment is often a vital step in the chemometric analysis of NIR spectral data. Data pretreatment can be defined as the mathematical transformation of NIR spectral data to enhance spectral features and/or remove or reduce unwanted sources of variation prior to using the spectrum. Calibration is the process of developing a mathematical relationship between NIR spectral response and properties of samples. Many suitable chemometric algorithms for data pretreatment and calibration exist; the selection should be based on sound scientific judgment and suitability for the intended application.

### Validation Parameters

Performance characteristics that demonstrate the suitability of NIR methods are similar to those required for any analytical procedure. A discussion of the applicable general principles is found in [Validation of Compendial Procedures](#) (1225). These principles should be considered typical for NIR procedures, but exceptions should be dealt with on a case-by-case basis. For qualitative NIR methods, see chapter (1225), Data Elements Required for Validation, Category IV assays. For quantitative NIR methods, see chapter (1225), Data Elements Required for Validation, Category I and Category II assays. Specific acceptance criteria for each validation parameter must be consistent with the intended use of the method. The samples for validation should be independent of the calibration set.

**Specificity**—The extent of specificity testing depends on the intended application. Demonstration of specificity in NIR methods is typically accomplished by using the following approaches:

**Qualitative**—Identification testing is a common application of qualitative NIR spectroscopy. Identification is achieved by comparing a sample spectrum to a reference spectrum or a library of reference spectra. The specificity of the NIR identification method is demonstrated by obtaining positive identification from samples coupled with negative results from materials that should not meet criteria for positive identification. Materials to demonstrate specificity should be based on sound scientific judgment and can include materials similar in visual appearance, chemical structure, or name.

**Quantitative**—Quantitative applications of NIR spectroscopy typically involve establishing a mathematical relationship between NIR spectral response and a physical or chemical property of interest. Demonstrating specificity against a physical or chemical property of interest is based on interpreting both NIR spectral attributes and chemometric parameters in terms of the intended application and may include the following:

- Spectral regions in the calibration model can be correlated to a known NIR spectral response associated with the property of interest.
- Wavelengths used by regression analysis for the calibration (e.g., for multiple linear regression [MLR] models) or the loading vector for each factor (e.g., for partial least squares [PLS] or principal component regression [PCR] models) can be examined to verify relevant spectroscopic information that is used for the mathematical model.
  - Variation in spectra from samples for calibration can be examined and interpreted as expected spectral observations.
- Variation in material composition and sample matrix may be shown to have no significant effect on quantification of the property of interest within the specified method range.

**Linearity**—Quantitative NIR methods generally attempt to demonstrate a linear relationship between NIR spectral response and the property of interest. Although demonstrating a linear response is not required for all NIR applications, the model chosen, whether linear or not, should properly represent the relationship.

Validation of linearity in NIR methods may be accomplished by examining a plot of NIR spectral response versus actual or accepted values for the property of interest. Many statistical methods are available for evaluation of the goodness of fit of the linear relationship. Other applicable statistics and graphical methods may be as appropriate.

The correlation coefficient,  $r$ , may not be an informative measure of linearity. The square of the (Pearson) correlation coefficient is a measure of the fraction of the data's variation that is adequately modeled by the equation. Linearity depends on the standard error of the calibration equation (and hence the reference method) and on the range of the calibration data. Thus, although values very near 1.00, such as 0.99 or greater, typically indicate a linear relationship, lower values do not distinguish between nonlinearity and variability around the line.

**Range**—The specified range of an NIR method depends on the specific application. The range typically is established by confirming that the NIR method provides suitable measurement capability (accuracy and precision) when applied to samples within extreme limits of the NIR measurement. Controls must be used to ensure that results outside the validated range are not accepted. In certain circumstances, it may not be possible or desirable to extend the validated range to include sample variability outside the validated range.

Extending the range of an NIR method requires demonstration of suitable measurement capability within the limits of the expanded range. Examples of situations in which only a limited sample range may be available are samples from a controlled manufacturing process and in-process samples. A limited method range does not preclude the use of an NIR method.

**Accuracy**—Accuracy in NIR methods is demonstrated by showing the closeness of agreement between the value that is accepted as either a conventional true value or an accepted reference value. Accuracy can be determined by direct comparison between NIR validation results and actual or accepted reference values. Suitable agreement between NIR and reference values is based on required measurement capability for a specific application. The purpose is to demonstrate a linear relationship between NIR results and actual values. Accuracy can be determined by agreement between the standard error of prediction (SEP) and the standard error of the reference method for validation. The error of the reference method may be known on the basis of historical data, through validation results specific to the reference method, or by calculating the standard error of the laboratory (SEL). Suitable agreement between SEP and SEL is based on required measurement capability for a specific application.

**Precision**—The precision of an NIR method expresses the closeness of agreement between a series of measurements under prescribed conditions. Two levels of precision should be considered: repeatability and intermediate precision. The precision of an NIR method typically is expressed as the relative standard deviation of a series of NIR method results and should be suitable for the intended application. Demonstration of precision in NIR methods may be accomplished using the following approaches:

**Repeatability**—Repeatability can be demonstrated by the following:

- Statistical evaluation of a number of replicate measurements of the sample without repositioning the sample between each individual spectral acquisition, or
- Statistical evaluation of multiple NIR method results, each result from a replicate analysis of a sample subsequent to re-positioning between spectral acquisitions

**Intermediate Precision**—Intermediate precision can be shown by the following:

- Statistical evaluation of a number of replicate NIR measurements of the same or similar samples in the Repeatability study by different analysts on different days.

**Robustness**—NIR measurement parameters selected to demonstrate robustness will vary depending on the application and the sample's interface with the NIR instrument. Critical measurement parameters associated with robustness often are identified and characterized during method development. Typical measurement parameters include the following:

- Effect of environmental conditions (e.g., temperature, humidity, and vibration)
- Effect of sample temperature
- Sample handling (e.g., probe depth, compression of material, sample depth/thickness, sample presentation)
  - Influence of instrument changes (e.g., lamp change, warm-up time)



#### Ongoing Method Evaluation

Validated NIR methods should be subject to ongoing performance evaluation, which may include monitoring accuracy, precision, and other suitable method parameters. If performance is unacceptable, corrective action is necessary. It involves conducting an investigation to identify the cause of change in method performance and may indicate that the NIR method is not suitable for continued use. Improving the NIR method to meet measurement suitability criteria may require additional method development and documentation of validation experiments demonstrating that the improved method is suitable for the intended application. The extent of revalidation required depends on the cause of change in method performance and the nature of corrective action required in order to establish suitable method performance. Appropriate change controls should be implemented to document ongoing method improvement activities.

Revalidation of a qualitative model may be necessary as a result of the following:

- Addition of a new material to the spectral reference library
- Changes in the physical properties of the material
  - Changes in the source of material supply
- Identification of previously unknown critical attribute(s) of material(s)

Revalidation of a quantitative model may be necessary as a result of the following:

- Changes in the composition of the test sample or finished product
  - Changes in the manufacturing process
- Changes in the sources or grades of raw materials
  - Changes in the reference analytical method
  - Major changes in instrument hardware

**Outliers**— Sample spectra that produce an NIR response that differs from the qualitative or quantitative calibration model may produce an outlier. This does not necessarily indicate an out-of-specification result; but rather an outlier indicates that further testing of the sample may be required and is dependent on the particular NIR method. If subsequent testing of the sample by an appropriate method indicates that the property of interest is within specifications, then the sample meets its specifications. Outlier samples may be incorporated into an updated calibration model subsequent to execution and documentation of suitable validation studies.

#### Method Transfer

Controls and measures for demonstrating the suitability of NIR method performance following method transfer are similar to those required for any analytical procedure. Exceptions to general principles for conducting method transfer for NIR methods should be justified on a case-by-case basis. The transfer of an NIR method is often performed by using an NIR calibration model on a second instrument that is similar to the primary instrument used to develop and validate the method. When a calibration model is transferred to another instrument, procedures and criteria must be applied to demonstrate that the calibration model meets suitable measurement criteria on the second instrument. The selection of an appropriate calibration model transfer procedure should be based on sound scientific judgment.

#### GLOSSARY

absorbance, A, is represented by the equation:

$$A = -\log T = \log (1/T)$$

where T is the transmittance of the sample. Absorbance is also frequently given as:

$$A = \log (1/R)$$

where R is the reflectance of the sample.

background spectrum is used for generating a sample spectrum with minimal contributions from instrument response. It is also referred to as a reference spectrum or background reference. The ratio of the sample spectrum to the background spectrum produces a transmittance or reflectance spectrum dominated by NIR spectral response associated with the sample. In reflection measurements, a highly reflective diffuse standard reference material is for the measurement of the background spectrum. For transmission measurement, the background spectrum may be measured with no sample present in the spectrometer or using a cell with the solvent blank or a cell filled with appropriate reference material.

calibration model is a mathematical expression to relate the response from an analytical instrument to the properties of samples.

diffuse reflectance is the ratio of the spectrum of radiated light penetrating the sample surface, interacting with the sample, passing back through the sample's surface, and reaching the detector to the background spectrum. This is the component of the overall reflectance that produces the absorption spectrum of the sample.

fiber-optic probes consist of two components: optical fibers that may vary in length and in the number of fibers and a terminus, which contains specially designed optics for examination of the sample matrix.

installation qualification is the documented collection of activities necessary to establish that an instrument is delivered as designed and specified, is properly installed in the selected environment, and that this environment is suitable for the instrument's intended purpose.

instrument bandwidth or resolution is a measure of the ability of a spectrometer to separate radiation of similar wavelengths.

multiple linear regression is a calibration algorithm to relate the response from an analytical instrument to the properties of samples. The distinguishing feature of this algorithm is the use of a limited number of independent variables. Linear-least-squares calculations are performed to establish a relationship between these independent variables and the properties of the samples.

operational qualification is the process by which it is demonstrated and documented that an instrument performs according to specifications and that it can perform the intended task. This process is required following any significant change such as instrument installation, relocation, or major repair.

overall reflectance is the sum of diffuse and specular reflectance.

partial least squares (pls) is a calibration algorithm to relate instrument responses to the properties of samples. The distinguishing feature of this algorithm is that data concerning the properties of the samples for calibration are used in the calculation of the factors to describe instrument responses.

performance qualification is the process of using one or more well-characterized and stable reference materials to verify consistent instrument performance. Performance qualification may employ the same or different standards for different performance characteristics.

photometric linearity, also referred to as photometric verification, is the process of verifying the response of the photometric scale of an instrument.

principal component regression (PCR) is a calibration algorithm to relate the response from an analytical instrument to the properties of samples. This algorithm, which expresses a set of independent variables as a linear combination of factors, is a method of relating these factors to the properties of the samples for which the independent variables were obtained.

pseudo-absorbance, A, is represented by the equation:

$$A = -\log R = \log (1/R)$$

where R is the diffuse reflectance of the sample.

reference spectrum—See Background Spectrum.

reflectance is described by the equation:



$$R = I/I_{IR}$$

in which  $I$  is the intensity of radiation reflected from the surface of the sample and  $I_{IR}$  is the intensity of radiation reflected from a background reference material and its incorporated losses due to solvent absorption, refraction, and scattering.

root-mean-square (rms) noise is calculated by the equation:

$$RMS = \sqrt{\frac{1}{N} \times \sum_i^N (A_i - \bar{A})^2}$$

in which  $A_i$  is the absorbance for each data point;  $A$  is the mean absorbance over the spectral segment; and  $N$  is the number of points per segment.

spectral reference library is a collection of spectra of known materials for comparison with unknown materials. The term is commonly used in connection with qualitative methods of spectral analysis (e.g., identification of materials).

specular (surface) reflectance is the reflectance of the front surface of the sample.

standard error of calibration (sec) is a measure of the capability of a model to fit reference data. SEC is the standard deviation of the residuals obtained from comparing the known values for each of the calibration samples to the values that are calculated from the calibration. SEC should not be used as an assessment tool for the expected method accuracy (trueness and precision of prediction) of the predicted value of future samples. The method accuracy should generally be verified by calculating the standard error of prediction (SEP), using an independent validation set of samples. An accepted method is to mark a part of the calibration set as the validation set. This set is not fully independent but can be used as an alternative for the determination of the accuracy.

standard error of cross-validation (SECV) is the standard deviation calculated using the leave-one-out method. In this method, one calibration sample is omitted from the calibration, and the difference is found between the value for this sample calculated from its reference value and the value obtained from the calibration calculated from all the other samples in the set. This process is repeated for all samples in the set, and the SECV is the standard deviation of the differences calculated for all the calibration samples. This procedure can also be performed with a group of samples. Instead of leaving the sample out, a group of samples is left out. The SECV is a measure of the model accuracy that one can expect when measuring future samples if not enough samples are available for the SEP to be calculated from a completely independent validation set.

standard error of the laboratory (sel) is a calculation based on repeated readings of one or more samples to estimate the precision and/or accuracy of the reference laboratory method, depending on how the data were collected.

standard error of prediction (sep) is a measure of model accuracy of an analytical method based on applying a given calibration model to the spectral data from a set of samples different from but similar to those used to calculate the calibration model. SEP is the standard deviation of the residuals obtained from comparing the values from the reference laboratory to those from the method under test for the specified samples. SEP provides a measure of the model accuracy expected when one measures future samples.

surface reflectance, also known as specular reflection, is that portion of the radiation not interacting with the sample but simply reflecting back from the sample surface layer (sample-air interface).

transflection is a transmittance measurement technique in which the radiation traverses the sample twice. The second time occurs after the radiation is reflected from a surface behind the sample.

transmittance is represented by the equation:

$$T = I/I_0 \text{ or } T = 10^{-A}$$

in which  $I$  is the intensity of the radiation transmitted through the sample;  $I_0$  is the intensity of the radiant energy incident on the sample and includes losses due to solvent absorption, refraction, and scattering; and  $A$  is the absorbance.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Gary E. Ritchie, M.Sc.</a> Scientific Fellow 1-301-816-8353	(GC05) General Chapters 05

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## 1120 RAMAN SPECTROSCOPY

### INTRODUCTION

Raman spectroscopy shares many of the principles that apply to other spectroscopic measurements discussed in [Spectrophotometry and Light-Scattering](#). Raman is a vibrational spectroscopic technique and is therefore related to infrared (IR) and near-infrared (NIR) spectroscopy. The Raman effect itself arises as a result of a change in the polarizability of molecular bonds during a given vibrational mode and is measured as inelastically scattered radiation.

A Raman spectrum is generated by exciting the sample of interest to a virtual state with a monochromatic source, typically a laser. Light elastically scattered (no change in wavelength) is known as Rayleigh scatter and is not of interest in Raman spectrometry, except for marking the laser wavelength. However, if the sample relaxes to a vibrational energy level that differs from the initial state, the scattered radiation is shifted in energy. This shift is commensurate with the energy difference between the initial and final vibrational states. This "inelastically scattered" light is referred to as Raman scatter. Only about one in 106–108 photons incident on the sample undergoes Raman scattering. Thus lasers are employed in Raman spectrometers. If the Raman-scattered photon is of lower energy, it is referred to as Stokes scattering. If it is of higher energy, it is referred to as anti-Stokes scattering. In practice, nearly all analytically useful Raman measurements make use of Stokes-shifted Raman scatter.

The appearance of a Raman spectrum is much like an infrared spectrum plotted linearly in absorbance. The intensities, or the number of Raman photons counted, are plotted against the shifted energies. The x-axis is generally labeled "Raman Shift/cm<sup>-1</sup>" or "Wavenumber/cm<sup>-1</sup>". The Raman shift is usually expressed in wavenumber and represents the difference in the absolute wavenumber of the peak and the laser wavenumber. The spectrum is interpreted in the same manner as the corresponding mid-infrared spectrum. The positions of the (Raman shifted) wavenumbers for a given vibrational mode are identical to the wavenumbers of the corresponding bands in an IR absorption spectrum. However, the stronger peaks in a Raman spectrum are often weak in an IR spectrum, and vice versa. Thus the two spectroscopic techniques are often said to be complementary.

Raman spectroscopy is advantageous because quick and accurate measurements can often be made without destroying the sample (solid, semisolid, liquid or, less frequently, gas) and with minimal or no sample preparation. The Raman spectrum contains information on fundamental vibrational modes of the sample that can yield both sample and process understanding. The signal is typically in the visible or NIR range, allowing efficient coupling to fiber optics. This also means that a signal can be obtained from any medium transparent to the laser light; examples are glass, plastics, or samples in aqueous media. In addition, because Raman spectra are ordinarily excited with visible or NIR radiation, standard glass/quartz optics may be used. From an instrumental point of view, modern systems are easy to use, provide fast analysis times (seconds to several minutes), and are reliable. However, the danger of using high-powered lasers must be recognized, especially when their wavelengths are in the NIR and, therefore, not visible to the eye. Fiber-optic probes should be used with caution and with reference to appropriate government regulations regarding lasers and laser classes.



In addition to "normal" Raman spectroscopy, there are several more specialized Raman techniques. These include resonance Raman (RR), surface-enhanced Raman spectroscopy (SERS), Raman optical activity (ROA), coherent anti-Stokes Raman spectroscopy (CARS), Raman gain or loss spectroscopy, and hyper-Raman spectroscopy. These techniques are not widely employed in pharmaceutical laboratories, and are not addressed in this general information chapter.

## QUALITATIVE AND QUANTITATIVE RAMAN MEASUREMENTS

There are two general classes of measurements that are commonly performed by Raman spectrometry: qualitative and quantitative.

### Qualitative Raman Measurements

Qualitative Raman measurements yield spectral information about the functional groups that are present in a sample. Because the Raman spectrum is specific for a given compound, qualitative Raman measurements can be used as a compendial ID test, as well as for structural elucidation.

### Quantitative Raman Measurements

For instruments equipped with a detector that measures optical power (such as Fourier transform [FT]-Raman spectrometers), quantitative Raman measurements utilize the following relationship between signal,  $SV$ , at a given wavenumber,  $V$ , and the concentration of an analyte,  $C$ :

$$SV = K\sigma V(V_L - V_B)^4 P_0 C$$

in which  $K$  is a constant that depends on laser beam diameter, collection optics, sample volume, and temperature;  $\sigma V$  is the Raman cross section of the particular vibrational mode;  $V_L$  is the laser wavenumber;  $V_B$  is the wavenumber of the vibrational mode; and  $P_0$  is the laser power. The Raman cross section,  $\sigma V$ , is characteristic of the nature of the particular vibrational mode. The sample volume is defined by size of the focus of the laser beam at the sample, the optic being used for focusing, and the optical properties of the sample itself. Spot sizes at the sample can range from less than 1  $\mu\text{m}$  for a microprobe to 6 mm for a large area sample system. For Raman spectrometers that measure the number of photons per second (such as charge-coupled device [CCD]-Raman spectrometers) the corresponding equation is:

$$SV = K\sigma V V_L (V_L - V_B)^3 P_0 C$$

From the above equations, it is apparent that peak signal is directly proportional to concentration. It is this relationship that is the basis for the majority of quantitative Raman applications.

## FACTORS AFFECTING QUANTIFICATION

### Sample-Based Factors

The most important sample-based factors that deleteriously affect quantitative Raman spectrometry are fluorescence, sample heating, absorption by the matrix or the sample itself, and the effect of polarization. If the sample matrix includes fluorescent compounds, the measured signal will usually contain a contribution from fluorescence. Fluorescence will be observed only if the laser excitation wavelength overlaps with an absorption band of a fluorescent compound. Fluorescence is typically observed as a broad sloping background underlying the Raman spectrum. Fluorescence can cause both a baseline offset and reduced signal-to-noise ratio. The wavelength range and intensity of the fluorescence is dependent on the chemical composition of the fluorescent material. Because fluorescence is generally a much more efficient process than Raman scattering, even very minor amounts of fluorescent impurities can lead to significant degradation of the Raman signal. Fluorescence can be reduced by using longer wavelength excitation sources such as 785 nm or 1064 nm. However,

it should be remembered that the strength of the Raman signal is proportional to  $(V_L - V_B)^4$ , so the advantage of using a long-wavelength excitation laser to minimize fluorescence is at least partially offset by the reduced strength of the Raman signal. The greatest signal-to-noise ratio will be obtained by balancing fluorescence rejection, signal strength, and detector response.

Fluorescence in solids can sometimes be mitigated by exposing the sample to the laser radiation for a period of time before measurement. This process is called photobleaching, and operates by degrading the highly absorbing species. Photobleaching is less effective in liquids, where the sample is mobile, or if the amount of fluorescent material is more than a trace.

Sample heating by the laser source can cause a variety of effects, such as physical form change (melting), polymorph conversion, or sample burning. The chance for sample heating is greatest when the spot size at the sample is the smallest, i.e., when a microprobe is being used. This is usually an issue for colored, highly absorbing species, or very small particles that have low heat transfer. The effects of sample heating are usually observable either as changes in the Raman spectrum over time or by visual inspection of the sample. Besides decreasing the laser flux, a variety of methods can be employed to diminish laser-induced heating, such as moving the sample or laser during the measurement or improving the heat transfer from the sample with thermal contact or liquid immersion.

Absorption of the Raman signal by the matrix or the sample itself can also occur. This problem is more prevalent with long-wavelength FT-Raman systems where the Raman signal can overlap with an NIR overtone absorption. This effect will be dependent on the optics of the system as well as on the sample presentation. Associated with this effect is variability from scattering in solids as a result of packing and particle-size differences. The magnitude of all of these effects, however, is typically less severe than in NIR because of the limited depth of penetration and the relatively narrower wavelength region sampled in Raman spectroscopy.

Finally, it should be recognized that laser radiation is polarized and the Raman spectra of crystalline materials and other oriented samples can differ significantly depending on the way that the sample is mounted. If the Raman spectrometer is capable of producing linearly polarized radiation at the sample then a polarization scrambler is recommended for routine sample analysis.

### Sampling Factors

Raman spectroscopy is a zero-background technique, in that the signal at the detector is expected to be zero in the absence of a sample. This situation can be contrasted with absorption spectrometry, where the signal at the detector is at a maximum in the absence of a sample. Zero-background techniques are inherently sensitive because small changes in sample concentration lead to proportionate changes in the signal level. The instrument will also be sensitive to other sources of light that can cause sample-to-sample variations in the measured signal level. In addition, a large background signal caused by fluorescence will lead to an increased noise level (photon shot noise). Thus it may be very difficult to use the absolute Raman signal for direct determination of an analyte. Other potential sources of variation are changes in the sample opacity and heterogeneity, changes in the laser power at the sample, and changes in optical collection geometry or sample position. These effects can be minimized by sampling in a reproducible, representative manner. Careful design of the instrumentation can reduce these effects but they cannot be eliminated entirely.

Use of an internal reference standard is the most common and robust method of eliminating variations caused by absolute intensity fluctuations. There are several choices for this approach. An internal standard can be deliberately added, and isolated peaks from this standard can be employed; or a band due to a moiety such as an aromatic ring, the Raman cross-section of which does not change with the way the sample is prepared, can also be used. For solution spectra, an isolated solvent band can be employed because the solvent will remain relatively unchanged from sample to sample. Also, in a formulation, an excipient peak can be used if it is in substantial excess compared to the analyte. The entire spectrum can also be used as a reference, with the assumption that laser and sample-orientation changes will affect the entire spectrum equally.

A second important sampling-based factor to consider is spectral contamination. Raman scattering is a weak effect that can be masked by a number of external sources. Common contamination sources include sample-holder artifacts (container or substrate) and ambient light. Typically, these issues can be identified and resolved by careful experimentation.

## APPARATUS

### Components

All modern Raman measurements involve irradiating a sample with a laser, collecting the scattered radiation, rejecting the Rayleigh-scattered light, differentiating the Raman photons by wavelength, and detecting the resulting Raman spectrum. All commercial Raman instruments therefore share the following common features to perform these functions:

1. Excitation source (laser)
  2. Sampling device
  3. Device to filter/reject light scattered at the laser wavelength
  4. Wavelength processing unit
  5. Detector and electronics
- excitation source (laser)

[Table 1](#) identifies several common lasers used for pharmaceutical applications or Raman spectrometry. UV lasers have also been used for specialized applications but have various drawbacks that limit their utility for general analytical measurements. As more applications for UV lasers are described, it is likely that they may become more common for Raman spectrometry.

Table 1. Lasers Used in Pharmaceutical Applications

Laser $\lambda$ , nm (nearest whole number)	Type	Typical Power at Laser	Wavelength Range, nm (Stokes Region, 100 cm $^{-1}$ to 3000 cm $^{-1}$ shift)	Comments
<b>NIR Lasers</b>				
1064	Solid state (Nd:YAG)	Up to 3 W	1075–1563	Commonly used in Fourier transform instruments
830	Diode	Up to 300 mW	827–980	Typically limited to 2000 cm $^{-1}$ ; Raman shift because of CCD spectral response; less common than the other lasers
785	Diode	Up to 500 mW	791–1027	Most widely used dispersive Raman laser
<b>Visible Lasers</b>				
632.8	He–Ne	Up to 500 mW	637–781	Relatively small fluorescence risk
532	Doubled (Nd:YAG)	Up to 1 W	535–632.8	High fluorescence risk
514.5	Ar+	Up to 1 W	517–608	High fluorescence risk
488–632.8	Ar+	Up to 1 W	490–572	High fluorescence risk

#### sampling device

Several sampling arrangements are possible, including direct optical interfaces, microscopes, fiber optic-based probes (either noncontact or immersion optics), and sample chambers (including specialty sample holders and automated sample changers). The sampling optics can also be designed to obtain the polarization-dependent Raman spectrum, which often contains additional information. Selection of the sampling device will often be dictated by the analyte and sample. However, considerations such as sampling volume, speed of the measurement, laser safety, and reproducibility of sample presentation should be evaluated to optimize the sampling device for any given application.

#### filtering device

The intensity of scattered light at the laser wavelength (Rayleigh) is many orders of magnitude greater than the Raman signal and must be rejected prior to the detector. Notch filters are almost universally used for this purpose and provide excellent rejection and stability combined with small size. The traditional use of multistage monochromators for this purpose, although still viable, is now rare. In addition, various filters or physical barriers to shield the sample from external radiation sources (e.g., room lights, laser plasma lines) may be required depending on the collection geometry of the instrument.

#### wavelength processing unit

The wavelength scale may be encoded by either a scanning monochromator, a grating polychromator (in CCD-Raman spectrometers) or a two-beam interferometer (in FT-Raman spectrometers). A discussion of the specific benefits and drawbacks of each of the dispersive designs compared to the FT instrument is beyond the scope of this chapter. Any properly qualified instruments should be suitable for qualitative measurements. However, care must be taken when selecting an instrument for quantitative measurements, as dispersion and response linearity might not be uniform across the full spectral range.

#### detector

The silicon-based CCD array is the most common detector for dispersive instruments. The cooled array detector allows measurements over the spectral range from 4500 to 100 cm $^{-1}$  Raman shift with low noise when most visible lasers, such as frequency-doubled neodymium-doped yttrium–aluminum–garnet (Nd:YAG) (532 nm) or helium–neon (632.8 nm) lasers, are used. When a 785-nm diode laser is used, the wavelength range is reduced to about 3100 to 100 cm $^{-1}$ . The most commonly used CCD has its peak wavelength responsivity when matched to the commonly used 632.8-nm He–Ne gas laser or 785-nm diode laser. FT instruments typically use single-channel germanium or indium–gallium–arsenide (InGaAs) detectors responsive in the NIR to match the 1064-nm excitation of a Nd:YAG laser.

#### Calibration

Raman instrument calibration involves three components: primary wavelength (x-axis), laser wavelength, and intensity (y-axis).

#### primary wavelength (x-axis)

In the case of FT-Raman instruments, primary wavelength-axis calibration is maintained, at least to a first approximation, with an internal He–Ne laser. Most dispersive instruments utilize atomic emission lamps for primary wavelength-axis calibration. In all instruments suitable for analytical Raman measurements, the vendor will offer a procedure of x-axis calibration that can be performed by the user. For dispersive Raman instruments, a calibration based on multiple atomic emission lines is preferred. The validity of this calibration approach can be verified subsequent to laser wavelength calibration by using a suitable Raman shift standard. For scanning dispersive instruments, calibration might need to be performed more frequently, and precision in both a scanning and static operation mode may need to be verified.<sup>1</sup>

#### laser wavelength

Laser wavelength variation can impact both the wavelength precision and the photometric (signal) precision of a given instrument. Even the most stable current lasers can vary slightly in their measured wavelength output. The laser wavelength must therefore be confirmed to ensure that the Raman shift positions are accurate for both FT-Raman or dispersive Raman instruments. A reference Raman shift standard material such as those outlined in ASTM E1840-96 (2002)<sup>1</sup> or other suitably verified materials can be utilized for this purpose. [note—Reliable Raman shift standard values for frequently used liquid and solid reagents, required for wavenumber calibration of Raman spectrometers, are provided in the ASTM Standard Guide cited. These values can be used in addition to the highly accurate and precise low-pressure arc lamp emission lines that are also available for use in Raman instrument calibration.] Spectrometric grade material can be purchased from appropriate suppliers for this use. Certain instruments may use an internal Raman standard separate from the primary optical path. External calibration devices exactly reproduce the optical path taken by the scattered radiation. [note—When chemical standards are used, care must be taken to avoid contamination and to confirm standard stability.]

Unless the instrument is of a continuous calibration type, the primary wavelength axis calibration should be performed, as per vendor procedures, just prior to measuring the laser wavelength. For external calibration, the Raman shift standard should be placed at the sample location and measured using appropriate acquisition parameters. The peak center of a



...ng, well-resolved band in the spectral region of interest should be evaluated. The position can be assessed manually or with a suitable, valid peak-picking algorithm. The software provided by the vendor might measure the laser wavelength and adjust the laser wavelength appropriately so that this peak is at the proper position. If the vendor does not provide this functionality, the laser wavelength should be adjusted manually. Depending on the type of laser, the laser wavelength can vary with temperature, current, and voltage. Wavelength tolerances can vary depending on the specific application.

#### signal level (y-axis)

Calibration of the photometric axis can be critical for successful quantification by using certain analytical methods (chemometrics) and method transfer between instruments. Both FT-Raman and dispersive Raman spectrometers should undergo similar calibration procedures. The tolerance of photometric precision acceptable for a given measurement should be assessed during the method development stage.

To calibrate the photometric response of a Raman instrument, a broad-band emission source should be used. There are two accepted methods. Method A utilizes a tungsten white light source.<sup>2</sup> The output power of such sources is traceable to the National Metrology Institute (NMI). In the United Kingdom, the National Physical Laboratory also provides calibrated light bulbs. Several other vendors also provide NIST-traceable irradiance calibration standards. This method is applicable to all common laser excitation wavelengths listed in [Table 1](#).

In Method B, NIST standard reference materials (SRMs) are utilized.<sup>3</sup> Several doped-glass fluorescence standards are currently available.

Method A— The source should be placed at the sample location with the laser off and the response of the detector measured (using parameters appropriate for the instrument). The output for the source used for calibration should be known. The ratio of the measured response to the true response should be determined and a correction file generated. This correction should be applied to all spectra acquired with the instrument. Most manufacturers will provide both appropriate calibration sources and software for this approach. If the manufacturer does not provide a procedure or method, the user can accomplish the task using a source obtained from NIST and appropriate software. If a manufacturer's method is used, attention must be paid to the calibration procedure and source validity. The user should obtain appropriate documentation from the manufacturer to ensure a qualified approach.

Method B— The fluorescence standard should be placed at the sample location. With the laser on, a spectrum of the SRM should be obtained (using parameters appropriate for the instrument). The output of the source used for calibration should be known. The ratio of the measured response to the true response should be determined and a correction file generated. This correction should be applied to all spectra acquired with the instrument. Most manufacturers will provide both appropriate calibration sources and software for this approach. If the manufacturer does not provide a procedure or method, the user can accomplish the task using a source obtained from NIST and appropriate software. If a manufacturer's method is used, attention must be paid to the calibration procedure and source validity. The user should obtain appropriate documentation from the manufacturer to ensure a qualified approach. [note—Method B is currently appropriate for systems with 785-nm (SRM 2241), 532-nm (SRM 2242), and both 514.5-nm and 488-nm (SRM 2243) laser excitation. NIST is currently developing other SRMs that will be wavelength-specific for 1064-nm (SRM 2244) and 632.8-nm excitation (expected to be available in 2006).]

#### external calibration

Detailed functional validation employing external reference standards is recommended to demonstrate instrumental suitability for laboratory instruments, even for instruments that possess an internal calibration approach. The use of external reference standards does not obviate the need for internal quality control procedures; rather, it provides independent documentation of the fitness of the instrument to perform the specific analysis or purpose. For instruments installed in a process location or in a reactor where positioning of an external standard routinely is not possible, including those instruments that employ an internal calibration approach, the relative performance of an internal versus an external calibration approach should be periodically checked. The purpose of this test is to check for changes in components that might not be included in the internal calibration method (process lens, fiber-optic probe, etc.), e.g., photometric calibration of the optical system.

### QUALIFICATION AND VERIFICATION OF RAMAN SPECTROMETERS

The suitability of a specific instrument for a given method is ensured by a thorough technology-suitability evaluation for the application; a routine, periodic instrument operational qualification; and the more frequent performance verification (see [Definition of Terms and Symbols](#)). The purpose of the technology-suitability evaluation is to ensure that the technology proposed is suitable for the intended application. The purpose of the instrument qualification is to ensure that the instrument to be used is suitable for its intended application and, when requalified periodically, continues to function properly over extended time periods. When the device is used for a specific qualitative or quantitative analysis, regular performance verifications are made. Because there are many different approaches to measuring Raman spectra, instrument operational qualification and performance verification often employ external standards that can be used on any instrument. As with any spectrometric device, a Raman instrument needs to be qualified for both wavenumber (x-axis) and shift from the excitation source) and photometric (signal axis) precision.

In performance verification, a quality-of-fit to an initial scan or group of scans (often referred to in nonscanning instruments as an accumulation) included in the instrumental qualification can be employed. In such an analysis, it is assumed that reference standard spectra collected on a new or a newly repaired, properly operating instrument represent the best available spectra. Comparison of spectra taken over time on identical reference standards (either the original standard or identical new standards, if stability of the reference standards is a concern) forms the basis for evaluating the long-term stability of a Raman measurement system.

#### Frequency of Testing

Instrumental qualification is performed at designated intervals or following a repair or significant optical reconfiguration, such as the replacement of the laser, the detector or the notch or edge filters. Full instrument requalification might not be necessary when changing between sampling accessories such as a microprobe, a sample compartment, or a fixed fiber-optic probe. Performance verification tests may be sufficient in these cases; instrument-specific guidance from the vendor on qualification requirements should be followed. Tests include wavelength (x-axis) and shift from the excitation source) and photometric (signal axis) precision. Instrument qualification tests require that specific application-dependent tolerances be met.

Performance verification is carried out on the instrument configured for the analytical measurements and is performed more frequently than instrument qualification. Performance verification includes measurement of the wavelength uncertainty and intensity-scale precision. Wavelength precision and intensity-scale precision tests may be needed prior to any data collection on a given day. Performance is verified by matching the current spectra to those collected during the previous instrument qualification.

#### Instrument Operational Qualification

It is important to note that the acceptance specifications given in both the Instrument Operational Qualification and Performance Qualification sections are applicable for general use; specifications for particular instruments and applications can vary depending on the analysis method used and the desired accuracy of the final result. ASTM standard reference materials are also specified, with the understanding that under some circumstances (specifically remote on-line applications) calibration using one of these materials may be impractical, and other suitably verified materials can be employed. At this juncture it is important to note that specific parameters such as spectrometer noise, limits of detection (LOD), limits of quantification (LOQ), and acceptable spectral bandwidth for any given application should be included as part of the analytical method development. Specific values for tests such as spectrometer noise and bandwidth will be dependent on the instrument chosen and the purpose required. As a result, specific instrument tests for these parameters are not dictated in this information chapter.

#### wavelength (x-axis) accuracy

It is important to ensure the accuracy of the wavelength axis via calibration to maintain the integrity of Raman peak positions. Wavelength calibration of a Raman spectrometer consists of two parts: primary wavelength axis and laser wavelength calibration. After both the primary wavelength axis and the laser wavelength are calibrated, instrument wavelength uncertainty can be determined. This can be accomplished using a Raman shift standard such as the ASTM shift standards or other suitably verified material. Selection of a standard with bands present across the full Raman spectral range is recommended so that instrument wavelength uncertainty can be evaluated at multiple locations within the spectrum. The tolerance of wavelength precision that is required for a given measurement should be assessed during the method-development stage. [note—For scanning dispersive instruments, calibration might need to be performed more frequently, and precision in both a scanning and static operation mode may need to be verified.]

#### photometric precision

Laser variation in terms of the total emitted photons occurring between two measurements can give rise to changes in the photometric precision of the instrument. Unfortunately, it is very difficult to separate changes in the photometric response associated with variations in the total emitted laser photons from the sample- and sampling-induced perturbations. This is one of the reasons why absolute Raman measurements are strongly discouraged and why the photometric precision specification is set relatively loosely. The tolerance of photometric



precision required for a given measurement should be assessed during the method-development stage.

#### performance qualification

The objective of performance qualification is to ensure that the instrument is performing within specified limits with respect to wavelength precision, photometric axis precision, and sensitivity. In certain cases when the instrument has been set up for a specific measurement (for example, installed in a process reactor), it might no longer be possible or desirable to measure the wavelength and photometric (signal) qualification reference standards identified above. Provided instrument operational qualification has shown that the equipment is fit for use, a single external performance verification standard can be used to reverify function on a continuing basis (for example, a routinely used process solvent signal, for both wavelength and photometric precision, following reactor cleaning). The performance verification standard should match the format of the samples in the current analysis as closely as possible and use similar spectral acquisition parameters. Quantitative measurements of an external performance verification standard spectrum check both the wavelength (x-axis and laser wavelength) and the photometric (signal) precision. Favorable comparison of a series of performance verification spectra demonstrates proper continued operation of the instrument.

#### wavelength precision

The wavelength precision should be measured by collecting data for a single spectrum of the selected Raman shift standard for a period equal to that used in the photometric consistency test. When appropriate, powdered samples should be repacked between each set of measurements. Peak positions across the spectral range of interest are used to calculate precision. Performance is verified by matching the current peak positions to those collected during the previous instrument qualification and should not vary with a standard deviation of more than  $\pm 0.3 \text{ cm}^{-1}$ , although this specification can be adjusted according to the required accuracy of the measurement.

#### photometric precision

The photometric precision should be measured by collecting data for a single spectrum of a suitably verified reference standard material for a specified time. After suitable baseline correction, the areas of a number of bands across the spectral range of interest should be calculated by means of an appropriate algorithm. The area of the strongest band is set to 1, and all other envelopes are normalized to this band. Performance is verified by matching the current band areas to the respective areas collected during the previous instrument qualification. The areas should vary by no more than 10%, although this specification can be adjusted according to the required accuracy of the measurement.

#### laser power output precision and accuracy

This test is applicable only to Raman instruments with automatic, internal laser power meters. Instruments without laser power measurement should utilize a calibrated laser power meter from a reputable supplier. The laser output should be set to a representative output, dictated by the requirements of the analytical measurement and the laser power measured. The output should be measured and checked against the output measured at instrument qualification. The power (in milliwatts or watts) should vary by no more than 25% compared to the qualified level. If the power varies by more than this amount, the instrument should be serviced (as this variation might indicate, among other things, a gross misalignment of the system or the onset of failure of the laser).

For instruments with an automatic, internal laser power meter, the accuracy of the values generated from the internal power meter should be compared to a calibrated external laser power meter at an interval of not more than 12 months. The internally calculated value should be compared to that generated by the external power meter. Performance is verified by matching the current value to that generated during the previous instrument qualification. The manufacturer might provide software to facilitate this analysis. If the instrument design prevents the use of an external power meter, then the supplier should produce documentation to ensure the quality of the instrument and provide a recommended procedure for the above analysis to be accomplished during a scheduled service visit.

### METHOD VALIDATION

Validation of Raman methods will follow the same protocols described in [Validation of Compendial Procedures \(1225\)](#) in terms of accuracy, precision, etc. However, several of these criteria are affected by variables specific to Raman spectrometry. Fluorescence is the primary variable that can affect the suitability of a method. The presence of fluorescent impurities in samples can be quite variable and have little effect on the acceptability of a material. The method must be flexible enough to accommodate different sampling regimes that may be necessary to minimize the effects of these impurities.

Detector linearity must be confirmed over the range of possible signal levels. Fluorescence might drive both the signal baseline and the noise higher than that used in the validation, in which case the fluorescence must be decreased, or the method modified to accommodate the higher fluorescence levels. This is also true for the precision, limit of detection, and limit of quantification of the method, as increased baseline noise will negatively impact all of these values. Because fluorescence can also affect quantification caused by baseline shifts, acceptable quantification at different levels of photobleaching, when used, should also be confirmed.

The impact of the laser on the sample must be determined. Visual inspection of the sample and qualitative inspection of the Raman spectrum for measurements with differing laser powers and exposure times will confirm that the sample is not being altered (other than by photobleaching). Specific variables to confirm in the spectrum are shifts in peak position, changes in peak height and band width, and unexpected changes in background intensity.

Method precision must also encompass sample position. The sample presentation is a critical factor for both solids and liquids, and must be either tightly controlled or accounted for in the calibration model. Sample-position sensitivity can often be minimized by appropriate sample preparation or sample holder geometry, but will vary from instrument to instrument based on excitation and collection optical configuration.

### DEFINITION OF TERMS AND SYMBOLS

calibration model is a mathematical expression that relates the response from an analytical instrument to the properties of samples.

instrument bandpass (or resolution) is a measure of the capability of a spectrometer to separate radiation of similar wavelengths.

operational qualification is the process by which it is demonstrated and documented that the instrument performs according to specifications, and that it can perform the intended task. This process is required following any significant change such as instrument installation, relocation, major repair, etc.

performance qualification is the process of using one or more well-characterized and stable reference materials to verify consistent instrument performance. Qualification may employ the same or different standards for different performance characteristics.

raman spectra<sup>4</sup> are plots of the radiant energy, or number of photons, scattered by the sample through the indirect interaction between the molecular vibrations in the sample and monochromatic radiation of frequency much higher than that of the vibrations. The abscissa is usually the difference in wavenumber between the incident and scattered radiation.

(normal) raman scattering<sup>4</sup> is the inelastic scattering of radiation that occurs because of changes in the polarizability, of the relevant bonds during a molecular vibration. Normal Raman spectra are excited by radiation that is not in resonance with electronic transitions in the sample.

raman wavenumber shift<sup>4</sup>,



is the wavenumber of the exciting line minus the wavenumber of the scattered radiation. SI unit: m<sup>-1</sup>. Common unit: cm<sup>-1</sup> = 100 m<sup>-1</sup>.



where  $\beta$  is the differential Raman cross section, is positive for Stokes scattering and negative for anti-Stokes scattering.

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1 ASTM E1840-96 (2002) Standard Guide for Raman Shift Standards for Spectrometer Calibration, ASTM International, 100 Barr Harbor Drive, PO Box C700, West Conshohocken, PA, USA 19428-2959.

2 NIST-traceable tungsten white light source statement: While the calibration of the Raman frequency (or Raman shift, cm<sup>-1</sup>) axis using pure materials and an existing ASTM standard is well accepted, techniques for calibration of the Raman intensity axis are not. Intensity calibrations of Raman spectra can be accomplished with certified white light sources.

3 NIST SRM 2241: Ray KG, McCreery RL. Raman intensity correction standard for systems operating with 785-nm excitation. *Appl. Spectrosc.* 1997, 51, 108-116.

4 Chalmers, J., Griffiths, P., Eds. *Handbook of Vibrational Spectroscopy*; John Wiley & Sons, Ltd: New York, 2002.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Gary E. Ritchie, M.Sc.</a> Scientific Fellow 1-301-816-8353	(GC05) General Chapters 05

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#### 1121 NOMENCLATURE

The USP (or NF) titles for monograph articles are legally recognized under the Federal Food, Drug, and Cosmetic Act as the designations for use in labeling the articles to which they apply.

The value of designating each drug by one and only one nonproprietary<sup>1</sup> name is important in terms of achieving simplicity and uniformity in drug nomenclature. In support of the U.S. Adopted Names program (see Mission and Preface in USP–NF), of which the U.S. Pharmacopeial Convention is a cosponsor, the USP Council of Experts gives consideration to the adoption of the U.S. Adopted Name, if any, as the official title for any compound that attains compendial recognition.

A compilation of the U.S. Adopted Names (USAN) published from the start of the USAN program in 1961, as well as other names for drugs, both current and retrospective, is provided in the USP Dictionary of USAN and International Drug Names. This publication serves as a book of names useful for identifying and distinguishing all kinds of names for drugs, whether public, proprietary, chemical, or code-designated names.<sup>2</sup>

A nonproprietary name of a drug serves numerous and varied purposes, its principal function being to identify the substance to which it applies by means of a designation that may be used by the professional and lay public free from the restrictions associated with registered trademarks. Teaching in the health sciences requires a common designation, especially for a drug that is available from several sources or is incorporated into a combination drug product; nonproprietary names facilitate communication among healthcare providers; nonproprietary names must be used as the titles of the articles recognized by official drug compendia; a nonproprietary name is essential to the pharmaceutical manufacturer as a means of protecting trademark rights in the brand name for the article concerned; and, finally, the manufacturer is obligated by federal law to include the established nonproprietary name in advertising and labeling.

Under the terms of the Drug Amendments of 1962 to the Federal Food, Drug, and Cosmetic Act, which became law October 10, 1962, the Secretary of Health and Human Services is authorized to designate an official name for any drug wherever deemed "necessary or desirable in the interest of usefulness and simplicity."<sup>3</sup>

The Commissioner of Food and Drugs and the Secretary of Health and Human Services published in the Federal Register regulations effective November 26, 1984, which state, in part:

"Sec. 299.4 Established names of drugs."

"(e) The Food and Drug Administration will not routinely designate official names under section 508 of the act. As a result, the established name under section 502(e) of the act will ordinarily be either the compendial name of the drug or, if there is no compendial name, the common and usual name of the drug. Interested persons, in the absence of the designation by the Food and Drug Administration of an official name, may rely on as the established name for any drug the current compendial name or the USAN adopted name listed in USAN and the USP Dictionary of Drug Names.<sup>4</sup>

It will be noted that the monographs on the biologics, which are produced under licenses issued by the Secretary of the U.S. Department of Health and Human Services, represent a special case. Although efforts continue toward achieving uniformity, there may be a difference between the respective title required by federal law and the USP title. Such differences are fewer than in past revisions of the Pharmacopeia. The USP title, where different from the FDA Center for Biologics Evaluation and Research title, does not necessarily constitute a synonym for labeling purposes; the conditions of licensing the biologic concerned require that each such article be designated by the name appearing in the product license issued to the manufacturer. Where a USP title differs from the title in the federal regulations, the former has been adopted with a view to usefulness, simplicity, and conformity with the principles governing the selection of monograph titles generally.



Change to read:

#### GENERAL NOMENCLATURE FORMS

Some monograph titles existing in the USP-NF do not conform to the formats outlined in this general information chapter. Typically, these monograph titles were adopted before the establishment of the title formats and nomenclature policies presented in this general information chapter. Such monograph titles may be subject to subsequent revision and should not be interpreted as precedents for other monograph titles.

Standardized forms of nomenclature have been devised in the interest of achieving uniformity for naming compendial articles. The general nomenclature forms that follow illustrate the terminology used throughout the official compendia for consistency in establishing titles of monographs on official pharmaceutical dosage forms and preparations. Examples are shown for the more frequently encountered categories of dosage forms.

For a variety of dosage forms, titles are in the following general form: [DRUG] [ROUTE OF ADMINISTRATION] [DOSAGE FORM].

Examples:

Calcium Carbonate Oral Suspension  
Cetylpyridinium Chloride Topical Solution  
Dexamethasone Ophthalmic Suspension  
Epinephrine Bitartrate Ophthalmic Solution  
Isosorbide Dinitrate Sublingual Tablets  
Miconazole Nitrate Topical Powder  
Triple Sulfa Vaginal Cream

▲The term "Vaginal Inserts", rather than "Vaginal Tablets", "Vaginal Capsules", or "Vaginal Suppositories" is used in the title of this type of vaginal preparation to avoid the potential for misuse of these products if the term "Tablets" or "Capsules" or "Suppositories" were to appear in the title.

Example:

Clotrimazole Vaginal Inserts  
▲USP32

The term for route of administration is omitted for those dosage forms for which the route of administration is understood. The general form then becomes simply [DRUG] [DOSAGE FORM]. Thus, capsules, tablets, and lozenges are administered via the oral route unless otherwise indicated by the title.

Examples:

Acetaminophen Capsules  
Aminophylline Delayed-Release Tablets  
Aspirin Extended-Release Tablets  
Hexylresorcinol Lozenges  
Meperidine Hydrochloride Tablets

Drugs that are injected may be administered via the intravenous, intramuscular, subcutaneous, etc., route; the route being specified in the labeling rather than in the name.

Examples:

Aurothioglucose Injectable Suspension  
Epinephrine Injection  
Fluorouracil Injection  
Hydrocortisone Acetate Injectable Suspension  
Phytonadione Injectable Emulsion

Creams, ointments, lotions, and pastes are applied topically, unless otherwise indicated by the name.

Examples:

Benzoyl Peroxide Lotion  
Betamethasone Dipropionate Cream  
Estradiol Vaginal Cream  
Nystatin Ointment  
Zinc Oxide Paste

▲The term "Suppositories" is used in the titles of preparations that are intended for rectal administration.

Example:

Aspirin Suppositories  
▲USP32

The term "for" is included in names, as appropriate, of preparations for which a solid drug substance must be dissolved or suspended in a suitable liquid to obtain a dosage form, and the general form becomes [DRUG] for [ROUTE OF ADMINISTRATION] [DOSAGE FORM].

Examples:

Ampicillin for Oral Suspension  
Epinephrine Bitartrate for Ophthalmic Solution  
Nafcillin for Injection  
Spectinomycin for Injectable Suspension

In some instances, the drug is supplied in one dosage form for the preparation of the intended dosage form.

Examples:

Aspirin Effervescent Tablets for Oral Solution  
Methadone Hydrochloride Tablets for Oral Suspension



## Papain Tablets for Topical Solution

Systems are preparations of drugs in carrier devices that are applied topically or inserted into body cavities, from which drugs are released gradually over extended times, after which the carrier device is removed. The general form for a system is [DRUG] [ROUTE] [SYSTEM].

Examples:

Nicotine Transdermal System  
Progesterone Intrauterine Contraceptive System  
▲▲USP32

Some drugs are available as concentrated solutions that are not intended for direct administration to humans or animals, but are to be diluted with suitable liquid vehicles to obtain the intended preparation. The general form for these preparations, which are not dosage forms, is [DRUG] [CONCENTRATE].

Examples:

Isosorbide Concentrate (used to prepare Isosorbide Oral Solution)  
Glutaral Concentrate (used to prepare Glutaral Disinfectant Solution)

For products intended for parenteral administration, the use of the word "Concentrate" in the monograph title is restricted to one specific monograph, Potassium Chloride for Injection Concentrate. The word "Concentrate" should not appear in the monograph title for any other parenteral product; rather, this issue is to be addressed in the product labeling.

Some drugs are supplied as preparations that may be intermediates used for convenience in formulating finished dosage forms. The general form for such preparations, which are not finished dosage forms, is [DRUG] [PREPARATION].

Examples:

Vitamin E Preparation  
Cranberry Liquid Preparation

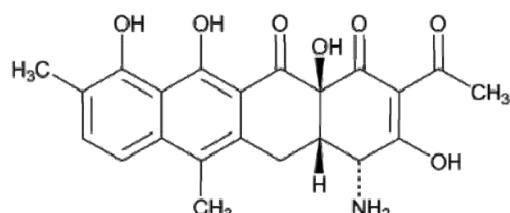
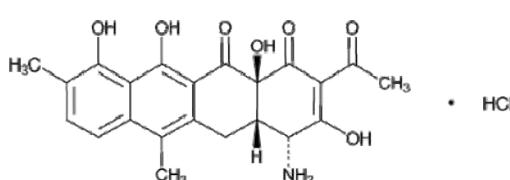
## MONOGRAPH NAMING POLICY FOR SALT DRUG SUBSTANCES IN DRUG PRODUCTS AND COMPOUNDED PREPARATIONS

The titles of USP monographs for drug products and compounded preparations formulated with a salt of an acid or base use the name of the active moiety, as defined below. The strength of the product or preparation also is expressed in terms of the active moiety.

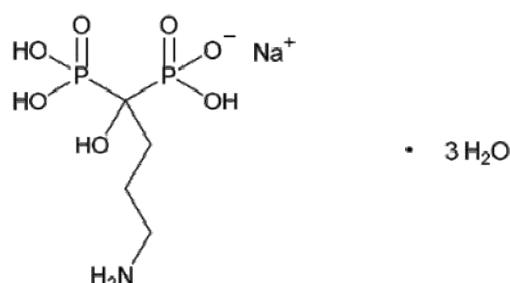
An active moiety is the molecule or ion, excluding those appended portions of the molecule that cause the drug to be an ester, salt (including a salt with hydrogen or coordination bonds), or other noncovalent derivative (such as a complex, chelate, or clathrate) of the molecule, responsible for the physiological or pharmacological action of the drug substance, without regard to the actual charged state of the molecule in-vivo.

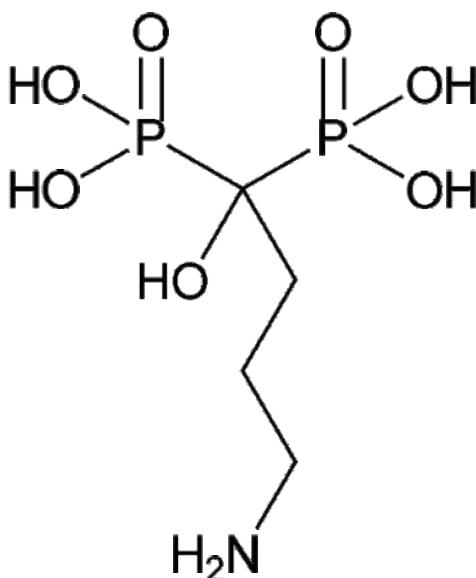
For example, the active moiety of a hydrochloride salt of a base will be the free base and not the protonated form of the base. The active moiety of a metal acid salt will be the free acid.

i. Example: Chelocardin Hydrochloride active moiety is Chelocardin



ii. Example: Alendronate Sodium active moiety is Alendronic Acid





This Policy is followed by USP in naming drug products and compounded preparations that are newly recognized in the USP. Revising existing monographs to conform to this Policy is not intended, except where the USP Council of Experts determines that, for reasons such as safety, a nomenclature change is warranted.

#### Related Issues

**Labeling**—The labeling clearly states the specific salt form of the active moiety that is present in the product/preparation, as this information may be useful to practitioners and patients. The names and strengths of both the active moiety and specific salt form (where applicable) are provided in the labeling.

**Exceptions**—In those rare cases in which the use of the specific salt form of the active moiety in the title provides vital information from a clinical perspective, an exception to this Policy may be considered. In such cases, where the monograph title contains the specific salt form of the active moiety, the strength of the product or preparation also is expressed in terms of the specific salt form.

#### POLICY FOR POSTPONEMENT SCHEDULES

It is the practice of USP to postpone the official dates of nomenclature and labeling revisions for a reasonable time primarily to allow for product label changes to be made and to allow health practitioners and consumers time to become familiar with the new terminology. A postponement period of 18 months is usually applied when only one or a small number of products is affected. A postponement period of 30 months is usually applied when names or labeling of multisource products or multiproduct lines of a firm's preparations are being changed. A postponement period of 60 months is usually applied for title and labeling changes that affect excipients, because such changes would require relabeling of very large numbers of prescription-only and OTC preparations.

There may be exceptions to this postponement schedule where a shorter time is needed in order to specify nomenclature and labeling changes in cases where public health and safety are a concern.

The assignment of a postponement schedule is handled by the USP Expert Committee on Nomenclature. The postponement schedules are presented below. USP's implementation of a postponement schedule is automatic, unless an exception is sought. Exceptions to the postponement schedule are rarely made, and must have suitable justification as well as the approval of the Expert Committee on Nomenclature. Any questions or concerns regarding this postponement schedule may be addressed to the USP staff liaison assigned to the Expert Committee on Nomenclature.

18 months—Schedule for title and labeling changes for a drug product. One or few companies are involved. Example: Sterile [Drug] change to [Drug] for Injection.

30 months—Schedule for title and labeling changes for prescription-only and OTC products.

1. Extensive product line for a company. Examples: syrups and elixirs.
2. Several companies are involved. Examples: syrups and elixirs; lotions; sunscreens.

60 months—Schedule for title and labeling changes for excipient monographs. Ingredient names in numerous multisource products are affected.

1 The term "generic" has been widely used in place of the more accurate and descriptive term "nonproprietary" with reference to drug nomenclature.

2 USP Dictionary of USAN and International Drug Names is obtainable on order from U.S. Pharmacopeia, Customer Service Department, 12601 Twinbrook Parkway, Rockville, MD 20852.

3 F.D.&C. Act, Sec. 508 [358].

4 53 Fed. Reg. 5369 (1988) amending 21 CFR § 299.4.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Andrzej Wilk, Ph.D.</a> Scientist 1-301-816-8305	(NOM05) Nomenclature 05

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1125 NUCLEIC ACID-BASED TECHNIQUES—GENERAL

#### SCOPE

Nucleic acid-based assays are used in a variety of settings, the most common of which include the detection of infectious agents (viruses, bacteria, etc.), and cellular materials, as well as disease profiling. More recently such assays have also been used for forensic purposes and for the detection of trace contamination in biological materials. The latter include



pharmaceutical development applications, such as viral clearance and adventitious agent testing in vaccine seed lots and tissue culture cell banks. This chapter introduces a series of general information chapters that provide techniques that support procedures for the detection and analysis of nucleic acids (see [Figure 1](#)). The assays using these techniques may be presented in a USP general chapter or in a private specification.

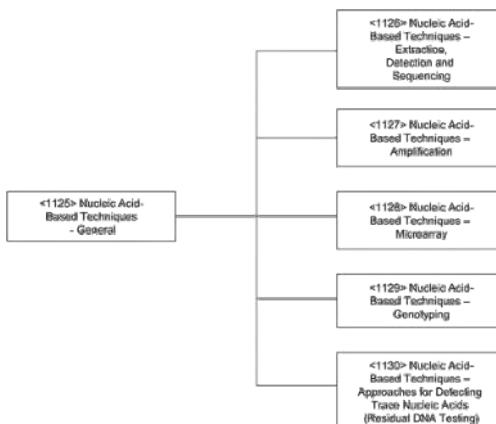


Figure 1

The major requirement for any nucleic acid analytical procedure is the availability of pure, intact nucleic acids for analysis. The information in [Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing](#) (1126) discusses procedures available for nucleic acid extraction and handling. Hybridization is the core mechanism underlying many molecular biology techniques, and in addition to the detection of nucleic acids by absorbance and fluorescence measurements and size measurement by gel electrophoresis, this chapter also covers blotting and identification of nucleic acid species by hybridization assays using labeled probes. Hybridization probes are oligonucleotides that have a sequence that is complementary to the target of interest. Probes contain radioactive, fluorescent, biotin, digoxigenin, or other tags that, upon binding of the probe to the target, allow visualization and identification of the target. Probes are capable of detecting target sequences that are present in concentrations too low to be detected by absorbance measurements or gel electrophoresis.

These analytical procedures require a minimum quantity of nucleic acid, typically in the nanogram to microgram range. However, in the vast majority of cases, e.g., in the detection of viruses or rare cellular RNA species, the nucleic acid under assay is present in minute quantities (in the picogram to femtogram range), and an amplification step must be performed before the nucleic acid can be detected and identified. The amplification step may be directed either at the signal used for detection (signal amplification), such as the branched DNA assay (bDNA assay), or at the target as in nucleic acid amplification technologies (NAT).

In 1983 a revolutionary yet simple process termed polymerase chain reaction (PCR) was developed for amplifying the number of specific nucleic acid fragments present in a sample, and in just a few years after its discovery PCR became the most frequently used procedure for amplifying nucleic acids, especially DNA. Since the inception of PCR, the number of applications has expanded rapidly, and the technique, which now includes quantitative and multiplex assays, is currently used in almost every field of research and development in biology and medicine. Numerous variations of assay procedures have been developed for specific analytes. The general information chapter, [Nucleic Acid-Based Techniques—Amplification](#) (1127), describes amplification procedures used for DNA and RNA analysis as well as qualitative and quantitative NAT assays. Signal amplification procedures in which the signals, typically fluorescent signals, are used to detect the nucleic acid of interest, are not very common. The major signal amplification procedure, the branched DNA or bDNA assay, is used predominantly for viral nucleic acid detection.

Quality assurance aspects of the methodology are also covered, together with a summary of current regulatory requirements for NAT assays. The need for globally comparable, accurate, and reliable results in the diagnostics field has driven the quest for, and development of, national and international standards within an increasingly sophisticated and metrologically sound, highly developed international regulatory environment devoted to the highest standards of regulatory science. Because NAT has become the most widely used of nucleic acid techniques, the majority of guidance documents and standards are related to NAT. The general information chapter, [Nucleic Acid-Based Techniques—Microarrays](#) (1128), addresses a still-emerging field that is of increasing relevance to molecular DNA analysis. Detailed treatment of various microarrays, including data analysis and validation, are excluded from (1128) at this time. The general information chapter, [Nucleic Acid-Based Techniques—Genotyping](#) (1129), focuses on the specific modifications of the techniques that are necessary to enable detection of single base differences and common genetic variations, e.g., single nucleotide polymorphisms (SNPs). The final general information chapter in the series, [Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids \(Residual DNA Testing\)](#) (1130), describes residual DNA testing in the context of pharmaceutical manufacturing. Applications relevant to viral adventitious agents, however, are discussed in the general information chapter [Virology Test Methods](#) (1237).

Two major uses of nucleic acid testing are excluded from this family of NAT chapters: viral testing for blood and blood product safety and genetic testing. The traditional perspective of USP is to develop public standards that can be applied to a particular final product without expressively defining a product and/or its production details. This chapter aims to specify when traditional methodologies or existing standards can be adapted. Novel methodologies for amplification and detection by NAT are also highlighted. As these new methodologies become mature and properly validated, they will be included in subsequent revisions.

Due to rapid development in the field, compendial and regulatory affairs scientists are advised to consult the current edition of USP and its Supplements regularly.

#### APPENDIX: REGULATIONS AND STANDARDS

Nucleic acid-based techniques have rapidly transformed almost every field of research, pharmaceutical development, and diagnostics. The need for globally comparable, accurate, and reliable results in the diagnostic field has driven the development of national and international standards as well as fostered a highly developed regulatory environment. Because NAT has become the most widely used of nucleic acid techniques, the majority of guidance documents and standards are related to NAT.<sup>1</sup> Virus-specific regulations and reference standards will be addressed in the Appendix to General Information chapter [Virology Test Methods](#) (1237). The following is a selective list of national guidance documents. For application-specific guidance the compendial user is referred back to the relevant regulatory agency for the most current guidance.

- FDA Center for Biologics Evaluation (CBER) "Review Criteria for Nucleic Acid Amplification-Based In Vitro Diagnostic Devices for Direct Detection of Infectious Microorganisms" (1993)
- FDA Center for Biologics Evaluation (CBER) "Guidance for Industry: Content and Format of Chemistry, Manufacturing and Controls Information and Establishment Description Information for a Biological In Vitro Diagnostic Product" (1999)
- FDA Center for Biologics Evaluation (CBER) "Guidance for Industry: In the Manufacture and Clinical Evaluation of In Vitro Tests to Detect Nucleic Acid Sequences of Human Immunodeficiency Viruses Types 1 and 2" (1999)
- FDA Center for Biologics Evaluation (CBER) "Guidance for Industry: Use of Nucleic Acid Tests on Pooled and Individual Samples from Donations of Whole Blood and Blood Components (including Source Plasma and Source Leukocytes) to Adequately and Appropriately Reduce the Risk of Transmission of HIV-1 and HCV" (2004)

#### GLOSSARY

3'-5' exonuclease activity—Enzymatic activity to remove a mispaired nucleotide from the 3' end of the growing strand. The reaction is a hydrolysis of a phosphoester bond. The presence of a 3'-5' exonuclease, or proofreading, activity improves the fidelity of the polymerization.



3' exonuclease activity— Enzymatic activity to remove a mispaired nucleotide from the 5' end of a polynucleotide strand. This activity is actually that of a single-strand-dependent endonuclease and is needed to remove RNA primers of Okazaki fragments, the RNA strand in the intermediate DNA–RNA heteroduplex during reverse transcription, and during DNA repair.

absorbance [Symbol: A] —The logarithm, to the base 10, of the reciprocal of the transmittance (T). [note—Descriptive terms used formerly include optical density, absorbancy, and extinction.]

accuracy— The accuracy of an analytical procedure is the closeness of test results obtained by that procedure to the true value.

allele— One of two or more alternative forms of a gene at a given position (locus) on a chromosome, caused by a difference in the sequence of DNA.

amplicon— A short segment of DNA generated by the PCR process whose sequence is defined by forward and reverse primers. Sometimes referred to as an amplimer.

amplification— The enzymatic in vitro replication of a target nucleic acid.

annealing— Hybridizing or binding of complementary nucleic acids, usually at an optimal temperature.

concatenation— The process in which a DNA segment composed of repeated sequences is linked end-to-end.

complementary dna (cdna)— DNA synthesized from an RNA template in an enzymatic reaction catalyzed by the enzyme reverse transcriptase.

denaturation— The process of separating double-stranded DNA into single strands by breaking the hydrogen bonds. This is typically accomplished by heating the DNA solution to temperatures greater than 90° or by treating it with strong alkali.

deoxyribonucleic acid (dna)— The genetic material that is passed from parent to daughter cells and propagates the characteristics of the species in the form of genes it contains and the proteins for which it codes. DNA contains the following four deoxyribonucleosides: dA, dC, dT, and dG.

deoxyribonucleotide triphosphate (dntp)— A base that is added to a primer during the PCR that comprises the newly synthesized strand. Examples of dNTPs are dATP, dUTP, dCTP, dGTP, and dTTP.

detection limit— It is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions.

dna polymerase— An enzyme that can synthesize new complementary DNA strands using a DNA template and primer. Several of these enzymes are commercially available, including Taq DNA polymerase and rTth DNA polymerase.

endonuclease— An enzyme that cleaves phosphodiester bonds in a polynucleotide chain.

energy transfer— This describes the process in which an excited state of one molecular entity (the donor) is deactivated to a lower-lying state by transferring energy to a second molecular entity (the acceptor), which is thereby raised to a higher energy state.

extension— Refers to the elongation of the DNA chain that is being synthesized using the parent DNA strand as the template for synthesis of that daughter strand. This is a natural process that occurs during DNA replication. Extension occurs during the PCR process with DNA polymerases.

extinction coefficient [Symbol: E] —The quotient of the absorbance (A) divided by the product of the concentration, expressed in moles/L, of the substance and the absorption path length, in cm. [note—Terms formerly used include molar absorbancy index; molar absorptivity; and molar absorption coefficient.]

fidelity— Fidelity is a measure of the accuracy of nucleic acid replication. The polymerase enzyme used is only one of the elements that influences fidelity. Other elements include buffer conditions, thermal cycling parameters, number of cycles, efficiency of amplification, and the sequence of the DNA being copied.

fluorophore— A functional group in a molecule that makes the molecule fluorescent by absorbing energy of a specific wavelength and re-emits the energy at another wavelength.

fluorescence— The emission of one or more photons by a molecule or atom activated by the absorption of a quantum of electromagnetic radiation. X-rays, UV, visible light, and IR radiations may all stimulate fluorescence. For details on the spectroscopic measurement of fluorescence, see [Spectrophotometry and Light-Scattering](#) 851.

genome— The complete genetic complement or the complete set of instructions for reproducing an organism and carrying out its biological function in life. The DNA in our cells comprises our genome. When our cells divide, the complete genome in our cells is duplicated for transmission to each of the remaining daughter cells.

genotype— The genetic constitution of an organism as revealed by genetic or molecular analysis, i.e., the complete set of genes, both dominant and recessive, possessed by a particular cell or organism.

genotyping— The process of assessing genetic variations present in an individual.

hairpin— Antiparallel duplex structure that forms by pairing of inverted repeat sequences within a single-stranded nucleic acid. The helical section is called the stem and the unpaired base segment at the end of the structure is called the loop.

hot-start pcr— Technique that is commonly used to improve the sensitivity and specificity of PCR amplification. A hot start is performed by withholding from the reaction mix a key component necessary for amplification until the reaction reaches a temperature above the optimal annealing temperature of the primers. The component withheld from the reaction mix can be primers, DNA polymerase, MgCl<sub>2</sub>, or dNTPs.

hybridization— The process of forming a double-stranded nucleic acid molecule, for example between a nucleotide sequence (probe) and a target.

ligation— The process of joining two or more DNA fragments.

melting temperature (T<sub>m</sub>)— The temperature at which 50% of the DNA becomes single-stranded.

microarray— Sets of miniaturized chemical reaction areas that are used to test DNA fragments, antibodies, or proteins. Usually the probes are immobilized on a chip and hybridized with target.

mismatch— Unconventional base pairing (other than C with G, and A with T or U). A mismatched base pair has lower bonding energy and decreases the stability of the DNA molecule.

nucleic acid— Linear polymers of nucleotides, linked by 3', 5' phosphodiester linkages. In DNA, deoxyribonucleic acid, the sugar group is deoxyribose, and the bases consist of adenine, guanine, thymine, and cytosine. RNA, ribonucleic acid, has ribose as the sugar, and uracil replaces thymine.

oligonucleotide— Linear sequence comprising as many as 25 nucleotides joined by phosphodiester bonds, generally used as a DNA synthesis primer.

photobleaching— Photobleaching is the irreversible destruction of a fluorophore in the excited state. Different fluorophores have different rates of photobleaching. For example, fluorescein photobleaches very easily. Often the rate of decomposition is proportional to the intensity of illumination. A simple and practical way to overcome this is to reduce the incident radiation.

polymerase— An enzyme that catalyzes the synthesis of nucleic acids on pre-existing nucleic acid templates, assembling RNA from ribonucleotides or DNA from deoxyribonucleotides.

polymerase chain reaction (pcr)— A laboratory technique that rapidly amplifies a specific region of double-stranded DNA, predetermined by the pair of primers used for amplification. Generally involves the use of a heat-stable DNA polymerase.

precision— The degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample.

primer— Nucleic acid polymerases link a mononucleotide to a chain of nucleic acids, which is called the primer. RNA polymerases are able to use a single nucleotide as a primer, but DNA polymerases always require an oligonucleotide.

probe— A specific DNA or RNA sequence that has been labeled by radioactive, fluorescent, or chemiluminescent tags and is used to detect complementary sequences by



hybridization techniques such as blotting or colony hybridization. In addition, probes can also be used for quantitation of amplicons as described for quantitative PCR assays. A more detailed description of such probes is given in the general information chapter, [Nucleic Acid-Based Techniques—Amplification](#) (1127).

processivity— The ability of an enzyme to repetitively continue its catalytic function without dissociating from its substrate.

proofreading activity— Literally to read for the purpose of detecting errors for later correction. DNA polymerase has a 3' to 5' exonuclease activity that is used during polymerization to remove recently added nucleotides that are incorrectly paired.

quantitation limit— It is the lowest amount of analyte in a sample that can be determined with an acceptable precision and accuracy under the stated experimental conditions.

quenching— The process of extinguishing, removing, or diminishing a physical property such as heat or light. Fluorescence quenching can be either collisional or static.

reverse transcriptase— An enzyme that requires a DNA primer and catalyzes the synthesis of a DNA strand from an RNA template. An enzyme that can use RNA as a template to synthesize DNA.

reverse transcription (rt)— The process of making cDNA using an RNA template.

real-time pcr— May often be referred to as Quantitative PCR or Real-Time Quantitative PCR but not RT-PCR and is a procedure for simultaneous DNA quantitation and amplification. The generation of amplicons monitored as they are generated by the use of a fluorescent reporter system and captured by sophisticated instrumentation.

real-time (rt-pcr)— The combination of real-time PCR and reverse transcription PCR.

reverse transcriptase polymerase chain reaction (rt-pcr)— A variation of the PCR technique in which cDNA is made from RNA via reverse transcription. The cDNA is then amplified using standard PCR protocols.

ribonucleic acid (rna)— A type of nucleic acid composed of a specific sequence of ribonucleotides linked together. RNA contains the following four ribonucleosides: A, C, G, and U.

robustness— The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in procedural parameters listed in the procedure documentation and provides an indication of its suitability during normal usage.

rTth dna polymerase— Recombinant thermostable DNA polymerase originally isolated from the bacterium *Thermus thermophilus*. rTth has optimal activity at 70°–80° and survives the denaturation steps of PCR. In addition to DNA polymerase activity, it has efficient reverse transcriptase activity in the presence of manganese.

specificity— The ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components.

Taq dna— Thermostable DNA polymerase that is originally isolated from the bacterium *Thermus aquaticus*. Taq has optimal activity at 70°–80° and is not degraded during the high-heat denaturation steps of PCR.

template— A master copy used to start the DNA or RNA replication process.

transcription— The synthesis of RNA using a DNA template.

#### ABBREVIATIONS

AABB	American Association of Blood Banks
ACD	acid citrate dextrose
ASO	allele-specific oligonucleotides
bDNA	branched DNA assay
BMA	bone marrow aspirate
CE-LIF	capillary electrophoresis and laser-induced fluorescence
CCD	charge-coupled device
cDNA	complementary DNA
CPR	cyclic probe reaction
CsCl	cesium chloride
Ct	cycle threshold
DEPC	diethylpyrocarbonate
DHPLC	denaturing high-performance liquid chromatography
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
DMSO	dimethyl sulfoxide
dNTP	dinucleotide triphosphate
DOP-PCR	degenerated oligonucleotide primed PCR
dsDNA	double-stranded DNA
ssDNA	single-stranded DNA
dUTP	2'-deoxyuridine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
ESI	electrospray ionization
EDTA	ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
FEN	flap endonuclease
FISH	fluorescent in situ hybridization
FFPE	formalin-fixed paraffin embedded
FRET	fluorescence resonance energy transfer
GLP	good laboratory practice
HCV	hepatitis C virus



HIV	human immunodeficiency virus
ICH	International Conference on Harmonization
LAPS	light-addressable potentiometric sensor
LCR	ligase chain reaction
LED	light-emitting diode
LNA	locked nucleic acid
MALDI	matrix-assisted laser desorption-ionization
MDA	multiple-displacement amplification
MOPS	3-[N-morpholino]propanesulfonic acid
MS	mass spectrometry
mRNA	messenger RNA
NAT	nucleic acid amplification technologies
NASBA	nucleic acid sequence-based amplification
NTP	nucleotide triphosphate
OLA	oligonucleotide ligation assay
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEP	primer-extension-preamplification
PPi	pyrophosphate
QA	quality assurance
QC	quality control
RCA	rolling circle amplification
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
RT	reverse transcriptase
RT-PCR	reverse transcription-polymerase chain reaction
rTth	recombinant <i>Thermus thermophilus</i>
SDS	sodium dodecyl sulfate
SNP	single nucleotide polymorphism
3SR	self-sustained sequence replication
SSCP	single-strand conformation polymorphism
STR	short tandem repeat
Taq	<i>Thermus aquaticus</i>
Tm	melting temperature; the temperature at which 50% of the double-stranded nucleic acid molecule becomes single-stranded
TMA	transcription-mediated amplification
TOF	time-of-flight
UNG	uracil-N-glycosylase
WGA	whole-genome amplification

1 Reference materials for nucleic acid-based techniques are available from National Institute of Standards and Technology (NIST),  
<http://ts.nist.gov/measurementservices/referencematerials/index.cfm>.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

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1126 NUCLEIC ACID-BASED TECHNIQUES—EXTRACTION, DETECTION, AND SEQUENCING

## NUCLEIC ACID EXTRACTION

### Introduction

The basic principles of nucleic acid amplification technology (NAT) and definitions of the various techniques are covered in [Nucleic Acid-Based Techniques—General](#) 1125. The current chapter covers general steps in the extraction and purification of nucleic acids from a variety of samples.

The expanding discipline of molecular biology in pharmaceutical and biomedical research and development is characterized by the rapid discovery of new markers for disease and technologies for their detection. Nucleic acid targets are isolated from a wide variety of specimens, and the quality and quantity of the extracted target are highly affected by specimen collection, handling, and choice of extraction procedure.

The analysis of complex organisms by molecular biological techniques requires the isolation of pure, high molecular weight genomic DNA and intact full-length RNA. The application of these techniques then allows the detection, identification, and characterization of the associated organism or adventitious agent. Recently developed tests employing purified human DNA enable genetic testing for the presence, predisposition, or carrier status of inherited diseases such as cystic fibrosis, hereditary hemochromatosis, or Tay–Sachs disease, to name



a few examples, or the analysis of single nucleotide polymorphisms (SNPs).

DNase and RNase are the major sources of nucleic acid instability. Although both enzymes are ubiquitous and are easily released during nucleic acid extraction, RNases are far more stable and harder to inactivate than are DNases because they generally do not require co-factors in order to function. Minute amounts of RNase are sufficient to destroy RNA, so great care should be taken to avoid inadvertently introducing these enzymes into the sample during or after the isolation procedure. If RNA is collected for the specific application of gene expression analysis, researchers should keep in mind that the sample collection process itself can alter the resulting expression profile.

Because of the ubiquity of RNases, measurement of intracellular RNA targets has lagged behind that of DNA targets in contributing to patient management and characterization of targets for pharmaceutical purposes. However, RNA represents the current status of the organism and is an important tool for correlating a phenotype with its associated genetic activity. The unstable nature of RNA has made standardization of NAT tests difficult, and false negative results can easily arise from a poorly handled sample because of target degradation rather than from the absence of disease or regulation of gene activity. Nevertheless, commercially available isolation and detection systems provide a high level of standardization and robustness, resulting in the implementation of RNA-based assays in recent years. The following sections discuss general steps in the extraction and purification of nucleic acids from a variety of samples, focusing on (1) collection, handling and storage of samples; (2) disruption of samples; (3) subsequent extraction and purification of nucleic acids; and (4) storage of purified nucleic acids.

#### Sample Source

The broad diversity of possible specimens requires different procedures for collection. For example, blood samples are collected in an appropriate anticoagulant- or additive-containing tube. Ethylenediaminetetraacetic acid (EDTA) and acid citrate dextrose (ACD) are the recommended anticoagulants for tests that require plasma or bone marrow aspirate (BMA) samples. When extraction from tissues is appropriate, the optimal amount of tissue is usually 1 to 2 g, depending on the type of tissue, because the amount of DNA and RNA per weight of tissue varies greatly from tissue to tissue. In general, more than 10 mg of tissue is required to obtain >10 µg of DNA or RNA. Because of the highly variable amounts and types of proteins and other contaminants present in different tissues, nucleic acid isolation protocols are tissue-specific, and a broad range of ready-to-use isolation systems are available from different manufacturers of kits for nucleic acid extraction. The tissue type also influences the stability of both DNA and RNA in specimens, and the two types of nucleic acid differ importantly with respect to sample preparation and downstream analysis. These issues are described later in the chapter.

#### Pre-Analytical Steps and Sample Collection

Although the genetic makeup of the organism remains mostly unchanged over time, the mRNA population represents the current status of a cell under any given set of conditions, and thus is highly dynamic. To prevent degradation of mRNA and/or to preserve the original transcription pattern of the cellular mRNA, tissue should be placed immediately on ice or snap-frozen in liquid nitrogen. However, freezing disrupts the cellular structure and releases RNases. Hence, for RNA isolation in general (mRNA, ribosomal RNA, viral RNA, etc.), thawing in an RNase-inactivating buffer is essential. A more convenient procedure employs a stabilizing agent at ambient temperature. Several reagents for different types of sample material (e.g., tissue or bacteria) are commercially available. Vanadium salts were once used to inhibit RNase activity, but they have been superseded by the use of chaotropic agents for the inhibition of RNase and stabilization of RNA. The sample can easily be collected in such reagents and stored for several days to weeks prior to RNA isolation.

For reliable gene-expression analysis, the immediate stabilization of the RNA expression pattern and of the RNA itself is an absolute prerequisite. Directly after the biological sample is harvested or extracted, changes in the gene-expression pattern occur because of specific and nonspecific RNA degradation as well as transcriptional induction. Such changes in the gene-expression pattern should be avoided for all reliable quantitative gene-expression analyses, such as biochip and array analyses and quantitative reverse transcription-polymerase chain reaction (RT-PCR).

The use of gloves while handling reagents and RNA samples is mandatory to prevent RNase contamination arising from contact with the surface of the skin or from laboratory equipment. In order to create and maintain an RNase-free environment, laboratory personnel should treat water or buffer solutions with diethylpyrocarbonate (DEPC), which inactivates RNases by covalent chemical modification. Care should be taken because DEPC is irritating to the eyes, skin, and mucous membranes and is also a suspected carcinogen. Alternatively, commercially available RNase-free solutions and reagents may be used. Commercially available RNase inhibitor proteins are also available for use in reactions but with different levels of effectiveness with respect to various RNase types. However, it should be noted that DEPC cannot be used with Tris-buffered solutions. Many scientists recommend the use of disposable vessels when working with RNA. Nondisposable glassware should be cleaned with a detergent, thoroughly rinsed, and oven baked at 240° for 4 or more hours before use (autoclaving alone will not fully inactivate many RNases). Alternatively, glassware can also be treated with DEPC. Nondisposable plasticware should be thoroughly rinsed with 0.1 M sodium hydroxide and 1 mM EDTA, followed by RNase-free water. Alternatively, chloroform-resistant plasticware can be rinsed with chloroform to inactivate RNases. The use of aerosol-resistant filter tips is also important for avoiding RNase contamination. These issues are not critical for DNA, and following the rules of Good Laboratory Practice (GLP) is generally sufficient for successful isolation of DNA.

As a general precaution, staff should follow all applicable safety precautions when handling tissue or body fluids (human or other). Some of these precautions (e.g., the use of disposable gloves) also prevent contamination of the sample. Applicable guidelines and standards for the collection and processing of human-derived materials have been published by the American Association of Blood Banks, the International Conference on Harmonization, and the FDA.

#### Sample Disruption and Homogenization

Prior to extraction, source material is disrupted and homogenized. Disruption is the complete breakage of cell walls and plasma membranes of solid tissues and cells in order to release all DNA and RNA contained in the specimen. This is usually done using a lysis buffer that also inactivates endogenous nucleases. In addition to disrupting tissues, homogenization shears high molecular weight DNA and cellular components. During RNA isolation, scientists often must reduce the viscosity of cell lysates (caused by the presence of high molecular weight DNA molecules) prior to final isolation in order to make the subsequent extraction steps easier and more efficient. Incomplete homogenization may interfere with subsequent RNA purification steps (e.g., inefficient binding of RNA to silica membranes) and therefore result in significantly reduced yields. A typical procedure to shear high molecular weight DNA and homogenize the sample is to repeatedly pass the lysate through a small-gauge needle. However, this procedure is time-consuming and is not suitable for high throughput of samples. Better procedures to achieve complete disruption and homogenization of cells and tissue include rapid agitation in the presence of beads and lysis buffer (bead milling) or rotor-stator homogenization.

During the bead milling process, disruption and simultaneous homogenization occur by the shearing and crushing action of the beads as they collide with the cells. Disruption efficiency is influenced by the size and composition of the beads, the speed and configuration of the agitator, the ratio of buffer to beads, the disintegration time, and the amount of starting material. These parameters must be determined empirically for each application. For disruption with mortar and pestle, the samples should be frozen in liquid nitrogen and ground to a fine powder under liquid nitrogen. Standard safety precautions and the use of safety clothing to protect the skin and eyes should be employed when working with liquid nitrogen. Rotor-stator homogenizers are able to disrupt and homogenize animal and plant tissues within 5 to 90 seconds, depending on the sample. The rotor turns at very high speed, causing the sample to be disrupted by a combination of turbulence and mechanical shearing. Other alternatives are commercial spin-column homogenizers in combination with silica-membrane technology, which provide a fast and efficient way to homogenize cell and tissue lysates without cross-contamination of samples.

In order to achieve complete disruption, different sample types require different procedures. Cells from tissue culture grown as a monolayer or in suspension are easily disrupted by the addition of a lysis buffer that typically contains a mixture of an anionic detergent, a protease, and a chaotropic agent in a buffered salt solution. In contrast, nucleic acid isolation from fibrous tissues such as skeletal muscle, heart, and aorta can be difficult to disrupt because of the abundance of contractile proteins, connective tissue, and collagen. Fresh or frozen tissue samples should be cut into small pieces to aid lysis. Blood samples, including those treated to remove erythrocytes, can be efficiently lysed using a lysis buffer and a proteinase.

In general, the same procedures are applicable for extraction of DNA and RNA. For DNA isolation more gentle procedures are preferable, but during RNA isolation, cells and tissues can be disrupted using a mixer mill because there is no risk of shearing the RNA. Certain downstream applications require high molecular weight DNA, and care should be taken not to shear the DNA molecules and thus render the DNA unsuitable for further analysis.

#### Extraction and Purification

Although several procedures are available for nucleic acid extraction, the suitability of a procedure depends on the starting material, the type and purity of nucleic acid isolated, and possibly the downstream application. The principal procedures are described below; several commercial kits are available to accommodate different sample types and applications.

Phase Extraction— The original technique for extraction of DNA and RNA from lysed samples is phase extraction, which involves nucleic acid extraction using a mixture of phenol and chloroform. Depending on pH and salt concentration, either DNA or RNA partitions in the aqueous phase. At neutral/basic pH, the DNA remains in the aqueous phase, and RNA

...ains in the organic phase or in the interphase (with the proteins). However, at acidic pH, DNA in the sample is protonated, neutralizing the charge and causing it to partition into the organic phase. RNA, which remains charged, partitions in the aqueous phase. The two phases are separated by centrifugation, and the aqueous phase is re-extracted with a mixture of phenol and chloroform, followed by extraction with chloroform to remove any residual phenol. The nucleic acid is recovered from the aqueous phase by precipitation with alcohol. For RNA, this procedure is often combined with a protease digestion, alcohol or lithium chloride precipitation, and/or cesium chloride (CsCl) density gradients. A potential problem is contamination of the recovered DNA or RNA with organic solvents that may interfere with enzymatic downstream applications or spectrometry readouts.

**Cesium Chloride Density Gradient Centrifugation**— For the isolation of high molecular weight genomic DNA, CsCl density gradient centrifugation is the traditional procedure. Cells are lysed using a detergent, and the DNA is isolated from the lysate by alcohol precipitation. The DNA is then mixed with CsCl and ethidium bromide and centrifuged for several hours at a high g force (typically 100,000  $\times g$ ). The DNA band, which can be visualized under UV light as a result of the intercalation of the ethidium bromide with the DNA, is collected from the centrifuge tube, extracted with isopropanol to remove the ethidium bromide, and then precipitated with ethanol to recover the DNA. This procedure allows the isolation of high-quality DNA, but it is time consuming and also a safety concern because of the high quantity of EtBr involved.

**Anion-Exchange Chromatography**— An alternative procedure for the purification of high molecular weight genomic DNA is anion-exchange chromatography based on the interaction between the negatively charged phosphate groups of the nucleic acid and positively charged surface molecules on the anion-exchange resin. Binding occurs under low-salt conditions, and impurities such as RNA, cellular proteins, and metabolites are washed away using medium-salt buffers. Pure DNA is eluted with a high-salt buffer and is desalted and concentrated by alcohol precipitation. This procedure yields DNA of a purity and biological activity equivalent to two rounds of purification in CsCl gradients, but in much less time. The procedure also avoids the use of toxic substances, and it can be adapted for different scales of purification. DNA up to 150 kilobases (kb) in length may be isolated using this procedure. Several kits are available for the isolation of DNA based on anion-exchange technology, and procedures vary in processing times and the quality and size of the isolated DNA.

**Silica Technology**— The current procedure of choice for most applications is based on silica technology and can be used for isolation of full-length RNA or DNA with an average size of 20 to 50 kb. However, higher molecular weight DNA exceeding 100 kb is not efficiently extracted by this technology. The procedure relies on the selective adsorption of nucleic acids to silica in the presence of high concentrations of chaotropic salts. Although both types of nucleic acid adsorb to silica, the use of specific buffers in the lysis procedure ensures that only the desired nucleic acid is adsorbed while other nucleic acids, cellular proteins, and metabolites remain in solution. The contaminants are washed away, and high-quality RNA or DNA is eluted from the silica using a low-salt buffer. The silica matrix can be used as particles in suspension, in the form of magnetic beads, or as a membrane. This technique is suitable for high throughput, and several kits and automated systems are commercially available. However, these aqueous lysis buffers (in contrast to lysis buffers based on an organic solvent such as phenol) are not ideally suited for difficult-to-lyse samples (e.g., fatty tissues). Kits designed to facilitate lysis of fatty tissues and to inhibit RNases are available. Silica-based kits provide a fast and reliable procedure for both DNA and RNA purification and are commonly used for nucleic acid extraction.

Although these procedures yield pure nucleic acids, for some applications in which even trace contaminations with either RNA or DNA may interfere, pretreatment with DNase or RNase may be necessary. Alternatively, procedures that use specific probe capture may be used. Relevant applications requiring such ultra-pure nucleic acids are discussed in

#### [Nucleic Acid-Based Techniques—Amplification \(1127\)](#)

##### Specific Applications for Hard-to-Extract Materials

**Extraction from Formalin-Fixed and Paraffin-Embedded Biopsies**— The nucleic acids in formalin-fixed paraffin embedded (FFPE) biopsies are usually heavily fragmented and chemically modified by formaldehyde. Although formaldehyde modification cannot be detected in standard quality control assays such as gel electrophoresis, formaldehyde modification does interfere with enzymatic analyses. Sufficient extraction and demodification for DNA can be achieved by prolonged digestion with protease, but this will lead to heavy fragmentation and degradation of RNA. Some isolation systems have been optimized to reverse as much formaldehyde modification as possible without further RNA degradation. Nevertheless, RNA purified from FFPE samples should not be used in downstream applications that require full-length RNA. Some applications may require modifications to allow the use of fragmented RNA (e.g., designing small amplicons for RT-PCR).

**Extraction from Bacteria and Pathogens**— Although Gram-negative bacteria are relatively easy to lyse, Gram-positive bacteria or yeasts typically need an enzymatic pretreatment to remove the cell wall for efficient lysis. This methodology can be applied only to DNA isolation because the enzymatic treatment will influence the expression profile of the organism, and therefore RNA isolation requires a more rapid lysis procedure. Another factor to consider is that microorganisms normally occur against the background of a host or an environmental matrix (e.g., soil), which makes detection by polymerase chain reaction (PCR) often difficult because of inhibitory components. This means that the isolation procedure has to be carefully adapted and optimized for the specific organism and sample type. Commercial kits are available, and most are based on the use of lysozyme for the removal of cell walls.

**Special Considerations for Limited Sample Amounts**— Multiple genetic testing techniques, including SNP analysis, short tandem repeat analysis, sequencing or genotyping using arrays, real-time PCR, and other procedures depend on the availability of high-quality DNA. Because human genomic DNA or samples of individual genotypes are often limited, a process to immortalize nucleic acid samples can overcome this limitation. Procedures applicable to genotyping are discussed in [Nucleic Acid-Based Techniques—Genotyping \(1129\)](#). Whole-genome amplification (WGA) has recently been employed to amplify limited genomic DNA from already purified DNA or directly from clinical or casework samples without any DNA purification. Two basic technologies for WGA are available and are PCR-based or rely on isothermal multiple-displacement amplification. These applications are described in

more detail in [Nucleic Acid-Based Techniques—Amplification \(1127\)](#).

##### Sample Handling and Long-Term Storage

DNA is a relatively stable macromolecule, and once isolated it can be kept at 2° to 8° for at least 1 year. However, where DNA is present in very small quantities, such as in a test of residual DNA, it may be advisable to store the DNA at less than or equal to -20°. Generally, DNA is stored in solution. Distilled water can be used if DNA will be used for PCR and/or endonuclease digestion within a few days after its isolation. However, Tris-EDTA at pH 7.5–8.5 is the preferred buffer for DNA storage because DNA degradation can occur in water because of the limited buffering capacity of this medium. Purified nucleic acids retain recognizable characteristics during long-term storage, provided the samples are stored as frozen solutions. The DNA solution should be stored as a primary stock solution frozen at -80°. DNA can also be lyophilized and stored dry without the need for refrigeration. In some cases DNA can be stored for years on special filter papers that bind DNA and allow storage in a dried state at ambient temperature.

The ubiquity of RNases requires extra precautions when handling RNA. Isolated RNA should be kept on ice when aliquots are pipetted. Filter tips that prevent RNase carry-over from the pipette and sterile, disposable polypropylene tubes are recommended throughout the procedure because these tubes are generally RNase-free and do not require any pretreatment to inactivate RNases. Purified RNA can be stored at -20° or -80° in water. Under these conditions no degradation is normally detectable. Unlike DNA, RNA does not benefit from basic buffer solutions during long-term storage because of its sensitivity to alkaline conditions. Generally, if nucleic acid samples are required for multiple testing, RNA and DNA samples should be frozen in multiple aliquots at -80° for subsequent analysis in order to avoid repeated freeze-thaw cycles that can lead to degradation, and also to minimize the possibility of contamination, which could result in analytical inaccuracy.

#### QUALITATIVE AND QUANTITATIVE EVALUATION OF NUCLEIC ACIDS

##### Introduction

This section describes procedures that assess the purity, integrity, and quantity of purified nucleic acids, including spectroscopic procedures, electrophoresis of nucleic acid fragments, and probe-based techniques. Detection and quantitation by amplification are discussed in [Nucleic Acid-Based Techniques—Amplification \(1127\)](#).

##### absorbance spectroscopy

The basic principles of spectroscopy are addressed in [Spectrophotometry and Light-Scattering \(851\)](#). For nucleic acids, absorbance is determined at 260 nm, but this procedure does not distinguish between DNA and RNA. Absorbance can also be used to estimate protein contamination in nucleic acids. Proteins maximally absorb at 280 nm, and nucleic acids maximally absorb at 260 nm. Thus the calculation of the A<sub>260</sub>/A<sub>280</sub> ratio is used as an estimation of protein contamination in nucleic acid preparations. A ratio of 1.8 to 2.0 is considered desirable. As an example, double-stranded DNA has an extinction coefficient of 20 for 1 mg per mL of DNA at 260 nm and a coefficient of 10 at 280 nm. In contrast, for 1 mg per mL of protein, the extinction coefficients are on the order of 1 at 280 nm (depending on tyrosine and tryptophan content) and 0.57 at 260 nm. Thus a large protein contamination could exist at a 260/280 ratio of greater than 1.8 because of the lower sensitivity of protein absorbance. In addition, the change of absorbance of DNA with wavelength ( $\Delta A/\Delta \lambda$ ) is steep at 280 nm, and this could lead to an incorrect determination if the spectrophotometer is out of calibration. The peak at 260 nm is broad, and thus readings are less



sensitive to calibration issues.

Information on contamination by nonproteinaceous materials can be provided by a scan of DNA from 220 nm to 320 nm. Pure DNA has a mostly symmetric peak around 260 nm, zero absorbance at 320 nm, and a minimum at 230 nm. Absorbance rises again from 230 nm to 220 nm. Interfering substances can co-purify with DNA and absorb in the lower UV range (around 230 nm). These substances can interfere with and lead to an overestimation of DNA content, thus showing the utility of a scan—or at least a measurement of absorbance—at 230 nm in addition to 260 nm and 280 nm. Absorbance above 300 nm can arise from other contaminants and particulate matter. Common reagents used in the isolation of DNA, particularly solvents such as phenol and alcohols if they are not completely removed, can interfere with DNA absorbance measurements. Analysts should be aware of the limitations of this type of measurement. Finally, the absorbance of DNA and the 260/280 ratio is dependent on ionic strength—a difference as large as 30% can exist. Absorbance of genomic DNA is higher, and the 260/280 ratio is lower in pure water when compared with the same DNA in a buffer or a salt solution.

For the purposes of quantitation of nucleic acids, the respective extinction coefficients for DNA and RNA are used. An absorbance of 1 in a 1-cm cuvette corresponds to 50  $\mu$ g per mL of double-stranded DNA [ $E$  (specific absorption coefficient) = 0.02 ( $\mu$ g per mL) $^{-1}$  cm $^{-1}$ ]. The specific absorption coefficient for RNA at 260 nm is  $E$  = 0.025 ( $\mu$ g per mL) $^{-1}$  cm $^{-1}$  (absorbance of 1.0 corresponds to 40  $\mu$ g per mL), and for single-stranded DNA  $E$  = 0.027 (absorbance of 1.0 corresponds to 37  $\mu$ g per mL). A solution of DNA is read against a blank of the same buffer solution in which the DNA is dissolved. Ideally, readings should fall within a range of 0.1 to 1.0 absorbance for adequate linearity. Absorbance above 1.0 becomes increasingly nonlinear as the absorbance rises. The accuracy of readings below 0.1 (5  $\mu$ g per mL DNA) depends on the quality and noise level of the spectrophotometer.

#### Fluorescence Protocols for DNA and RNA Quantitation

Cyanine dye derivatives are used for the quantitation of nucleic acids because they specifically interact with nucleic acids (DNA, RNA, and oligonucleotides) and fluoresce only upon binding. The exact mechanism of interaction is not always fully understood but may involve intercalation in double-stranded DNA and surface binding.

Measurements can be performed using a fluorometer or a plate reader. The sensitivity of fluorescence with these dyes is much higher than that of absorbance, which gives these dyes great utility when DNA concentration is low (down to 25  $\mu$ g per mL). The dye must be protected from light to avoid photobleaching. Linearity is maintained over three to four orders of magnitude. Calf thymus and Lambda phage DNA are often used as calibrants to construct a standard curve. Some of these dyes have been optimized to bind double-stranded DNA or single-stranded RNA and oligonucleotides. A DNA-binding dye will also bind to single-stranded DNA and RNA but at low ionic strength, and the signal is about 10% or less than that seen with double-stranded DNA for an equivalent mass of material. Thus, this methodology is preferred for DNA measurements when no effort has been made to remove RNA from the preparation. Another fluorescent dye is available and is optimized for RNA measurements. Using two different concentrations of this dye, analysts can detect RNA in amounts as low as 1 ng per mL and as high as 1  $\mu$ g per mL. The dye also fluoresces with DNA but does not display an equivalent ability to minimize binding by the use of particular conditions (e.g., with DNA and the double-strand binding dye). Quantitation may be affected by contaminating nucleic acid (e.g., DNA in an RNA preparation and vice versa). Treatment with a DNase is needed if DNA is present in the RNA preparation. Proteins are unlikely to interfere with these dyes, but some detergents as well as phenol result in loss of fluorescence.

Nucleic acid extraction reagents should thus be checked for effect on subsequent fluorescent assays.

Bisbenzimidole fluorochrome dyes such as (2'-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole) represent another option for measuring DNA. Researchers have studied the binding of these dyes to the minor groove of DNA and have found that sequences of adenine or thymine in the DNA sequence provide a minor groove dimension that binds the dyes best. Thus the fluorescent signal can show DNA sequence dependence, and the calibrant DNA should have a nucleotide composition that is similar to that of the DNA to be measured. These dyes are not as sensitive as cyanine dyes but are more sensitive than absorbance measurements. Low dye concentrations and high ionic strength are required in order for analysts to distinguish double-stranded DNA from RNA. Low ionic strength conditions are required in order to differentiate double-stranded DNA from single-stranded DNA.

#### Detection by Size

**Agarose Gel Electrophoresis**— Agarose gel electrophoresis provides a simple and accurate procedure for separating nucleic acids by fragment size. The technique can be adapted to separate fragments over a large range of sizes and can be used in a preparative or analytical fashion. For example, gel electrophoresis can be used to verify that a product of a PCR reaction is of the correct size. DNA fragments can be retrieved from a gel slice and provide a sufficiently pure PCR product for cloning or sequencing. The general integrity of an RNA preparation can be determined by gel electrophoresis as well. The stoichiometry of the nucleic acid fragment size (in base pairs) and negative charge from the phosphate provide the basis for the separation. With the exception of plasmids, electrophoresis is generally free of DNA conformation-induced effects. Supercoiled plasmid DNA will migrate ahead of linear or open-circle/nicked plasmid, which is useful for determining the conformation of a plasmid preparation. In contrast, denaturing gels are used for RNA because RNA's tendency to form inter- and intramolecular secondary structures.

Agarose gel electrophoresis utilizes a horizontal setup wherein the gel is cast in a box and placed on a bridge between two buffer compartments that are filled with the buffer of choice.

The gel is also covered with a thin layer (~1 mm) of buffer. Although the main electrical resistance resides in the gel itself, there is sufficient charge on the nucleic acids to move fragments through the gel toward the anode. The fragments move in proportion to size, the smallest moving the fastest. The parameters that most affect electrophoresis are gel pore size, buffer concentration, and the voltage gradient. The ability to separate the fragments of choice is largely a function of the gel pore size, which depends on agarose concentration.

Generally the agarose concentration is in the range of 0.5% to 1.0% for DNA fragments of <100 to 25,000 base pairs, and the higher concentration is used when it is important to distinguish the smallest fragments. Lowering the agarose concentration in the gel results in the resolution of larger fragments but also in a loss of resolution of small fragments. For the largest fragments pulsed, (reversed)-field electrophoresis is utilized.

To achieve uniform electrophoresis, all of the agarose must be completely melted. Electrophoresis-grade agarose is dissolved in the same buffer that will be used for electrophoresis. The buffers most commonly used for DNA separations are TBE (tris-borate-EDTA) or TAE (tris-acetate-EDTA). TBE has a higher buffering capacity than TAE, but TAE should be used if the DNA is going to be retrieved from the gel. Denaturing RNA gels use MOPS buffer (40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA, pH 7.0). Melting the agarose is conveniently achieved with the assistance of a microwave oven. The agarose will easily come to a boil, but this may not result in complete melting of the agarose, which may require bringing the solution to a boil several times, with intermittent mixing and holding periods, until the agarose is completely melted. Agarose particles transform from white to transparent before melting. Any partially melted agarose can be detected by swirling the flask while holding it up to the light. If the solution does not appear uniform, then it requires additional heating. The agarose is poured into the gel box after partial cooling but before setting up. Commercially available ready-to-use gels suitable for a particular application can also be used. For RNA-denaturing gels, formaldehyde is added under a fume hood to the melted agarose to a final concentration of 2.2 M or 6.7%. Before the agarose has hardened, the analyst places a comb in the gel to provide wells for the samples and size standards. Once solidified, the gel is placed in the electrophoresis box, and buffer is added until both sides are filled and there is a layer of buffer across the surface of the gel. Then 10X tracking buffer (40% sucrose with 0.25% bromophenol blue or 0.25% xylene cyanol or both) is added to each DNA sample to increase the sample density and to provide a tracking dye that is used to assess when the electrophoresis is finished. The increased density allows the sample to be transferred into the well and to remain there until it migrates into the gel during electrophoresis.

One or more lanes should be used for a DNA size standard containing fragments in the range that is relevant to the samples and agarose concentration. Size standards in various ranges are readily available. Bracketing the samples in wells between standards is useful to determine whether the electrophoresis gradient has been uniform over the width of the gel. However, in the case of eukaryotic RNA preparations, the 18S and 28S ribosomal RNAs that are co-extracted from prominent bands (corresponding to 1900 and 4700 nucleotides) can also be used as size standards. In addition, the rRNA provides information on the RNA integrity because missing or fuzzy rRNA bands indicate problems with the quality of the RNA preparation. Once the wells are filled, the cover is placed over the gel box, and the box is connected to the power supply. The indicator dye in the tracking buffer added to the samples and size standard allows the easy determination of how far the electrophoresis has proceeded. Bromophenol blue will migrate with DNA fragments of <500 base pairs, and xylene cyanol will migrate with fragments of 5000 base pairs.

The power supply is frequently run under conditions of constant voltage (1 to 10 V per cm) of gel length. Elevated voltage can cause high current, resulting in the generation of damaging heat and exhaustion of the buffer.

**Pulsed-Field Electrophoresis**— This variation on agarose gel electrophoresis is used to separate a range of large DNA fragments and is most useful when resolution of 50,000 to 200,000 base-pair fragments is needed. The main difference is the addition of an alternating-field device that controls the power supply operating under constant voltage. Large fragments of DNA change conformation in order to move through the agarose pores, and the larger pieces take longer to readjust when the field is reversed and thus move more slowly than do smaller fragments. This allows resolution of fragments over the period of hours that the pulsed-field procedure operates. A commonly used ratio of forward to reverse is 3:1, and, in addition, the procedure typically calls for a stepwise increase in the unit time between reverses of the field. Electrophoresis may continue for 10 to 16 hours to avoid fluctuation in gel temperature, viscosity, and other properties that may cause artifacts.

**Polyacrylamide Gel Electrophoresis (PAGE)**— The format for performing PAGE is quite different from that for agarose gel electrophoresis. The general procedure for PAGE is



described in [Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis](#) (1056). For resolution of small fragments of DNA in the 10 to 500 base-pair range, nondenaturing polyacrylamide gel electrophoresis is more suitable than agarose gel electrophoresis because separation of fragments of this size requires much smaller pore size than is achievable in agarose gels. The gel is prepared by polymerization of acrylamide monomers. The percentage of acrylamide dictates the range of fragment sizes that can be best resolved. For example, 20% acrylamide is suitable for the 10–100 base-pair range, and 5% acrylamide is useful in the 100–500 base-pair range. Commercially available ready-to-use polyacrylamide gels suitable for the particular size discrimination can also be used. The separated nucleic acids are visualized by staining with, for example, silver nitrate solution rather than with ethidium bromide or cyanine dye. However, staining with silver nitrate is laborious and time-consuming and not suitable for preparations that contain a large amount of protein, because proteins will also stain with silver nitrate.

**Capillary Electrophoresis and Laser-Induced Fluorescence (CE-LIF)**—CEF has been used for many years to separate DNA fragments (for the general principles of CE, see [Biotechnology-Derived Articles—Capillary Electrophoresis](#) (1053)). The procedure relies on a principle similar to that underlying agarose gel electrophoresis. CE can utilize the cross-linked buffer systems applied in gel electrophoresis, but the technique can also use polymer-containing solutions (e.g., polymethylcelluloses) that are designed to create pores that entangle proteins. These polymer solutions may be added to the capillary between injections, allowing a “fresh” gel prior to each run. In addition, capillaries can be used for more injections than are possible for polymerized gel-filled capillaries. The resolving power of the separation depends on the size of the pores, which is based on the composition of the gel. Kits are available to separate fragments into the desired size ranges. Fragment sizes outside the resolution window can possibly be separated, but the separation may not be reliable or reproducible when the gel capability is exceeded.

Fragments can be detected by a variety of mechanisms. Detection utilizing UV absorbance is possible, but the preferred and most common detection procedure is laser-induced fluorescence (LIF). Fluorescence offers improvements over UV detection in terms of selectivity and sensitivity. In addition, the detection limits for fluorescence are two to three orders of magnitude better than those for UV. Although DNA is intrinsically fluorescent, the background fluorescence and complex laser spectroscopy required preclude routine use. The most common way to label DNA is described in the section above on fluorescent protocols for RNA and DNA quantitation. This system is widely employed because of its simplicity (the dyes are added to the sample or into the reaction buffer) and effectiveness. The advantages of CE include speed of analysis, sensitivity using minimum sample volumes, and the potential for automation. These are achieved mainly by the inherent miniaturization of the gel. Automated systems allow robust analysis of the quality, quantity, and fragment size of both RNA and DNA. CE applications have been especially important for evaluating the integrity of RNA because of the instability and progressive degradation of RNA caused by ubiquitous RNases, and new technologies that compare the ratios of 28S and 18S are improving the capabilities of these procedures.

#### FILTER HYBRIDIZATION AND IN VITRO LABELING OF PROBES

##### Introduction

Hybridization techniques were used early in molecular biology to identify individual nucleic acids and to estimate the degree of similarity between species. Hybridization is widely used in the procedures described in this and other chapters to visualize and identify nucleic acid sequences (see [Nucleic Acid-Based Techniques—Amplification](#) (1127), [Nucleic Acid-Based Techniques—Genotyping](#) (1129), and [Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids \(Residual DNA Testing\)](#) (1130)). With the advent of restriction endonuclease digestion of DNA and electrophoretic separation by molecular mass, hybridization using labeled probes provided a way to visualize the organization of genes within a specific genome.

The hybridization techniques described are dot and slot blotting, Northern blotting, Southern blotting, in situ hybridization, and fluorescent in situ hybridization (FISH). All these techniques rely on the use of nucleic acid probes. Probes are oligonucleotides with specific DNA or RNA sequences that have been labeled with radioactive, fluorescent, chemiluminescent, chemical tags or enzymes (reporter molecules). Hybridized probes bind to complementary sequences on the target nucleic acids and are used to visualize and characterize targets, as described below.

##### Dot and Slot Blotting

Dot blotting is the simplest and quickest of the hybridization techniques. The nucleic acids are directly applied to a support membrane, which may be a nitrocellulose or nylon membrane, without prior separation of the nucleic acid species by agarose gel electrophoresis. The nucleic acids are spotted onto the filter using a micropipettor or an apparatus such as a dot blot or slot blot apparatus. This consists of a membrane frame with a membrane sandwiched in between the two pieces of the frame. The bottom frame plate is connected to a vacuum manifold, and the top piece of the frame has slots through which the nucleic acids are loaded. The samples are loaded under vacuum and pulled through the membrane by vacuum, with the nucleic acid binding to the membrane, and then the filter is air-dried. The nucleic acids are fixed to the filter either by heating to 80° for nitrocellulose membranes or by exposure to UV light for a predetermined time for nylon filters. Hybridization with a labeled probe provides confirmation of the identity of the nucleic acid but does not provide any information about the number or sizes of the species. The nucleic acid species of interest can be quantitated by spotting known concentrations of the purified nucleic acid on the filter and comparing the signal generated by the unknown samples with those of the standard preparations.

##### Southern Blotting

Southern blotting refers to the transfer of DNA from an agarose or polyacrylamide gel to a nitrocellulose or nylon membrane. Small, single-stranded DNA probes can then be used to visualize and identify the DNA species of interest. Southern blot analysis is based on a transfer and immobilization methodology developed in 1975, coupled with the electrophoretic separation of fragmented DNA. More specifically, the procedure typically is used to identify specific nucleic acid sequences in the context of a defined genetic topography, such as a restriction endonuclease map. The position of genes within the viral genome can be accurately mapped using a variety of restriction endonucleases in combination with Southern blot analysis. The procedure requires that DNA be obtained in sufficient quantity for analysis. Fragmented DNA is separated according to size using agarose gel electrophoresis. Double-stranded DNA fragments must be denatured before they are transferred and immobilized on a membrane by capillary action. The immobilized DNA is then cross-linked to the filter, which may be composed of nitrocellulose or nylon, as described above. However, the use of positively charged nylon membranes eliminates the need to fix the DNA to the nylon membrane. Nitrocellulose membranes are more fragile and may be probed up to 3 times with separate probes. Nylon membranes are more robust and may be probed 10 to 12 times, but they may present more background noise, particularly when they are used with chromogenic probes.

##### Northern Blotting

Northern blot analysis comprises a series of steps for the separation, transfer, and immobilization of RNA in a manner similar to the treatment of DNA using Southern blot analysis. Denaturation of the RNA is required to reduce secondary structure to ensure that the RNA separates in the agarose uniformly according to length. Denaturation of RNA is accomplished either prior to electrophoresis using glyoxal or dimethyl sulfoxide (DMSO) or during electrophoresis by means of gels that contain formaldehyde. Transfer is achieved in a manner identical to that used for Southern blotting. However, in the case of Northern blotting, it is unnecessary to denature the RNA prior to transfer because denaturation is accomplished before electrophoretic separation of the RNA species. The immobilized RNA is cross-linked to the membrane in a manner similar to the cross-linking of DNA.

##### In Situ Hybridization and Fluorescent In Situ Hybridization (FISH)

Hybridization of a nucleic acid in situ classically refers to determining the location of that nucleic acid sequence in its natural state—in tissue, in individual cells, or on a chromosome. In situ hybridization probes are designed to bind to complementary nucleic acid sequences, whether they be DNA or RNA. The purpose of these hybridization procedures is to discover where in a tissue a gene is being expressed, in which case the target is RNA, or to map a specific DNA sequence to its location on a chromosome, in which case the target is DNA.

Chromosome mapping of DNA sequences is accomplished by chemically attaching silver grains to the probe sequences and then counting the density of the grains in a metaphase chromosome spread. Although, historically, these procedures worked well, sensitivity was always an issue. The solution was to use a reporter that was more sensitive and safer than the other reporters, namely, fluorescence used in the technique of fluorescent in situ hybridization (FISH). FISH has an additional benefit in that the different colors available in fluorescence afford the ability to observe multiple hybridization events simultaneously, a feature not available with other detection systems.

##### Detection of DNA and RNA in Hybridization Assays Using Labeled Probes

Visualization and location of individual nucleic acid species of interest are achieved by the specific hybridization of DNA or RNA probes that are labeled for easy visualization. The filter or sample (fixed cells or tissues in the cases of in situ hybridization and FISH) is incubated with the labeled probe at an appropriate temperature and salt concentration that allows hybridization of desired stringency. This is followed by washing with buffers of varying detergent and salt concentrations and at varying temperatures in order to minimize background signal due to nonspecific hybridization. The labeling and types of probes are discussed below.



obes can be RNA probes generated in vitro or DNA probes, either double-stranded fragments, plasmids, or single-stranded oligonucleotides containing moieties to facilitate detection of fragments that contain portions of the gene of interest. Probes can be labeled with radioactive tracers such as  $^{32}\text{P}$  or  $^{35}\text{S}$  by incorporation of a labeled nucleotide in the probe sequence or with a nonradioactive label such as biotin by incorporation of a modified base, such as adenine monophosphate linked to biotin. Radioactive probes are visualized with X-ray film placed over the blot. Biotin-labeled probes are detected with a conjugate of streptavidin–alkaline phosphatase. An enzymatic reaction is run with alkaline phosphatase and a substrate that yields an insoluble colored product at the site of the probe. Variations on nonradioactive probes utilize other modifications to the DNA and linked antibody–alkaline phosphatase, as well as chemiluminescent probes that are detected on film.

Nucleic acids can be synthesized and manipulated by either enzymatic or chemical means. These same systems can be used to modify nucleic acid structure and to introduce foreign moieties to create unique molecules that can provide an advantage to the detection of limiting viral nucleic acids against a background of host nucleic acids. The chemical synthesis of nucleic acids and their purification has become routine, and high-quality synthesis and purification are commonly achieved. Moreover, larger segments can be synthesized, and when even larger segments are required, the subsections can be designed for concatenation and ligation.

Custom synthesis of DNA oligonucleotides is readily achievable in the laboratory using commercially available reagents and equipment. Alternatively, probes can be custom ordered from numerous commercial providers. Size-exclusion procedures for purification generally are used to eliminate incomplete oligonucleotides. RNA oligonucleotides also may be chemically synthesized or generated in vitro using complementary cloned DNA fragments under the control of various prokaryotic RNA polymerase promoter sequences. The use of DNA probes is much more common, but there may be some applications in which the increased association of RNA–RNA or RNA–DNA hybrids is advantageous.

The principal procedures of labeling DNA are direct labeling using a kinase reaction to attach a labeled nucleotide to the end of each DNA strand, by incorporating labeled nucleotides into a nicked DNA by utilizing the DNA repair function of the Klenow fragment of *Escherichia coli* DNA polymerase I enzyme (nick translation), and by PCR. This last procedure generates a relatively higher yield of internally labeled probe because each round of thermal cycling doubles the amount of labeled probe, whereas the former procedures result in a ratio of less than one probe molecule per template molecule. The PCR procedure also is used to generate unique probes with a variety of moieties located at the termini.

## NUCLEIC ACID SEQUENCING

### Introduction

The first DNA sequencing procedure, described in 1977, utilized chemical cleavage to specifically introduce chain breaks in a DNA sequence (Maxam and Gilbert sequencing). The procedure proved to be of significant utility in the early years of molecular biology, but it has not been used to perform high-volume sequencing and therefore is not discussed in detail here. The majority of sequencing performed today is based on the dideoxysequencing procedure, also described in 1977 (Sanger sequencing). This procedure fundamentally changed sequencing by exploiting the enzymatic specificity of polymerases that introduce strand interruptions at specific bases. This is the most widely recognized sequencing procedure and is considered a routine assay in molecular biology laboratories. Innovations in instrumentation, sample preparation and collection, data management, data analysis, and sequence assembly have relied on this sequencing procedure as their fundamental sequence generator.

High-throughput sequencing takes all the elements of the sequencing procedures and applies them to a mass collection of sequence data, typically for larger genomes, but high-throughput sequencing certainly may be used for smaller projects as well. Obtaining the final sequence information includes all processes associated with sample preparation, sequencing, data assembly, and data finishing. The technology to achieve these individual objectives includes the instrumentation, disposables, protocols, and procedures.

### Sequencing Reaction

The dideoxysequencing procedure takes advantage of specificity of the Klenow enzyme to introduce chain-terminating nucleosides, called dideoxynucleotides, intermittently during the polymerase extension process. The sequencing of each sample requires four separate reactions (one for each base). The resulting mixture of various nucleotide chain lengths is then separated on the basis of individual molecular masses. The incorporation of radioactively labeled nucleotides during the sequencing reaction permits the detection of the nucleotide chains.

Improvements in biotechnology have led to the discovery of more robust enzymes with high fidelity, improved stability, and other attributes that have led to longer reads and improved sequence fidelity. These improvements have made possible the introduction of cycle sequencing, which is now commonly used. The principle of the cycle sequencing procedure is a combination of Sanger sequencing and aspects of PCR amplification, whereby dideoxynucleotides are incorporated into the amplified DNA. Cycle sequencing leads to a higher concentration of labeled fragments covering a wider range of sizes than does Sanger sequencing, leading in turn to a higher read length.

### Separation Procedures for DNA Sequencing Fragments

The previous sections of this chapter deal with the treatment of intact DNA and RNA molecules; the following sections address the challenges of separating the fragments that result from the sequencing reactions, notably slab gel sequencing and capillary electrophoresis. Subsequent sections address detection technologies and sequence integrity.

### Slab Gel Sequencing

Polyacrylamide gel electrophoresis, frequently referred to as slab gel electrophoresis, was the first separation mechanism employed for the separation of DNA sequencing fragments. As described above, the electrophoretic separation of DNA fragments is driven by the size of the fragments in the reaction mixture. However, for slab gel sequencing the pore sizes are chosen so that single-base resolution for many hundreds of bases is possible. In addition to the polyacrylamide in the gel, a denaturant such as urea is frequently included to ensure denaturation of the fragments. Until the implementation of multicapillary sequencing systems, the separation power and throughput of slab gel separation mechanisms were often considered state of the art.

### Capillary Electrophoresis Sequencing

As noted above, capillary electrophoresis offers significant advantages over gel-based separations. However, as with slab gel sequencing, the pore sizes are chosen so that single-base resolution for many hundreds of bases is possible. Multicapillary systems that utilize 8 to 384 capillaries are commercially available. These systems are the primary systems used for large-scale DNA sequencing, and, theoretically, they yield more than 1.1 billion base pairs of DNA sequences per year.

### Detection

**Radioactivity**— The first detection strategies for DNA sequencing reactions utilized radioactive isotopes such as  $^{32}\text{P}$  or  $^{35}\text{S}$ , primarily because these were practical for gel separations. The advantages are that detection is universal, low limits of detection are possible, mobility shifts are eliminated, and fidelity differences for the DNA polymerases do not occur. Disadvantages include the high disposal and safety costs, the inability to multiplex (ultimately limiting throughput), and the need for 24 to 36 hours of exposure time (i.e., no real-time detection).

**Fluorescence**— Fluorescence dyes have largely replaced radioactive isotopes as detection tools during DNA sequencing, mainly because they do not have the disadvantages of radioactive probes. Because the dyes can be discriminated by means of their emission maxima, multiplexing is possible, so four sequencing reactions per sample can be replaced by a single reaction using four different labels. Thus a single lane can be used rather than the four separate lanes that were necessary with radioactive probes. Additional advantages are higher throughput and automated data collection in real time.

**Mass Spectrometry**— Mass spectrometry (MS) has revolutionized the field of biochemistry and has significant potential in the area of nucleic acid sequencing. Soft-ionization techniques such as electrospray ionization and matrix-assisted laser desorption–ionization have expanded the potential application of MS to DNA sequencing. MS offers some advantages over other detection methodologies, including speed of fragment detection (signal acquisition is in the range of microseconds versus hours for conventional approaches) and accuracy (e.g., the molecular mass of each fragment can be determined with a high degree of accuracy). The Sanger procedure makes use of mass differences of the fragments generated as part of the polymerization reaction. MS is sufficiently precise to resolve fragment sizes that differ by only one base pair. Unfortunately, the sensitivity of MS detection suffers as fragment length increases, and the 100-base-pair barrier has yet to be crossed.

More recently, other sequencing technologies have emerged that are based on massively parallel sequencing techniques that attempt to achieve low-cost sequencing. These techniques are based, for example, on solid-phase sequencing or they make use of highly parallel and miniaturized pyrosequencing, which is described in [Nucleic Acid-Based Techniques—Genotyping](#) (1129).



## Sequence Integrity

A prerequisite for automated data collection and interpretation is that the data must be of good quality, which means minimizing human intervention and allowing the system to make base identifications following detection steps. It is a critical step to ensure accurate base identification by minimally sequencing both strands of the DNA several times. In addition, other tactics may be employed, such as using primers at different sequence positions, which can improve the accuracy of the developed consensus sequence. This task can be facilitated by the use of specialized software packages that are commercially available. More recent technology developments have produced alternative sequencing platforms that are more amenable to large-scale sequencing projects. These techniques include array-based platforms on which short stretches of target are sequenced on a chip that supplies raw data to sophisticated computational programs that reconstruct the sequence. Other sequencing approaches have been developed for the rapid sequencing of short nucleic acid sequences such as oligonucleotides of short PCR products. These technologies include MS-based and pyrosequencing platforms, the latter of which is described in [Nucleic Acid-Based Techniques—Genotyping \(1129\)](#).

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1127 NUCLEIC ACID-BASED TECHNIQUES—AMPLIFICATION

## INTRODUCTION

The basic principles of nucleic acid amplification technologies (NAT) and definitions of the various techniques are covered in [Nucleic Acid-Based Techniques—General \(1125\)](#). The current chapter covers major techniques that result in amplification of targeted nucleic acid sequences. The most common NAT assay is the polymerase chain reaction (PCR), which was first described by Kary Mullis. This procedure has been further refined to amplify a DNA fragment starting from RNA (reverse transcription-PCR, or RT-PCR). Initially, PCR was used in a qualitative manner to amplify and detect DNA molecules because its exquisite sensitivity paired with its high specificity made it a useful tool for the detection of nucleic acid targets. Since its inception, the number of PCR applications has expanded rapidly, and the technique, which now includes quantitative and multiplex assays, is currently used in almost every field of research and development in biology and medicine. In addition to the changes and improvements to the original design of the PCR procedure, alternatives to PCR are techniques used to amplify target nucleic acids to generate RNA instead of DNA amplicons. The most commonly used techniques are nucleic acid sequence-based amplification (NASBA) and the transcription-mediated amplification (TMA) which are described here in detail. In contrast to PCR, which relies on incubating the sample at three different temperatures, NASBA and TMA are based on isothermal conditions.

In addition to amplification of the target nucleic acid, the amplification step also can be directed at the signal used for detection (signal amplification). The most commonly employed technique is the branched DNA (bDNA) assay, in which the signal, typically a fluorescent probe that binds to the target sequence, is amplified. The bDNA assay is used predominantly for viral nucleic acid detection and quantitation.

This chapter describes the main assay components necessary for a PCR procedure and includes a discussion of the general optimization of PCR assays. The various PCR assay formats, including PCR, nested PCR, and RT-PCR are covered, and a discussion of the detection of the resulting amplicons follows. Although all these assays are essentially qualitative procedures, they can be modified for semiquantitation, and the various modifications are described. For accurate and reliable quantitation, real-time PCR has now replaced the methods listed above; real-time PCR and real-time RT-PCR are described in the NAT Assays section. The same section includes a discussion about probes and dyes that are an essential component of real-time PCR and the methods of quantitation using the generation of standard curves. The next PCR technique discussed is multiplex PCR, which is used for simultaneous detection of multiple targets or for normalization of assay results. Apart from PCR, the major alternative NAT tests that are used routinely, primarily in blood screening and clinical diagnostic screening are NASBA and TMA. The final technique described is whole-genome amplification, wherein the complexities of amplification require modifications to the PCR procedures. The chapter concludes with a discussion about the evolution of instrumentation used in NAT assays and the quality assurance and quality control issues associated with NAT because this is probably one of the most highly regulated biological techniques, especially when applied to blood screening.

## ASSAY COMPONENTS

## Enzymes

The essential components for NAT assays—polymerases, reaction buffers which include desoxynucleotides, ions, primers, probes, and fluorescent dyes—can be chosen from a broad selection of commercially available NAT reagent kits and vendors. Polymerases suitable for NAT applications can, in principle, be grouped into Taq DNA polymerases or DNA I polymerases from other *Thermus* species that are polymerases with features that are similar to those of Taq DNA polymerase. In addition, so-called proofreading polymerases are available (e.g., from *Pyrococcus* species) that display a 3'-5' exonuclease activity capable of removing wrongly incorporated DNA bases from the growing DNA strand under amplification conditions. Taq DNA polymerase is the standard NAT enzyme and is the one most often used in NAT assays. Modifications of Taq DNA polymerase, such as deletions of the 5'-3' exonuclease domain (Klenow fragment, Stoffel fragment) or point mutations for improved incorporation of dideoxynucleotides are also employed (e.g., for PCR-based sequencing reactions). Proofreading DNA polymerases or mixtures of Taq DNA polymerase with a proofreading polymerase are used if either fidelity of the NAT product is critical (e.g., for DNA cloning experiments) or longer NAT products are to be amplified. For RT-PCR, a reverse transcriptase is necessary to first convert the RNA target to copy DNA (cDNA) that can subsequently be amplified. For TMA reverse transcriptase with an RNase H activity is needed to convert the RNA target to double-stranded template DNA, while for NASBA exogenous RNase H is added to the reaction mixture. Depending on the reaction environment, two types of enzymes can be used to generate cDNA: a reverse transcriptase isolated from retroviral sources or a DNA polymerase that can function both as reverse transcriptase and DNA polymerase. Finally, chemical modification of the polymerase, resulting in an inactive enzyme at temperatures below 90°, is now typically used to prevent mispriming of templates at sub-optimal temperatures (see section on Assay Optimization).

## Reaction Buffers

Reaction buffers vary with respect to ion composition, pH, and additives and are sometimes specifically adopted for particular applications such as multiplex PCR, real-time PCR, RT-PCR, TMA and NASBA. An important component of the reaction mixture is Mg<sup>2+</sup> ions, or, in the case of polymerases with both reverse transcriptase and DNA polymerase functions, such as *Thermus thermophilus* (Tth), Mn<sup>2+</sup> ions. Other additives that enhance the sensitivity and specificity of the assay may be present. The concentration of the four deoxynucleotide triphosphates (dNTPs) must be optimized.

## Primers

Primer sets are oligonucleotides with sequences that are designed specifically to prime the amplification of a portion of a target nucleic acid of interest. Synthetic oligonucleotide primers for both standard PCR and for real-time or quantitative PCR are designed for the specific recognition of and binding to a single DNA or RNA sequence. Such specificity is achieved through design that involves both the length and the sequence of the primers. Length and sequence specifications have separate criteria that must be simultaneously met in order for the primers to perform properly. The length of a primer is a statistical issue that relates to the issue of the minimum length of a specific sequence necessary to guarantee that the desired target sequence is unique, regardless of the size or complexity of the genome. As an example, in the case of the human genome, with its 3.2 billion DNA bases, that length is 17 bases. For this reason the vast majority of PCR primers are between 20 and 25 bases long. The specificity of a primer should be determined by comparison with sequences in all known databases. Tools available on the Web facilitate such comparisons.

In terms of primer sequence, the issues to consider are Tm (the temperature at which 50% of the double-stranded nucleic acid molecule becomes single-stranded) and secondary structure. Every DNA has its own characteristic Tm, determined by length, sequence composition, and reaction environment. PCR primers are designed to bind to a perfectly complementary DNA sequence via guanine-cytosine (G-C) and adenine-thymine (A-T) base pairing. The Tm of the two PCR primers used in a reaction should be as close as possible. In terms of secondary structure, the formation of secondary structures by intra- or intercomplementarity should be minimized. Interaction between different primers can result in primer-



users that will compromise assay sensitivity and specificity. All of the design issues presented are accounted for in any one of the dozens of primer design software packages that are available and can be found on the Internet.

#### Assay Optimization

NAT assay optimization is necessary for successful amplification that is sensitive and specific. Parameters that should be optimized include the thermocycling conditions, both temperatures and cycling times (that depend to a large extent on the target, primer, and probe sequences), concentrations of template, concentrations of NAT reagents, sample matrix and the number of amplification cycles. In the case of multiplex PCR, a compromise among elements of the reaction conditions is usually necessary because of the difficulties in optimizing the conditions for all the primer and probe sets. Recent changes have been made to improve sensitivity and specificity of NAT assays. One change is hot-start PCR, in which the addition of one of the essential components of the NAT assay, typically the DNA polymerase, is temporarily withheld. When this occurs during reaction setup, the initial nucleic acid denaturation step prevents nonspecific amplification due to mispriming at suboptimal temperatures. Early hot-start procedures made use of wax barriers that effectively separated essential components into two liquid phases that were mixed only when the wax melted. However, this procedure has been replaced by two important hot-start technologies that do not require physical separation of the components by inconvenient additional handling steps. In the first procedure, antibodies directed against the DNA polymerase are complexed with the enzyme and lose their binding avidity at elevated temperature at the start of the reaction. The second procedure uses chemical modification of the polymerase, resulting in an inactive enzyme. At temperatures above 90°, typically in the first denaturation step, the modifier dissociates from the enzyme, and the enzymatic activity is restored. The advantage of an antibody-mediated hot start is the immediate release of enzyme activity at the start of the reaction by a very short heat incubation step. However, antibody-mediated hot-start chemistries tend to be less stringent when compared with chemically activated enzymes if there is a large excess of active polymerase molecules.

#### NAT ASSAYS

This section describes the basic techniques of PCR, nested PCR, and RT-PCR and procedural modifications that allow semiquantitation.

##### Polymerase Chain Reaction

The PCR technique is based on a three-step process: denaturing double-stranded DNA (dsDNA) into single strands (ssDNA), annealing primers to the ssDNA, and enzymatic extension of primers that are complementary to the ssDNA templates. Each step is usually carried out at a different temperature. By cycling the temperature steps many times (usually 30 to 45 times), a billion-fold amplification of the target nucleic acid can be achieved, but the optimal number of cycles should be determined empirically. In some cases, especially where sensitivity is more important than false positive results due to excessive cycling, such as in blood screening, extra sensitivity can be gained by increasing the number of cycles to 60 to ensure that extremely low levels of target are detected. In a typical reaction, PCR product (amplicon) doubles at each cycle of amplification (exponential amplification). The increase in amplification in the early cycles follows a sigmoidal curve. In later cycles, the concentrations of the template strands and amplicons favor template strands re-annealing instead of PCR primer annealing to the template. At this point the concentration of the PCR product no longer doubles after each cycle, and the curve begins to plateau. A thermostable enzyme such as Taq-polymerase is a prerequisite because temperature cycling at 95° (the typical temperature step used to denature double-stranded templates) would inactivate a thermolabile polymerase.

##### Nested PCR

An early variation of the PCR assay was nested PCR, which was designed to increase the assay's sensitivity and specificity. In this procedure amplicons from the initial PCR reaction are subjected to a second round of amplification using a different set of primers. This set of primers is specific to the amplicon sequence but is within the first set of primers (nested primers). The advantage of amplification with two sets of target-specific primers is increased specificity (any nonspecific amplification during the first amplification round would be reduced) and increased sensitivity (due to initial amplification of the target in the first amplification round). In addition, amplification of a product of the expected size is taken as confirmation of the presence of the target. However, a major drawback of this procedure is the high likelihood of cross-contamination due to the increased manipulation of amplicons generated in the first round of amplification. The use of highly specific primers and probes and the optimization of reaction conditions have resulted in the diminished applications of this procedure for routine testing, but the procedure is sometimes used for samples that are difficult to amplify by conventional PCR.

##### RT-PCR

In amplifying RNA targets, analysts prepare cDNA before the amplification step (RT-PCR). One-step and two-step RT-PCR procedures are available. In one-step RT-PCR the reverse transcription of RNA into cDNA and the subsequent amplification step are carried out in a single reaction without intermediate procedures. Therefore the reaction mixture for one-step RT-PCR includes the gene-specific amplification primers that are used for both reverse transcription and amplification. The advantage of this procedure is the overall reduction in handling time, increased throughput, and reduced contamination risk because reopening the reaction vessel is not necessary. In contrast, in two-step RT-PCR the reverse transcription and amplification are performed as two separate steps. In general, random primers or oligo-d(T) primer rather than gene-specific primers are used for the reverse transcription step. An aliquot of the cDNA synthesis reaction is then transferred into the NAT reaction for subsequent amplification. The advantage of this procedure is the standardization of the reverse transcription reaction, which can be used as a single source for the analysis of multiple transcripts in gene expression analysis.

##### Detection of Amplicons

Following amplification, analysts can employ a variety of procedures for detection of the amplicon as described in detail in the general information chapter, [Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing \(1126\)](#). These include agarose gel electrophoresis with ethidium bromide or other dyes, capillary electrophoresis, and laser-induced fluorescence and hybridization followed by chromogenic detection such as streptavidin horseradish peroxidase detection, chemiluminescence, or fluorescent detection using labeled probes.

**Quantitation**— The original PCR and RT-PCR assays were qualitative and detected amplicons at the end of the reaction. Such detection is not easy to quantitate because at this stage the amplification is in a plateau phase at the end of the assay, and the amount of amplicon is not necessarily directly related to the quantity of the starting template. Several approaches have been deployed to attempt to overcome the shortcoming of PCR to produce reliable, quantitative results. Initial attempts at quantitation relied on assessing the amount of amplified DNA during the early or exponential part of the assay, but this procedure was fraught with problems because the aliquots had to be taken from the reaction mixtures at regular intervals, thus greatly increasing the risk of cross-contamination. One of the earliest and most straightforward approaches to quantifying PCR products was to measure the amount of amplicons that were generated during the exponential phase of the reaction by comparing this to a serially diluted external control. Several aspects, including variability in sample preparation and variations in reaction conditions, however, hampered this approach. Because of the exponential amplification of NAT procedures, even small errors or variances can lead to distinct differences.

Compared with dilution procedures, competitive PCR proved to be a much more precise approach to achieving reliable estimates of the originally present target molecules. This procedure relies on the simultaneous co-amplification of a specific target sequence in the presence of increasing concentrations of an exogenous target molecule (control) which shares the primer binding sites with the target sequence but whose sequence is slightly modified or shortened in order to facilitate discrimination from wild-type amplicons. In addition, the concentration of the control is known. The close sequence homology and similar size of the control and target amplicons are designed to ensure that the template and internal control are amplified with comparable efficiency. The relative strength of the amplicon bands of template and control can be assayed, for example, on ethidium bromide-stained agarose gels, giving a relatively precise quantitation of the wild-type target. A drawback of this approach is that the internal control and the template should be present in the reaction in approximately the same quantity in order to yield correct results. The development of real-time, quantitative PCR has eliminated the variability associated with quantitative PCR, thus allowing the routine and reliable quantification of PCR products.

##### Real-Time PCR and Real-Time RT-PCR

Although gene quantitation by quantitative PCR was a widely used procedure, its applications were expanded by the advent of real-time PCR and real-time RT-PCR. Real-time PCR displays the same advantages as standard quantitative PCR—sensitivity, specificity, and a wide dynamic range—but the real-time procedure offers the additional advantage of requiring no post amplification processing because it combines amplification and detection in a single step. Real-time PCR collects data throughout the amplification process by measuring a fluorescence signal created as amplification progresses. A multitude of fluorescence chemistries allows the correlation of generated PCR product to fluorescence intensity. In principle, fluorescence intensity will increase with every cycle performed. Once the intensity is greater than background fluorescence, the so-called cycle threshold (C<sub>t</sub>) value is achieved. This value, which represents the first cycle in which there is a detectable increase in fluorescence above the background level, is used to measure relative or absolute target quantities. The C<sub>t</sub> value is inversely proportional to the number of target molecules in the sample and thus provides a means to quantitate the amount of target in the



starting material (i.e., the greater the number of target molecules present, the lower the Ct value).

The reaction conditions for real-time PCR applications have to take into account the presence of the probe(s) and will require optimization. The most commonly used probes currently are hydrolysis probes, although hybridization probes are an alternative. In most cases, the amplification and detection steps can be combined into a two-step cycling reaction, but these conditions have to be optimized. In contrast, DNA-binding dyes which may also be used for amplicon detection require separation of the annealing and extension steps since the dye binding occurs during the extension step which is usually done at 72°.

A fluorescent DNA intercalating dye is used for detection of the PCR product in real-time mode. This dye emits light when bound to double-stranded DNA and the subsequent increase in fluorescence can be detected by real-time PCR instruments. Dyes that bind to dsDNA bind not only to the specific PCR product but also to artifacts such as nonspecific PCR products and primer-dimers. Analysts have observed substantial differences in the specificity of dsDNA-binding dyes in use with real-time PCR kits. Therefore, some analysts recommend verifying the presence of a single PCR product by gel electrophoresis to determine the correct size of the PCR product. Also, a melting curve analysis is advisable to ensure the absence of artifacts that could contribute to the fluorescent signal and thereby lead to misinterpretation of quantitative data. Alternatively, sequence-specific labeled probes can be employed. A wide variety of fluorescence-labeled probes and primers exist for use in real-time PCR and are described in the next section.

**Real-Time PCR Probes**— The difference between conventional PCR and real-time PCR is the presence of a third chemically synthesized oligonucleotide, the probe, which, for the most basic hybridization probes, contains some type of reporter molecule, usually a fluorescent molecule or fluorophore. Non-nucleic acid materials can be added to chemically synthesized DNAs that are then incorporated into oligonucleotide probes for real-time PCR. Other applications include hybridization probes such as those used for fluorescence *in situ* hybridization (FISH) and microarrays and probes designed to capture other nucleic acids. A challenge arises in using fluorescent probes for real-time PCR because the unbound or free probe is not removed before detection, thus requiring a means to distinguish between signal obtained from bound and free probe. In contrast, FISH assays involve washing away free probe following hybridization.

All of the issues associated with primer design for conventional PCR apply to real-time PCR primers as well as to the probe sequence. As a general rule only two additional considerations apply to the probe sequence. One of these is thermodynamic, and the other specifically concerns the reporter moieties themselves. Thermodynamically, a good probe molecule that is designed to bind in the sequence somewhere between the two PCR primers will have a Tm that is about 5° higher than that of the two primers. In the large majority of cases the amplicon will be between 100 and 500 DNA bases in length, although for real-time PCR a smaller amplicon between 100 and 150 bases long results in a more efficient reaction. Thus it is rarely a problem to find a sequence inside a PCR amplicon that meets the necessary criteria.

Current probe designs overcome the problems of background from unbound probe using simple hybridization probes. In the original design, two probes that hybridize to adjacent sequences on the target nucleic acid are labeled. The reporter moiety is a fluorescent molecule attached to the 3' end of the upstream probe sequence, and a second fluorescent molecule is attached to the 5' end of the second probe. Excitation of the 5' fluorophore with light energy of the proper wavelength results in absorption of that energy, followed by emission of light energy of a slightly longer or less energetic wavelength (Stoke's Law). This emitted energy then excites the 3' fluorophore if it is close enough to the emitter and compatible with it in the sense that the emitted energy from the 5' fluorophore can excite the 3' fluorophore. When this occurs, the observed fluorescent light wavelength will be that of the acceptor molecule and not that of the donor. Fluorescence absorption and emission spectra are readily available for all of the commonly used fluorophores, and the only applicable rules are that the two fluorescent molecules must be fewer than 40 DNA bases apart and that the emission spectrum of the donor must overlap the absorption spectrum of the acceptor. Thus hybridization of the two probes, also known as hybridization probes or FRET probes (Fluorescence Resonance Energy Transfer), results in the emission of a fluorescent signal by the acceptor, and the latter signal can be detected. In the absence of hybridization, the probes are sufficiently separated in solution so that energy transfer cannot occur, and only background fluorescence is emitted by the donor.

Issues of fluorophore compatibility have been resolved by the increased use of a special class of molecule called a quencher. Quenchers are fluorescent molecules that absorb fluorescence energy over a wide range of wavelengths. Instead of re-emitting that energy as light they simply dissipate it as heat. Thus, if a quencher molecule is placed at the 3' end of a probe and a fluorophore at the 5' end, the probe will remain dark even when excitation energy is present so long as the molecule remains intact (hydrolysis probes). These probes utilize the 5' nuclease activity of the DNA polymerase to hydrolyze a probe bound to its target amplicon. Cleavage results in separation of the reporter and quencher and permits fluorescence of the reporter. This reduces much of the work of optimization of the assay conditions (since only a single probe is used) and background noise generated with two probes.

A variation on hydrolysis probes involves placing the reporter and quencher molecules on a single oligonucleotide that is constructed so that, in the unbound state, the quencher and reporter are in close proximity, resulting in efficient quenching of the reporter. When the probe hybridizes to its complementary sequence on the amplicon, the probe undergoes a conformational change that forces the quencher and reporter apart, permitting fluorescence of the reporter. A variation on these kinds of probes is a combined primer and probe in which, again, the quencher and reporter are in close proximity in the native probe, thus resulting in no signal. Priming and subsequent elongation of the primer-probe results in hybridization to the newly synthesized DNA strand, causing spatial separation of the quencher and reporter and resulting in the generation of a signal.

**Probe Labeling**— Modern synthetic oligonucleotide modification chemistries permit the manufacture of oligonucleotides with non-nucleic acid materials. Placement of modifications is carried out in one of two ways: during synthesis or after synthesis. For the former, modifications are constructed in such a way that they behave like the four DNA or RNA bases that are routinely placed in the sequence. The modification is then presented in the desired location during the synthesis as if it were just another base in the series. In the latter, usually employed when more than one modification occurs, the synthesis contains a linker, such as an amino group, to which the desired modification is then attached. This process is often called "hand-tagging."

Perhaps the best-known example of hand-tagging is the conventional dual-labeled probe used in real-time PCR. The quencher is placed at the 3' end of the sequence during synthesis, and the fluorescent reporter molecule is hand-tagged to an amino modification at the 5' end of the sequence after the synthesis is finished and has undergone purification. Some modifications, such as biotins, are designed so that multiple modifications can be carried out in a single synthesis. Thus, it is possible to modify a synthetic DNA or RNA sequence to contain a number of different non-nucleic acid molecules. A cost is associated with such modifications insofar as alterations often are achieved with a loss of mass due either to an inherently lower efficiency of modifications to bind to the oligonucleotide as compared with standard DNA or RNA bases or to the requirement that the synthesis must be purified before modification, after modification, or both.

The benefits of modifying synthetic DNAs or RNAs usually outweigh the costs. The standard, quenched, dual-labeled, real-time PCR probe has permitted precise quantification of gene expression. Fluorescently labeled DNA oligonucleotides are also essential components of *in situ* hybridizations and microarrays. Some modifications confer increased thermal stability when synthetic DNAs or RNAs are hybridized to complementary DNAs or RNAs by comparison with unmodified DNA-DNA and DNA-RNA duplexes. These analogues include peptide nucleic acids, 2'-fluoro N3-P5'-phosphoramidates, and 1', 5'-anhydrohexitol nucleic acids. Although such analogues succeed to varying degrees in achieving increased thermal stabilities, they fail to provide enhanced target recognition. Another approach is to use base analogues such as locked nucleic acid, which is an analogue that contains a 2'-O, 4'-C methylene bridge. This bridge restricts the flexibility of the ribofuranose ring and locks the structure into a rigid bicyclic formation, conferring enhanced hybridization performance and stability.

The modification of a probe typically is governed by its intended use. Generally, fluorescent reporters are used in real-time PCR and for *in situ* hybridization. The range of available fluorescent reporters covers the spectrum from 517 nm to 778 nm. For hybridization probes, base modifications are preferred because these primarily alter thermodynamic interactions between bases, leading to improved specificity. Amino attachment groups, both with and without C-spacers, are used to attach other modifications to DNA sequences and to attach DNA sequences to solid surfaces such as glass slides. An example is the attachment of biotin molecules to DNA sequences. Biotin forms a strong bond with streptavidin-coated materials such as magnetic beads, allowing capture of specific nucleic acids that may themselves be hybridized to other molecules.

**Quantitation**— PCR products may be quantified using a standard curve drawn from replicate serial dilutions of a reference reagent or standard for the nucleic acid sequence of interest. The concentration of the nucleic acid in the reference reagent is known. Real-time PCR quantitation based on a standard curve may utilize plasmid DNA or other forms of DNA. However, the efficiency of PCR must be the same for the standards and the target samples. Performing PCR from purified targets can in some cases be more efficient than performing PCR with complex nucleic acid mixtures. The cycle threshold (Ct) values and concentrations of the dilutions of the reference reagent can be used to construct a standard curve from which the concentration of the unknown sample can be estimated. When the assay run conditions have been well standardized and the standard curve for a particular target has been well calibrated, in subsequent assay runs it may be sufficient to co-amplify only two dilutions of a reference reagent (usually dilutions containing known amounts of nucleic acid at high and low concentrations). These dilutions, or calibrators, can then be used to quantitate any unknown samples by comparison of the Ct values.

**Multiplex PCR**— Multiplex PCR describes the simultaneous amplification of several nucleic acid targets in a single assay reaction. This is a particularly demanding variation of PCR



Because it requires the use of a single set of reaction conditions for the amplification of multiple targets with different sequence characteristics. Additional complications can arise due to the increased chance of nonspecific amplification products arising from multiple primer interactions. In addition, the differing individual target amplification efficiencies can result in weaker reactions being out-competed by stronger, more efficient reactions.

Both qualitative and quantitative applications of multiplex PCR have been described in the literature, as have multiplex RT-PCR assays. Quantitative multiplex PCR relies on either the generation of multiple standard curves to enable quantitation of each target in the assay, or the inclusion of internal competitor sequences that can be used as calibrants.

Hybridization kinetics of primers and probes may be significantly different, even when designed using the same algorithm. This leaves the analyst with very limited room to optimize reaction conditions. However, optimization may include adjustment of DNA polymerase amount, Mg<sup>2+</sup> to increase hybridization efficiency, or primer concentration. Especially in real-time PCR, optimization of primer concentration is critical for quantitative co-amplification of target genes. These are contained in the sample at significantly different amounts.

Increasing hybridization efficiency of the primer–probe system can be achieved by providing sufficient reagents, such as Mg<sup>2+</sup>, as well as adding a “molecular crowding” reagent that increases the effective concentration of all reaction components in the mixture. Multiplex PCR is not only used for genotyping applications, but also for quantitative real-time PCR because it offers several advantages over standard single real-time PCR reactions. Some of these advantages are a minimized amount of sample used, increased precision through the use of an internal control (e.g., housekeeping gene) co-amplified with the target gene in the same reaction, no separate pipetting steps, and cost-effectiveness.

Most PCR assays, however, suffer from a common problem—that of minimizing differences in extractions or amplifications between different samples. Multiplex PCR is useful in cases where it is critical to ensure that variability in quantitation of different samples is not due to differences in nucleic extraction or amplification measurements (usually when one measures the production of an mRNA species). Certain precautions and techniques can be employed to minimize these challenges; they are discussed in the next section on normalization of assay results.

**Normalization of Assay Results**—To minimize the effects of assay variables, analysts sometimes use a relative quantitation procedure that normalizes the target transcript level to a control that can be employed and compared for all samples included in the gene expression study. Probably the most reliable and most frequently used relative quantitation procedure relies on the measurement of “housekeeping” or control genes to normalize the expression of the target gene in a multiplex PCR format. This procedure is preferred because the quantitation of both the housekeeping gene and the target gene are influenced by varying cDNA synthesis efficiencies or the presence of enzyme inhibitors contained in the sample. However, it should be noted that the efficiency of conversion of target RNA to cDNA is not necessarily consistent even within a single-tube reaction but is a function of primer design, target sequence, etc. which may differ between target and housekeeping genes. The selection of appropriate control genes can cause problems because they may not necessarily be equally expressed across all unknown samples and may vary under experimental conditions. Normalizing measurements to a set of housekeeping genes in order to avoid the problem of variability may circumvent this concern. Alternatively, analysts can establish a thorough evaluation of housekeeping genes that do not alter gene expression levels under the experimental conditions.

All the NAT techniques described thus far are variations on the PCR assay, which is the most widely used of the NAT techniques. However, isothermal assays that are based primarily on the amplification of RNA are used for routine purposes. This is known as the transcription-mediated amplification (TMA) assay, which is closely related to the nucleic acid sequence-based amplification (NASBA) assay. Both assays are described in more detail in the following section.

#### Nucleic Acid Sequence-Based Amplification and Transcription-Mediated Amplification

Both NASBA and TMA rely on in vitro isothermal amplification for detection and amplification of nucleic acids, also referred to as self-sustained sequence replication or 3SR. The major difference between the assays is that NASBA uses three enzymes—reverse transcriptase (RT), RNA polymerase, and RNase H—whereas TMA uses only two enzymes: RT and RNA polymerase. The complete procedure generally is performed at 41° to 42° using two primers. Both NASBA and TMA are especially suited to amplifying RNA analytes, including rRNA, mRNA, pathogens that have RNA as their genetic material, as well as DNA targets.

One of the primers that has a promoter sequence for the RNA polymerase at the 5' end binds to the RNA target and is extended via the DNA polymerase activity of the RT. The product of this reaction is an RNA–DNA hybrid. RNase H activity then specifically digests the RNA strand of the hybrid, leaving only the cDNA to which the second primer can bind. A complementary strand of DNA is then synthesized by the RT, resulting in a dsDNA molecule with a T7 promoter at the 5' end. The T7 RNA polymerase then transcribes multiple copies of the RNA amplicon. The RNA copies may undergo the same cycle to create new duplex DNA molecules with a T7 promoter from which many molecules of RNA are transcribed. Thus, unlike the action of PCR, the amplicon amplified in this case is of an RNA species.

Some of the characteristics of this technology are that only relatively short target sequences can be amplified efficiently (around 100–250 nucleotides); it uses a single temperature, which eliminates the need for special thermocycling equipment; the fidelity of the technique is comparable to that of other amplification processes; and the RNA amplicons are exponentially amplified. Carryover contamination is minimized because of the labile nature of the RNA amplicon in the laboratory environment. Containment procedures built into the assay procedure further help to minimize contamination. Detection of amplicons is typically achieved by the use of labeled probes and, in TMA technology, a common method is detection of chemiluminescent signals from hybridized probes that remain intact during the subsequent alkaline hydrolysis step used to destroy free probe.

The NAT techniques described, both PCR and TMA, are optimized for amplifying specific, small fragments of a genome. In cases when whole genome amplification is desirable, such as for mutation analysis or identity testing, modifications of the PCR procedure are necessary in order to ensure adequate sequence representation of genetic loci, as described in the following section.

#### Whole Genome Amplification

Historically, whole genome amplification (WGA) has been performed using modified PCR procedures. These procedures have relied on the nonspecific amplification of the genome using primers that bind under low-stringency conditions to the DNA template. PCR-based approaches differ mainly in terms of the type of primer employed in the reaction: in primer-extension-preamplification (PEP), short 15 base random primers are used in an initial cycling reaction at low stringency to make multiple random copies of segments of the genome.

This product is then used as target for the specific PCR reaction. Amplification bias of favorable sequence contexts leading to uneven representation of the genome is the major drawback of this technique. The generation of increasingly shorter fragments during each round of amplification is a further drawback. Another procedure called degenerate oligonucleotide primed-PCR (DOP-PCR) uses tagged primers and low stringency amplification for the first few cycles of amplification followed by an increase in annealing stringency in later cycles. The tagged primers are characterized by defined sequence tags at the 3' and 5' ends and a random sequence in the centre of the primer. Under the later, more stringent conditions, the target DNA fragments generated during the first cycles containing the amplification tag sequences are amplified preferentially without any further shortening of the fragment length. PCR-based WGA typically employs Taq-like polymerases that possess the disadvantage of introducing variations into the amplified DNA due to their relatively low processivity and fidelity which become compounded by the very high number of amplification cycles used in these methods. This may cause problems in downstream applications such as genotyping analysis. These limitations as well as the relatively poor sequence representation of genomic loci inherent to PCR-based WGA can be overcome by an isothermal reaction called multiple displacement amplification (MDA).

The enzyme that is used for MDA comprises a high processivity polymerase with proofreading and strand-displacement activity. The isothermal reaction is performed at 30° without any change in reaction temperature. The reaction starts with the annealing of multiple random primers to the target DNA and elongation of the primers using a DNA polymerase from the *Bacillus subtilis* phage Phi29. Because the polymerase is able to displace DNA strands in a 5'–3' direction, the polymerase reaction is not stopped when the elongating strands meet downstream DNA strands. The displaced DNA strand serves again as a target for multiple primed elongation reactions so that the DNA template is amplified exponentially in a branched-like manner, yielding high molecular weight DNA with a good representation of the genomic loci. Compared with PCR-based WGA, the error rate is very low. In particular, the mutation rate of repetitive sequence structures is low because of the limited strand-displacement activity of Phi29-polymerase. This permits reliable genotyping of genomic DNA (e.g., SNP analysis, mutation analysis, identity testing, or analysis of case work samples) on different platforms such as real-time PCR or array analysis.

#### INSTRUMENTATION

The development of the numerous and varied NAT techniques described in this chapter has been facilitated by the evolution of instrumentation that has served to automate these complex procedures. A general description of the major changes in instrumentation is discussed in this section.

The continuous control of the temperature steps necessary to achieve exponential amplification for PCR assays is carried out by fully automated thermocyclers that consist of a heating block in which the temperature can be rapidly cycled. Temperature changes are induced by water, or more recently, by using the Peltier effect. These instruments may be coupled to a fluorometer apparatus if they are used for real-time PCR analysis. In the latter case certain instruments are equipped with a rotor device that is heated and cooled by air instead of a



...etal block that typically is used as a heating module. In the case of endpoint PCR, PCR products are usually analyzed according to size on agarose or polyacrylamide gels, or by capillary electrophoresis using fluorophore-labeled primers. They may also be analyzed by an array-based approach or other hybridization procedures.

Because no post-PCR processing or label-separation steps are required, real-time PCR assays are simple to perform, making them useful for high-throughput applications. Real-time PCR instruments combine the properties of a thermocycler and a fluorometer to allow determination of PCR products by fluorescence measurement. In each PCR cycle, either one or several fluorescence readouts are taken to monitor the PCR reaction for generation of amplicons, usually at the extension step of the PCR reaction.

Real-time PCR instruments vary with regard to simultaneous sample throughput (32–384 reaction vessels), sample volume (5–100  $\mu$ L), excitation source, and detector used. These compositions define the suitable range of fluorescent dyes for multiplex real-time PCR as well as size and heating/cooling principle (see above). The excitation source of real-time thermocyclers is either a laser-based system, halogen bulbs, or light-emitting diodes (LED). Optical filters are used to select the wavelength of interest. In most instruments, the emitted light is detected by a charge-coupled device (CCD) that consists of an array of light-sensitive cells. Light projected onto the CCD is converted to an electric charge, resulting in a signal that is proportional to the light intensity.

The versatility of the PCR assay has resulted in the widespread and diverse use of this technique. With the advent of real-time PCR, it has been possible to design high-throughput instrumentation for automated testing. Similarly, the TMA assay has also been automated. Such technology is used by laboratories doing high-throughput, highly regulated testing, typically blood screening for hepatitis C virus (HCV) or human immunodeficiency virus-1 (HIV-1) because automated tests are ideal in a regulated environment where minimum human intervention is required. The use of NAT in a highly regulated environment has resulted in the development of guidances for managing the quality assurance (QA) and quality control (QC) aspects of testing, as well as the validation of systems and assays as described in the following section.

#### QUALITY ASSURANCE AND QUALITY CONTROL FOR NAT

This section serves as a general guidance for the development of laboratory- and procedure-specific QC and QA procedures for NAT. Aspects such as waste management, management of radioactive material, or working with hazardous material are not covered. NAT is a technology that offers extreme sensitivity with its ability to generate millions of amplicons from as little as a single nucleic acid template, resulting in a detectable signal. The advantages of this technology can be offset by the necessity of establishing complex assay protocols and the requirement to follow carefully very stringent QC/QA protocols. Deviation from these protocols can cause major problems, such as false positive results due to the contamination of templates by amplicons generated in previous assay runs. Similarly, failure to control inhibitors could lead to suboptimal amplification and possible false negative results. Given the myriad factors that can greatly influence the outcome of a NAT assay, all aspects concerning NAT need to be covered by appropriate and stringent QC/QA procedures. This requires careful facility design, workflow, and selection of equipment suitable to the purpose. Data recording, record keeping, and data interpretation are other aspects that should be covered by QC/QA. Thus, QA for NAT assays includes assay validation, establishment of acceptance criteria and specifications, and adherence to good manufacturing/laboratory practices. These aspects are also described in this section. In addition, reference should be made to other published guidelines such as the ICH Guideline Validation of Analytical Methods: Methodology (Q2B) and the NCCLS Guidelines.

##### Laboratory QC/QA

An NAT laboratory should be designed and operated in a manner that prevents contamination of reactions with products from previous amplifications (carry-over) as well as cross-contamination between samples. Historically, the application of PCR required strict separation of the various steps of the assay in order to prevent cross-contamination of PCR by amplicons. This was necessary because early procedures for analysis of PCR products involved the transfer of the product, which potentially could lead to contamination. Therefore, in an open system the best measure to prevent contamination has been the strict separation of working areas for individual process steps. This includes individual areas for template preparation, master mix setup, distribution of the master mix to individual reaction wells and addition of template, space for cycling the PCR assays and, optionally, a separate work space for PCR product analysis. These requirements are not necessary with closed systems. With both open and closed systems it is still necessary to take additional precautions. These safety measures include UV illumination of work spaces overnight to inactivate residual DNA by crosslinking. In case of contamination, laboratory benches and pipettes can be decontaminated by cleaning with a 10% solution of commercial bleach, which usually contains about 5% sodium hypochlorite, taking appropriate safety measures such as wearing gloves and eye protection. Afterwards, benches and pipettes should be rinsed with distilled water. A unidirectional workflow will reduce the opportunity for contamination to occur. Also, no materials, supplies, or equipment should be exchanged between designated working areas or rooms.

##### Equipment QC/QA

Other good laboratory practices that are related to the prevention of carry-over contamination include the use of suitable and clean equipment. Generally, disposable consumables (tubes, pipette tips, etc.) are highly preferable to reusable equipment. The use of disposable tips containing hydrophobic filters is another very effective measure to minimize cross-contamination. All samples, primer, probes, etc. must be labeled with relevant information such as identity of the content, date of use or preparation, expiration date, concentration, and storage information. Dedicated laboratory coats or disposable lab coats should be available in each room (or section) of the NAT laboratory. Appropriate gloves should be used during all processing steps to prevent sample contamination. The gloves should be changed frequently. Because heat sterilization does not completely destroy DNA, PCR products may lead to detectable contamination of, for example, glass surfaces. Following unique sterilization procedures for different materials such as waste and glass laboratory equipment is advisable.

##### Carry-Over Prevention with Uracil-N-Glycosylase

Contamination by PCR product carry-over can be mitigated by using the commercially available uracil-N-glycosylase (UNG) procedure. The procedure involves substituting 2'-deoxyuridine 5'-triphosphate (dUTP) for 2'-deoxythymidine 5'-triphosphate (dTTP) in the PCR setup and treating all PCR mixtures with UNG prior to PCR amplification, which can be easily incorporated as a first step into PCR cycling programs. Incorporating dUTP into the amplicon makes the PCR products biochemically distinct from the native DNA template. The enzyme UNG cleaves the deoxyuridine-containing PCR products by opening the deoxyribose ring at the C1 position. When the deoxyuridine-containing DNA is heated during the first thermal cycle, the amplicon DNA chain breaks at the position of the deoxyuridine at the alkaline pH of the PCR reaction mixture and thereby renders the carried-over PCR product nonamplifiable. Thus, any previously generated U-containing amplicon that might have contaminated another sample will become nonamplifiable. As a consequence, false positive results can be avoided. However, it should be noted that UNG has concentration limits above which it does not fully remove PCR carry-over products.

##### Validation of NAT Systems

###### Assay validation is achieved by

1. ensuring the quality and consistency of assay components, including primers, probes, and enzymes; (including shelf life and contamination control) and
2. establishing the performance characteristics of the NAT assay in terms of reproducibility, accuracy, ruggedness, robustness, specificity, precision, and analytical and clinical sensitivity.

The analytical sensitivity of an assay is defined as the minimum concentration of a reference reagent or standard detected by the test while the clinical sensitivity of a test is determined by testing clinical specimens and determining the 95% LOD. The clinical sensitivity of a test is not necessarily the same as the analytical sensitivity. The closer the reference or standard material is to the samples being tested the closer the correlation.

###### The principal steps of assay validation are

1. sample preparation;
2. consistent production of critical reagents;
3. use of controls, calibrators, and quantitation standards;
4. specimen and reagent stability;
5. functionality of instruments and software;
6. operator training; and
7. laboratory surveillance for proficiency.

Following assay validation, further QA is necessary to monitor specifications and functional characteristics that have been established by the use of well-characterized reagents of known potency.

##### Quality Control of Reagents



—DNA Templates— The test specimens used are usually, but not limited to, whole blood, plasma, and serum. Specimen preparation is a key step in the NAT assay and has a major influence on the performance and variability of the assay. Specimen collection is the first step in sample preparation. QC/QA staff should carefully evaluate the effects on the integrity of DNA of collection tubes and temperatures during sample transport. To prevent cross-contamination during specimen collection, aseptic techniques should be used along with closed sampling systems in order to avoid specimen contamination. The use of appropriate sample handling techniques, temperature conditions, and anticoagulants or preservatives should help reduce the risk of contamination. Anticoagulants such as heparin or EDTA may interfere with the NAT assay.

Sample Extraction— The buffers, reagents, and detergent or chaotropic agents used for nucleic acid extraction should be evaluated for inhibitory effects on the NAT assay. Extraction controls, including spiked materials, should be included to monitor the efficiency and reproducibility of the extraction method. Reproducibility of the sample preparation method should be determined under the specimen processing conditions, including sample handling, storage, and shipping conditions. DNA is generally stable, but personnel should take care to avoid storage at refrigerated temperatures for extended periods of time to avoid sample degradation. Repeated freeze-thaw cycles can sometimes cause DNA fragmentation. In the case that the target is RNA, it should be noted that RNA is very unstable and specimens should be frozen.

Primers— Primers and probes should be qualified in terms of purity, identity, and functional potency. Purity can be assessed by use of HPLC or mass spectrometry; identity can be established by sequencing; and functionality can be established by the use of reference reagents. However, in many cases, these methods may not be available for in-house testing. In these cases, it may be sufficient to compare lot-to-lot variation of purity and functional potency using relevant methods available in-house coupled with the use of reference reagents.

DNA Polymerases— The functionality of enzymes should be determined using reference materials. Enzyme preparations should be tested for other enzymatic activities; for example, exonucleases and DNA- and RNA-dependent polymerase activities and specifications should be established. Lot-to-lot comparison, as well as comparison with the manufacturer's CoA should also be done. Storage conditions recommended by the manufacturer should be strictly followed, and appropriate controls should be used to monitor the stability of enzymes.

#### Run Controls

The use of controls affords the operator assurance that the assay has performed within accepted specifications. In PCR testing, several steps in the testing process, as outlined above, should be monitored and verified. Multiple controls or controls that serve multiple purposes may be needed for a PCR assay. Controls should reflect the specific technology under development but should typically allow monitoring of ultracentrifugation, extraction, amplification, hybridization, quantitation, contamination, etc. Controls should be similar to the specimen type whenever feasible, although spiked controls may be acceptable.

A negative control is one that does not contain the target sequence or pathogen that is being tested. It should resemble as closely as possible the sample matrix under testing. Multiple negative controls should be examined, including nontarget sequences and nucleic acid-free controls to monitor for false positives resulting from contamination. Because of the high sensitivity of amplification assays, QC/QA personnel highly recommend that sponsors include control measures for the prevention of contamination events.

A positive control is one that contains the target sequence of interest. It should resemble as closely as possible the specimen matrix being tested and should contain an appropriate and defined amount of target sequences. (e.g., kit control).

Specifications for both positive and negative controls should be provided, as well as validation data supporting the proposed assay cut-off/reporting threshold value or the assay's limit of detection. The laboratory should define the source of the controls and calibrators and have a plan for their continued renewal. Controls can be infectious or non-infectious. In the latter case, validation of viral inactivation should be provided.

Reagent controls are often referred to as blanks and could include samples that have no target sequence, no enzyme, no primers, etc. These controls provide additional information about problems encountered in PCR assays.

An internal control is added to each specimen to ensure the overall validity of the individual test results. Internal controls are used to verify sample extraction, amplification, and detection.

#### External Quality Assessment and Proficiency Testing

Quality assessment of the laboratory is achieved by participation in periodic competency assessment and laboratory proficiency programs. The latter should include the testing of reference reagents and well-characterized panels to measure the technical proficiency of operators. Therefore, care should be taken to prevent cross-contamination, to monitor workflow, and to ensure careful specimen and test sample handling. Evaluation of operator proficiency should include participation in competency and quality assessment programs.

Each operator in a particular laboratory should participate in such programs and should demonstrate comparable results.

#### Data Management

Complete and consistent documentation of all activities performed and all data generated is necessary. Such documentation does not only require the maintenance of records of the data generated through sample testing but also information about reagents and equipment calibration and maintenance. Moreover, any alteration in the assay procedure needs to be introduced through a planned change control process and documented in such a way that change can be assessed by an independent party.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

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1129 NUCLEIC ACID-BASED TECHNIQUES—GENOTYPING

#### INTRODUCTION

This chapter outlines techniques for detecting single-base DNA differences and other types of polymorphic DNA sequences that occur in the three billion bases that make up the human genome. The most common genetic variation is a single nucleotide polymorphism (SNP), which is a simple change in one base of the gene sequence. SNPs occur on average every 1000 bases and account for a significant amount of inter-individual variability. SNPs can predispose individuals to disease or influence their response to a drug. Approximately 1.8 million human SNP loci have been identified, and more are likely to be discovered in the coming years.<sup>4</sup>

Common approaches for detecting SNPs and other types of polymorphic DNA sequences are described in the following sections. These approaches encompass a variety of techniques, such as nucleic acid amplification techniques (NAT), real-time NAT, and microarrays, the principles of which are covered in more detail in related chapters. This chapter focuses on the specific modifications of the techniques that are necessary to enable detection of single base differences.

#### SNP Genotyping Technologies

Although the usefulness of studying SNPs for gene mapping and disease association studies is apparent, a single standardized procedure for SNP genotyping has not been adopted.

Various approaches for performing SNP genotyping have been developed to meet a wide range of needs, including throughput capacity, ease of assay design, accuracy, and reliability. Available procedures can also be divided according to whether they are based on identifying known SNPs or whether they can be used to screen for unknown SNPs. To identify the most appropriate SNP genotyping procedure for a specific application, the throughput requirements in terms of the number of SNPs to be analyzed per sample (multiplexing level) and the sample throughput need to be determined because different approaches may work best depending on these requirements.

Most procedures used for genotyping SNPs depend on polymerase chain reaction (PCR) amplification of the genomic regions that span the SNPs followed by the actual genotyping reaction. PCR provides the required sensitivity and specificity for distinguishing between heterozygous and homozygous genotypes in large, complex genomes. The difficulty of



Designing and carrying out multiplex PCR reactions limits the throughput of many of the current SNP genotyping assays. The following sections outline several of the major approaches currently in use for SNP genotyping. In many cases the underlying technology can be modified to meet the specific application requirements in terms of sample throughput and number of SNPs detected. In general, real-time PCR-based procedures are better suited to higher sample numbers, and array-based procedures are better suited to the simultaneous detection of many SNPs. Newer technologies based on multiplexed array formats are also emerging and will be suitable for high sample numbers and many SNP applications.

### Sequencing

Sequencing is the definitive procedure for DNA analysis, and its use for SNP detection allows unambiguous identification of base changes (see [Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing](#) 1126 for nucleic acid sequencing). The standard technology is expensive, and the procedure is time consuming and labor intensive and suffers from low sample throughput. Sequencing is a useful confirmatory tool, and it has applications in situations when other technologies are not appropriate, but is not the most cost-effective solution for the majority of SNP genotyping applications that require the identification of only one or a few bases.

### Restriction Fragment Length Polymorphism Analysis

The first widely used procedure for the detection of polymorphisms exploited alterations in restriction enzyme sites caused by SNPs, leading to the gain or loss of cutting events. PCR-restriction fragment length polymorphism (RFLP) analysis comprises PCR amplification of a fragment of interest and subsequent digestion with a restriction enzyme. The fragments produced are typically analyzed by a size fractionation procedure, usually gel electrophoresis. Because of its simplicity, the procedure has been and still is extensively used, although it entails certain limitations: only a subset of polymorphisms that reside in an endonuclease restriction site can be studied with the conventional procedure; incomplete digestion due to suboptimal processing can produce misleading digestion patterns; and the procedure is less amenable to automation than are other SNP genotyping procedures.

### Probe Hybridization

The basis of many SNP genotyping procedures are DNA hybridizations that make use of the stronger binding of a DNA probe to a perfectly matched complementary target than to a target that contains a single base mismatch. The ability of hybridization with allele-specific oligonucleotides (ASO) to detect a single base mismatch was first shown in the late 1970s and subsequently was used to detect the sickle-cell mutation in the beta-globin gene by Southern blot hybridization. The invention of PCR facilitated the further development of probe-based assays for genotyping SNPs in complex genomes.

The thermal stability of a hybrid between an ASO probe and its SNP-containing target sequence is not only determined by the stringency of the reaction conditions but also by the secondary structure of the target sequence and the nucleotide sequence flanking the SNP. Therefore, prediction *a priori* of the reaction conditions or the sequence of the ASO probe that will allow optimal discrimination between two alleles using ASO hybridization is difficult. These parameters should be established empirically and separately for each SNP. Consequently, there is no single set of reaction conditions that would be optimal for genotyping all SNPs, which makes the design of multiplex assays based on hybridization with ASO probes an extremely difficult task.

One approach to counter the problem of assay design is to carry out multiplex ASO hybridization reactions on arrays that carry multiple probes for each SNP that will be analyzed. This involves using probe sets in which the SNP occurs at different positions along the probes. It becomes feasible to include large numbers of ASO probes per SNP when one uses high-density arrays that can carry as many as 106 probes per cm<sup>2</sup>.

Another approach is to use base analogues such as locked nucleic acid (LNA), which is described in detail in [Nucleic Acid-Based Techniques—Amplification](#) 1127. For applications that involve few SNPs but many samples, homogeneous real-time PCR approaches have been developed. These include the use of fluorescent probe chemistries such as hydrolysis probes, stem-loop probes, and FRET (fluorescence resonance energy transfer) hybridization probes. The principle of these assays is discussed in more detail in [Nucleic Acid-Based Techniques—Amplification](#) 1127. For SNP detection, the basis of many assays is the selective binding of the ASO probe to its perfectly matched target sequence, resulting in energy transfer and generation of a fluorescence signal. Probes designed with specific secondary structures tend to form a stem-loop structure that destabilizes mismatched hybrids, increasing their power of allele distinction as compared with that of linear ASO probes. Hydrolysis probes modified with minor groove-binder molecules that increase target affinity show improved powers of allele discrimination. The use of two probes, each labeled with a different reporter fluorophore, allows both SNP alleles to be detected in a single tube. Limited multiplexing can be achieved by using probes labeled with different fluorophores. In the fluorescent probe-based assays, the increase in fluorescence due to accumulating PCR product is usually monitored in real time in 96-well or 384-well microtiter plates. Alternatively, the fluorescence generated from the two alleles can be measured after completion of the PCR. In this case the results are expressed as a signal ratio that reflects the hybridization of the two oligonucleotides to the target sequence, and so differences in amplification efficiency between samples do not affect interpretation of the genotyping results.

A third approach involves heating the reaction after PCR has been completed in order to disassociate the probe from the target. Each duplex has its own specific T<sub>m</sub>, which is defined as the temperature at which 50% of the DNA becomes single stranded. The T<sub>m</sub> depends on the stability of the probe-target duplex. Perfectly matched probe-target duplexes have a greater stability and hence a higher T<sub>m</sub> than does the same duplex containing a single base mismatch. By continuously monitoring the fluorescence during the heating phase, analysts generate a "melt curve" that measures the changes in fluorescence that result when the probe denatures, or "melts," away from the amplicon. This approach can be used only for systems that do not rely on hydrolysis of the probe to generate a signal and is therefore not suitable for hydrolysis probe assays.

Because no post-PCR processing or label-separation steps are required, homogeneous real-time PCR assays are simple to perform, making them useful for high-throughput genotyping applications. The optimal probes must be designed individually for each SNP, and the assays are therefore most efficient when a limited number of SNPs is analyzed. The cost of probes modified with fluorescent and quenching moieties may also be a limiting factor in the high-throughput application of the assays.

### Primer Extension

In this technique, an oligonucleotide is used to prime DNA synthesis by a polymerase enzyme, as performed in a standard PCR or sequencing reaction. Variations of the technique exist. Allele-specific PCR uses two primers, each fully complementary to one of the SNP alleles, with the SNP position being at the 3' end of the primer, and with a common reverse PCR primer to selectively amplify the SNP alleles. Because only perfectly matched oligonucleotides will prime DNA polymerase extension, product will be detected only from the reaction containing the perfectly matched primer.

Agarose gel electrophoresis is used to detect the amplified products, although homogeneous, real-time, allele-specific PCR approaches have also been developed using primers labeled with different fluorophores or a fluorescent dye that intercalates with the double-stranded PCR products or by performing amplicon detection using probes such as hydrolysis and hairpin (stem-loop) probes. When using intercalating dyes or labeled allele-specific PCR primers without a consecutive target-specific detection reaction or size-separation step, one may find that the specificity of the procedure may be compromised owing to primer-dimers and other spurious amplification products that will not be distinguished from the actual PCR product. A limitation of all variants of allele-specific PCR is that the reaction conditions or primer design for selective allele amplification must be optimized empirically for each SNP. Like the hydrolysis and hairpin probe assays, the homogeneous allele-specific PCR procedures are best suited for the analysis of a limited number of SNPs in large sample collections. Array-based approaches for greater SNP multiplexing have also been developed.

In procedures based on single nucleotide primer extension (sometimes known as minisequencing), allele discrimination is based on the high accuracy of nucleotide incorporation by DNA polymerase. A primer is used, and its 3' end is positioned on the base just preceding the SNP to be tested. The DNA polymerase is then used to incorporate labeled ddNTPs, each labeled with different fluorescent dyes. After the labeled oligonucleotides are separated from the nonincorporated ddNTPs, the results can be scored on a fluorescence plate reader. In addition to fluorescent tags, ddNTPs may be labeled with biotin or haptens and then detected indirectly through antibodies conjugated to alkaline phosphatase or peroxidase using colorimetric or chemiluminescent markers in ELISA formats.

Multiplexing of this procedure has also been described to reduce costs and improve throughput. In these procedures, the different loci genotyped simultaneously are separated either by gel electrophoresis or by hybridization to arrayed tags. Primer extension directly on a solid support such as a microarray is also possible. The immobilization of the single-stranded primers on the solid support may be through biotin-avidin-streptavidin reaction or covalently via 5' disulfide groups.

Mass spectrometry using techniques such as matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) can also be used to determine the identity of the ddNTP incorporated based on mass. A difficulty with MALDI-TOF MS is that the primer extension products must be rigorously purified before measurement to avoid background from biological material present in the sample. Such enzyme-assisted procedures have proven to be more robust and to provide more specific allele discrimination than does ASO hybridization at similar reaction conditions. These features are advantageous for high-throughput applications because the effort required for assay design and optimization is minimized.



### Ligation

In the oligonucleotide ligation assay (OLA), oligonucleotides are designed so that they meet at the position of the SNP to be tested. Enzymatic joining, using a DNA ligase, occurs only when the match is perfect. The test is usually performed by designing two oligonucleotides specific for each allele and labeled differently on one side of the SNP, and one common oligonucleotide on the other. Detection of the alleles can be performed directly in the microplate wells by colorimetric approaches. Multiplexing and the use of gel separation have also been described.

OLA has also been used in microarray formats with one of the ligation probes immobilized or with immobilized single stem-loop probes. Alternatively, ligation can be carried out in solution followed by capture of the ligation products on microarrays or on microparticles that carry a generic set of oligonucleotides that are complementary to a "tag" sequence on one of the ligation probes. In practice, thermostable ligases are frequently used for genotyping SNPs in combination with PCR before allele-specific ligase detection reactions. Because the reaction mechanisms for PCR and ligation are different, the reagents for both reactions can be combined. This feature is used in a homogeneous, real-time PCR assay with ligase-mediated genotyping and detection by FRET. Compared with DNA-polymerase-assisted primer extension procedures, a drawback of the OLAs is that detection of each SNP requires three oligonucleotides, which increases the costs of these assays.

Padlock probes are linear oligonucleotides, the ends of which are complementary to the target and have a central stretch of random sequence. When perfectly hybridized to their target sequence, padlock probes can be circularized by ligation, whereas a mismatch with the target sequence prevents ligation. Circularized oligonucleotides can act as templates for DNA-polymerase-assisted rolling circle amplification (RCA). RCA can be used to amplify the ligated circularized padlock probes to a level required for detecting single-copy sequences. A homogeneous, isothermal assay for genotyping individual SNPs in a microtiter plate format has been devised by combining exponential amplification of ligated padlock probes using a branched rolling circle amplification reaction with detection by energy-transfer-labeled hairpin primers.

### Displacement

The invader assay uses the property of flap endonucleases (FENs) for removing redundant portions (flap) from the 5' end of a downstream DNA fragment overlapping an upstream (invader) DNA fragment. An invader oligonucleotide is designed with its 3' end on the SNP to be tested. Two oligonucleotide signal probes are also designed, overlapping the polymorphic site and each corresponding to one of the alleles. After displacement of the signal probes by the invader probe, FEN-mediated cleavage occurs only for the perfectly matched allele-specific signal probe. Generation of the cleaved fragment is monitored by using it in a second reaction as an invader probe to cleave a FRET probe. This assay does not require PCR amplification of the locus to be tested, and scoring can be done using a simple fluorescence plate reader.

### Pyrosequencing

In the pyrosequencing procedure, primer extension is monitored by enzyme-mediated luminometric detection of pyrophosphate (PPi), which is released on incorporation of deoxynucleotide triphosphates. The genotype of an SNP is deduced by sequential addition and degradation of the four nucleotides using apyrase in a dedicated instrument that operates in a 96-well or 384-well microtiter plate format. Using pyrosequencing, the apparatus can determine short 30 to 50 bp sequences of DNA that flank an SNP. A limitation of the procedure is that the sequential identification of bases prevents genotyping of several SNPs per reaction in diploid genomes. An advantage of the procedure is that any new polymorphism will be detected. However, specific equipment is needed for the injection of the nucleotides.

### Single-Strand Conformation Polymorphism and Heteroduplex Analysis

Single-strand conformation polymorphism (SSCP) and heteroduplex analysis were among the first procedures established for the detection of SNPs. Conventional SSCP analysis involves denaturing PCR-amplified fragments and subsequent formation of sequence-specific secondary and tertiary structures of the single strands during nondenaturing gel electrophoresis. The electrophoretic mobility then depends on the 3-D shape of the single-stranded molecules. One single base difference in DNA fragments of up to 300 bp will usually change the conformation in a way that can be detected by nondenaturing PAGE.

The traditional polyacrylamide gels and 32P-labeled fragments are frequently being replaced by fluorescently labeled fragments and automated capillary electrophoresis. The simplicity of the procedure, combined with automation and short analysis time, contribute to high-throughput analysis at relatively low cost. If the denatured PCR products are allowed to slowly re-nature, they form DNA duplexes. The duplexes with the same sequence on both strands (homoduplexes) or with a single base pair mismatch on one strand (heteroduplexes) have different electrophoretic mobility in a native gel. In the case of a single base pair substitution, the heteroduplex can easily be separated from a homoduplex.

In other versions of the technique, denaturing high-performance liquid chromatography (DHPLC) is used for the separation of the heteroduplex and homoduplex strands. The mutation analysis with DHPLC can be almost totally automated with an autosampler on one end and a fraction collector on the other. Analysis is rapid (about 5 minutes per sample), and simple evaluation of data distinguishes between simple and multiple peaks in the elution profiles, allowing lengths as large as 1.5 kb of DNA to be analyzed. A disadvantage may be the recommended use of Pfu DNA polymerase, which, as a high-fidelity enzyme, allows sharper peaks but may be less successful in amplifying some regions.

### Short Tandem Repeat Profiling

A short tandem repeat (STR) is a type of DNA polymorphism that occurs when a pattern of two or more nucleotides is repeated and the repeated sequences are directly adjacent to each other. The pattern can range in length from 2 to 10 bp (e.g., CATGn in a genomic region) and is typically in the noncoding intronic, or upstream/downstream regions. By examining several STR loci and counting how many repeats of a specific STR sequence there are at a given locus, one can create a unique genetic profile of an individual. Currently more than 10,000 STR sequences in the human genome have been published. STR analysis has become the prevalent analysis procedure for determining genetic profiles in forensic cases. STR analysis in the field of forensics came into popularity in the mid to late 1990s. The STRs in use for forensic analysis are tetra- or penta-nucleotide repeats (4 or 5 repeat units) because these give a high degree of error-free data while being robust enough to survive degradation in nonideal conditions. Shorter repeat sequences tend to suffer from artifacts such as stutter and preferential amplification; several genetic diseases are associated with tri-nucleotide repeats, including Huntington's disease. Longer repeat sequences suffer more highly from environmental degradation and do not amplify by PCR as well as do shorter sequences.

The analysis is performed by extracting nuclear DNA from the cells of a forensic sample of interest and then PCR amplifying specific polymorphic regions of the extracted sample. Once these sequences have been amplified, they are resolved either by gel electrophoresis or capillary electrophoresis, which allow the analyst to enumerate the repeats of the STR sequence in question. If the DNA is resolved by gel electrophoresis, the DNA can be visualized either by silver staining or an intercalating dye such as ethidium bromide or, as in most modern forensics labs, by fluorescent dyes. Instruments built to resolve STR fragments by capillary electrophoresis also use fluorescent dyes. In the United States, 13 core STR loci have been selected as the basis by which an individual genetic profile can be generated. These profiles are stored in local, state, and national DNA databanks such as the Combined DNA Index System (CODIS).

Forensic reference materials are available. The DNA Profiling Standard is composed of well-characterized human DNA in two forms: genomic DNA and DNA to be extracted from cells spotted onto filter paper.

### ASSAY VALIDATION CONSIDERATIONS

The difficulty in reproducing and validating existing and emerging SNP genotyping assays due to factors such as variation in performance of PCR thermal cyclers, efficiency of different enzymes, personnel, and the presence of PCR inhibitors in the sample matrix (discussed in more detail in [Nucleic Acid-Based Techniques—Amplification](#) (1127) for general NAT assays) can hamper appropriate implementation of the technologies. Also, in the clinical laboratory the use of in-house assay formats often makes comparisons between laboratories difficult. Incorrect diagnosis of a genetic mutation can have significant consequences, so accuracy of 99.99% or higher is essential for such assays. To determine the accuracy of a technology, the new procedure should be validated on multiple samples in which the genotype has been previously determined with a gold standard procedure, such as sequencing.

Even with the most accurate procedure of analysis, sample preparation and amplification and detection procedures must be optimized to eliminate any potential inaccuracies.

Some genotyping errors can be minimized by careful planning of the laboratory procedures, the inclusion of well-defined controls, and increased automation. However, errors due to the processes used for genotyping are sometimes difficult to overcome and need to be taken into account. The types of errors and the frequency with which they occur differ between different approaches. Situations in which preferential amplification of one allele or nonspecific probe hybridization occur can all result in SNP miscalls. Additional unanticipated polymorphisms present within the primer/probe sequences can lead to amplification bias, highlighting the need for careful assay design and validation using alternative techniques.

Limited and degraded samples can also result in preferential allelic amplification due to chance PCR priming events at low copy number.



It is preferable to have a no-call result, which would require the test to be repeated, than a miscall that provides incorrect results that are subsequently reported. Performance of replicate assays may also help to ensure accuracy. Data interpretation can also affect accuracy. Wild-type, heterozygous, and homozygous mutant results should be clearly distinguished from one another, and a well-defined measure of uncertainty should be attributed to them. Proficiency testing schemes and ring-trials go some way toward ensuring that individual assays are fit for the purpose for which they are intended for specific applications and that the staff performing them are competent. Sharing of technical information for assay design and sample preparation will also help. The availability of reference panels of well-characterized samples aids assay design and evaluation and allows sound interlaboratory comparisons to be made.

1 Database of Single Nucleotide Polymorphism (db SNP) Build 128 is available from National Center for Biotechnology Information (NCBI),  
<http://www.ncbi.nlm.nih.gov/projects/SNP/index.html>.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

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1130 NUCLEIC ACID-BASED TECHNIQUES—APPROACHES FOR DETECTING TRACE NUCLEIC ACIDS (RESIDUAL DNA TESTING)

## INTRODUCTION

The basic principles of nucleic acid amplification technologies (NAT) and definitions of the various techniques are covered in [Nucleic Acid-Based Techniques—General 1125](#). This chapter covers the analytical procedure used to quantify residual DNA in biopharmaceuticals.

Quantification of residual DNA impurities in biopharmaceuticals is based on safety concerns. The cells used to produce biopharmaceuticals can be sources of a range of complex, heterogeneous, and potentially unsafe impurities, and host cell DNA is among these. Much of the safety concern associated with residual DNA in biopharmaceuticals lies in the possibility that host cell DNA, particularly continuous-cell-line DNA, may result in tumors or adverse reactions. Cells used to produce biopharmaceuticals may possibly carry viruses or harbor harmful nucleic acid, and the residual DNA in a given biopharmaceutical product may be infectious. Although animal testing has shown that extraneous DNA can cause tumors or infections, no reports to date have demonstrated this risk in humans. Therefore, some regulatory agencies have allowed a target of 100 pg or less of residual DNA per dose in biopharmaceuticals, and levels up to 10 ng of residual DNA per dose may be considered, depending on the source of the residual DNA and the product's route of administration.

One can address residual DNA in pharmaceutical processes in two ways: by validating clearance during process validation or by monitoring residual DNA levels by routine testing of the drug substance. The level of concern regarding residual DNA can be tied to the potential source of the residual DNA (e.g., infectious viral DNA) and the route of administration, so the residual DNA specification and procedure for monitoring DNA clearance for a given product should be developed in consultation with regulatory agencies. Regardless of whether routine testing of a drug product is used to determine residual DNA content or whether DNA clearance is demonstrated by process validation, analytical procedures for the quantification of residual DNA are required. The analytical procedures used to determine the residual DNA content of biopharmaceuticals can include hybridization, instrumentation based on DNA-binding protein, quantitative PCR (q-PCR), or other DNA amplification methods. The expectation is that the analytical procedure used to quantify residual DNA in biopharmaceuticals has a detection limit approximating 10 pg per dose. The assays based on hybridization, DNA-binding protein, and q-PCR are typically the techniques of choice because they can meet the sensitivity expectation.

## SAMPLE PRETREATMENT

Analysis of residual DNA requires accurate quantification of pg levels of DNA in mg (or larger) quantities of product. The sample itself, whether it is a protein or other chemical entity, can create sample matrix effects that must be overcome in order to yield a useful assay. Protein samples may require only digestion with proteinase (e.g., Proteinase K, Pronase) to allow the analytical method to quantitatively recover the residual DNA. Treating the sample with a detergent may be required to dissociate the residual DNA from the sample matrix. Traditionally, extraction methods based on phenol and chloroform, followed by ethanol precipitation, have been applied to the purification of DNA in molecular biology research. The phenol/chloroform extraction technique may be a useful pretreatment for residual DNA samples prior to analysis. Because of the typically low levels of residual DNA present in samples, quantitative DNA recovery with ethanol precipitation may be difficult. For this reason, a carrier molecule (e.g., glycogen) may be necessary to aid in DNA recovery if this technique is used.

A commercial kit is available<sup>1</sup> and has been used successfully for pretreatment of residual DNA samples. The commercial kit uses a chaotrope (sodium iodide) and a detergent (sodium N-lauroyl sarcosinate) to disrupt the association of the DNA with the sample. The DNA is then co-precipitated using glycogen as the carrier molecule in the presence of isopropanol.

Each of these pretreatment techniques may yield acceptable results, or analysts may combine the techniques to obtain acceptable recovery of the residual DNA from the sample. Sample extraction is an extra handling step that may cause the incomplete recovery of the residual DNA or may introduce environmental DNA into the sample, so great care must be taken during any sample manipulations. Addition of DNA-spiked samples in the residual DNA assay is a common practice. A recovery of 80% to 120% of the spiked DNA is an acceptance criterion often applied to residual DNA assays to ensure that the assay yields acceptable results. When sample characteristics (e.g., matrix effects, sample preparation method) make achieving a recovery acceptance criterion of 80–120% impractical, then correcting the observed DNA concentration by the load recovery percentage is also an acceptable approach. During the qualification of a residual DNA assay, some scientists treat the samples with DNase I to degrade the DNA in the sample in order to demonstrate that the assay response was due to DNA and not some other sample component.

## HYBRIDIZATION-BASED RESIDUAL DNA ASSAY

The first residual DNA assays were based on DNA hybridization, wherein a DNA probe created from host cell DNA detects and quantifies the amount of complementary DNA present in the product under assay. Double-stranded host cell DNA consists of two complementary strands of DNA that are held together by hydrogen bonding. The double-stranded DNA in the test sample is denatured to single strands and immobilized to a membrane, typically a nitrocellulose or nylon membrane. The sample is probed using host cell DNA that has been denatured and labeled. The host cell DNA probe is not a specific sequence but is prepared by a random labeling procedure during which a radioactive or fluorescent label is introduced into the host cell DNA to produce the probe. When the denatured labeled DNA probe is brought into contact with the membrane-immobilized DNA, the probe will bind to complementary sequences of the host cell DNA. If the probe is radioactive, the membrane is placed against autoradiography film for a sufficient length of time, the film is developed, and a dark spot will be observed where the test DNA was immobilized. If the probe has a fluorescent label, the intensity of the spots is determined using a phosphor- or fluorescence-imaging system. The intensity of the spot is proportional to the amount of probe that was hybridized to the test DNA and therefore is proportional to the amount of residual DNA in the sample. The intensity of the spot can be compared visually with the intensity of spots that correspond to a standard curve yielding semi-quantitative results (i.e., visual quantitation), or the intensity can be determined using an instrument (e.g., densitometer) to create a quantitative value that is compared with the values obtained from the standard curve.

## DNA-BINDING PROTEIN-BASED RESIDUAL DNA ASSAY

Instrumentation is commercially available for the quantitation of residual DNA in biopharmaceuticals. The instrumentation requires reagents that use DNA-binding protein and antibodies targeted for DNA in a four-step analytical procedure. The first step requires that the DNA be denatured into single-stranded DNA by sample heating. The denatured DNA is mixed with a single reagent that contains DNA-binding protein that is conjugated with streptavidin and a monoclonal anti-DNA antibody that is conjugated to urease. The DNA-binding



protein and the monoclonal antibody are specific for single-stranded DNA but do not have any sequence specificity. This liquid phase facilitates the formation of reaction complexes that contain DNA, streptavidin, and urease. During the second step the sample is filtered through a biotinylated membrane that binds to the streptavidin and captures the complexes on the membrane, which is washed to remove any reagents that are not bound to the membrane. During the third step the membrane is inserted into a sensor on the instrument, where the urease in the DNA complex reacts with a urea solution in the sensor, producing ammonia and a change in pH that is detected using a light-addressable potentiometric sensor (LAPS). The change in pH directly correlates with the amount of DNA in the sample. In the fourth step the raw data from the instrument are analyzed using the appropriate software to determine the residual DNA content of the sample.

#### QUANTITATIVE PCR-BASED RESIDUAL DNA ASSAY

Real-time q-PCR is a procedure that is well-adapted to fast sample throughput and has applications in many areas of biopharmaceutical manufacture (e.g., copy number detection, virus detection). The technique can quantify the amount of a nucleic acid target sequence in DNA from a variety of samples. The DNA probe used in the analysis is the key to the procedure. The probe has a reporter dye attached to one end and a quencher dye attached to the other end. A DNA primer is also added to the reaction. During the amplification reaction, DNA polymerase I attaches where the DNA primer binds to the single-stranded sample (template) DNA and moves along the sample DNA synthesizing new complementary DNA. While following the template DNA, DNA polymerase I cleaves any complementary DNA in the path. If DNA polymerase I encounters the labeled DNA probe it will cleave the reporter dye from the probe. The reporter dye is released into solution and, in the absence of the quencher dye, can be quantitated as a fluorescent measurement. Repeating the reaction cycle results in an amplification of the fluorescent signal. The number of cycles required for the fluorescent measurement to exceed a threshold value correlates to the amount of starting residual DNA in the sample. By comparing with a standard curve the fluorescence obtained from a sample, analysts can quantify the residual DNA in the sample.

#### PRACTICAL APPLICATIONS OF RESIDUAL DNA TESTING

Analysts choosing hybridization, DNA-binding protein, or q-PCR techniques for residual DNA analysis should consider how the assay will be used, the structure of the DNA available (e.g., fragment length), and regulatory issues. The cost of analysis can be significant and should be considered when evaluating an assay format. Traditionally, hybridization assays were performed using 32P-labeled DNA and autoradiography. Because 32P decays quickly, probes prepared with 32P have a limited shelf life, and the precautions necessary for handling radioactive material can be cumbersome.

These issues with 32P labeling may make fluorescence labeling of the hybridization probe a more desirable option. If the hybridization assay is assessed visually, this represents a semiquantitative assay, but if the intensity of the spots is determined using a densitometer or other image system, the results can be quantitative. DNA-binding protein assays and q-PCR give quantitative results. Quantitative assays are typically preferred instead of semiquantitative assays because the results are considered more accurate and precise, which allows better process monitoring and control.

Due to sample interference, a sample pretreatment step is often required to obtain accurate and reproducible results. Pretreatment steps can influence the recovery of DNA, so it is often necessary to design the assay with a spike-recovery control and an acceptance criterion to ensure assay performance. Commercial sources of host cell and vector DNA are typically not available to prepare in-house controls. In-house controls are usually prepared in the laboratory and quantified by UV spectroscopy, using standard techniques employed in molecular biology, to determine the DNA content and purity. Additionally, it is a good practice to evaluate in-house residual DNA controls by agarose gel electrophoresis to demonstrate that the DNA is of a proper size for the assay employed and has not degraded.

The hybridization assay uses genomic and/or vector DNA, labeled randomly throughout the DNA, as the hybridization probe reagent. For this reason the hybridization assay is specific for the source of DNA but is not specific for a given sequence. A synthesized probe, specific for a specific sequence, can be prepared and used in the hybridization assay if this level of specificity is desirable. The DNA-binding protein residual DNA assay is not sequence-specific and hence not specific for the host DNA. Therefore, laboratory personnel should avoid contaminating samples for this assay with environmental DNA before denaturing the DNA; otherwise the DNA result may be falsely elevated. The q-PCR probe is sequence-specific, which creates some special challenges for development of a q-PCR residual DNA assay. The q-PCR-specific sequence must be a stable sequence within a highly conserved region of DNA. The recovery of the probe target sequence must consistently represent the recovery of all the residual DNA. As a guideline, for a DNA fragment to be detected by hybridization, q-PCR, and DNA-binding protein assays, it must have no fewer than 50, 150, and 600 base pairs, respectively. A bioprocess typically may have operations that shear DNA into smaller fragments, and this must be taken into consideration when selecting an assay. Procedures exist to determine whether the DNA fragments in a sample are too small for adequate residual DNA recovery with a given assay. As noted, residual DNA assays are extremely sensitive. Detection limits as low as <1, 3, and 6 pg of DNA per sample have been reported for q-PCR, DNA-binding protein, and hybridization assays, respectively.

Although safety concerns regarding residual DNA impurities are not as prominent as they once were, the levels of residual DNA in any bioprocess remain a key quality attribute and help define the process.

1 DNA Extractor Kit, Wako Chemicals.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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#### 1136 PACKAGING— UNIT-OF-USE

##### INTRODUCTION

This chapter provides guidance in the use and application of unit-of-use packaging and is intended for use by drug manufacturers, repackagers, and pharmacists. Suppliers of packages and packaging components may find the information useful, as well.

The General Notices defines a unit-of-use container as one that contains a specific quantity of a drug product that is intended to be dispensed as such without further modification except for the addition of appropriate labeling.

Unit-of-use packaging, when provided by the manufacturer, offers some of the following attractive advantages. (1) A dosage form can be dispensed to a patient in the manufacturer's original container, a practice that recognizes that the suitability of the container has been established on the basis of the manufacturer's stability studies. (2) The counting and repackaging of dosage units in the pharmacy is eliminated, thereby reducing the possibility of human error. (3) The pharmacist is able to affix the label for the patient onto the unit-of-use package and is free to use the manufacturer's expiration date as the beyond-use date. (4) The number of dosage units in a single unit-of-use package may be determined on a case-by-case basis. (5) Patient compliance is improved. (6) The unit-of-use package can protect against counterfeiting because traceability of product is ensured through bar coding techniques and NDC numbers.

Unit-of-use packaging, when provided by repackagers, offers the same attractive advantages as those offered by the manufacturer. However, unit-of-use repackagers should conform to all requirements as presented in [Good Repackaging Practices](#) (1178). There are a number of reasons why repackagers produce unit-of-use packaging: for example, (1) requests from institutions, (2) better inventory control, (3) reduced dispensing times, and (4) variations in some drug therapies.

The packaging of a unit-of-use system may be a multiple container or a single-unit container. A unit-of-use system may contain a drug product in a liquid, semisolid, or solid dosage form (see also FDA Guidance for Industry on Container Closure Systems for Packaging Human Drugs and Biologics). [note—The terms "unit-of-use package" and "unit-of-use



container" may be used interchangeably.]

The Poison Prevention Packaging Act (PPPA) of 1970 requires in certain cases the use of special packaging—child-resistant and senior-friendly. Child-resistant packaging protects children from serious injury or illness resulting from ingesting or handling hazardous products including drugs.

Because drugs packaged in unit-of-use packaging are intended to be dispensed to the consumer without repackaging by the pharmacist, the manufacturer or repackager is responsible for the special packaging of PPPA-regulated substances in unit-of-use containers (16 CFR 1701.1).

#### TYPES OF CONTAINERS FOR UNIT-OF-USE

Unit-of-use containers are required to be child-resistant if they are intended to be dispensed directly to the patient pursuant to a prescription. Unit-of-use packaging intended for institutional or hospital use may or may not be required to be child-resistant. Unit-of-use containers that are child-resistant single-unit containers include supported blisters, such as separate, peel, push, and tear notch, and enclosed or in-card blisters, such as pull tabs and slide packs. Blister packaging is discussed in the general chapter [Packaging Practice—Repackaging a Single Solid Oral Drug Product into a Unit-Dose Container](#) (1146). Unit-of-use containers that are multiple-unit containers include glass and plastic containers.

##### Single-Unit Container

A single-unit container is one that is designed to hold a quantity of drug product intended for administration as a single dose or a single finished device intended for use promptly after the container is opened. Preferably, the immediate container and/or the outer container or protective packaging shall be so designed as to show any evidence of tampering with the contents. Each single-unit container shall be labeled to indicate the identity, quantity, and/or strength, name of the manufacturer, lot number, and expiration date of the article.

##### Unit-Dose Container

A unit-dose container is a single-unit container for articles intended for administration by other than the parenteral route as a single dose, directly from the container.

##### Single-Dose Container

A single-dose container is a single-unit container for articles intended for parenteral administration only. It is labeled as such.

##### Multiple-Unit Container

A multiple-unit container is a container that permits withdrawal of successive portions of the contents without changing the strength, quality, or purity of the remaining portion.

#### PACKAGING FABRICATION MATERIALS

Packaging fabrication materials include substances used to manufacture packaging containers such as glass, plastics (including high-density polyethylene (HDPE), low-density polyethylene (LDPE), polyethylene terephthalate, polyethylene terephthalate G and polypropylene (PP), other resins, and other materials as listed in the general test chapter [Containers—Glass](#) (660), [Containers—Plastics](#) (661), and in the FDA Guidance for Industry on Container Closure Systems for Packaging Human Drugs and Biologics.

##### Glass

Any glass packaging material used in the immediate container should meet the glass test requirements for Limits for Glass Types and Chemical Resistance—Glass Containers: Powdered Glass Test, Water Attack at 121°, and Arsenic under general test chapter [Containers—Glass](#) (660).

##### Plastic

Any plastic packaging material used in the immediate container should meet the plastic test requirements for Plastics in the general test chapters [Containers—Plastics](#) (661) and [Containers—Performance Testing](#) (671). Depending on the type of plastic packaging material used, the packaging material meets the requirements for Biological Tests—Plastics and Other Polymers, Physicochemical Tests—Plastics, Polyethylene Containers, Polyethylene Terephthalate Bottles and Polyethylene Terephthalate G Bottles, and Polypropylene Containers under general test chapter [Containers—Plastic](#) (661).

The test for moisture vapor transmission may be carried out as described in the general test chapter [Containers—Performance Testing](#) (671) for multiple-unit and unit-dose containers.

#### PACKAGING CLOSURE TYPES

Reclosables and nonreclosables may be used for solid, semisolid, and liquid dosage forms. Both must be packaged in compliance with the 16 CFR 1700.15 standards.

##### Reclosables

Reclosables are containers with suitable closures that may incorporate tamper evidence and child-resistance capabilities. Reclosables may be used for glass or plastic containers.

##### Nonreclosables

Nonreclosables are containers with closures that are nonreclosable, such as blisters, sachets, strips, and other single-unit containers. Nonreclosables may include packs such as cold-formed foil blisters, foil strip packs, and PVC/Aclar combining multilayer materials that are thermo-formed or cold-formed foil blisters (see [Packaging Practice—Repackaging a Single Solid Oral Drug Product into a Unit-Dose Container](#) (1146)). Nonreclosables may be child resistant depending on the intended use and place of use. Household nonreclosables are subject to the PPPA as defined in 16 CFR 1700.14. However, because of some unit-dose designs, not all unit-dose packages comply with the PPPA.

#### LABELING

The unit-of-use containers are labeled to include expiration dates, the manufacturer's lot number, the NDC designation, and bar codes as provided in the Labeling section of the General Notices and Requirements under Preservation, Packaging, Storage, and Labeling and in [Good Repackaging Practices](#) (1178). Some of the advantages of having bar codes on the label include reduced medication errors, improved inventory control, and improved access to medication identity. The labeling covers information placed in the container by the manufacturer (see General Notices and Requirements). Acceptable labeling can range from the full labeling as for multiple-unit containers to an abbreviated labeling when the container is too small to include all the text. Full labeling may also be provided on the carton if it is not present on the immediate container.

#### REPACKAGING AND REPROCESSING

Unit-of-use containers are reprocessed or repackaged as instructed by the manufacturer or as directed in the general test chapters [Containers—Glass](#) (660) and [Containers—Plastics](#) (661) or in the general information chapter [Packaging Practice—Repackaging a Single Solid Oral Drug Product into a Unit-Dose Container](#) (1146). A unit-of-use package that is a blister package may not be reprocessed by a pharmacist once it has been deblistered from a unit-dose container (see General Notices and Requirements for application of the appropriate beyond-use date for a multiple-unit or unit-dose container). Deblistering is the process of removing medication from a blister-type container. However, under current Good Manufacturing Practices (cGMPs) and tight quality controls, the manufacturer or contract repackager may repackage and reprocess unit-of-use containers.

#### INFORMATION FROM MANUFACTURERS

The manufacturer should provide appropriate stability information that can be used to determine appropriate labeling, storage, and shipping statements that will properly inform patients and practitioners. The manufacturer may make other assurances based on product information on packaging and distribution arrangements. In the event that a product is not to be

packaged, the manufacturer may so state in the labeling. The manufacturer also includes labeling and information suitable for optimal handling by the practitioner and the patient. The labeling and information should be bar coded to eliminate medication error and promote medication traceability.

#### RESPONSIBILITY OF THE DISPENSER

##### Labeling

The labeling on a unit-of-use container also includes a label added at the dispensing stage by the pharmacist. Prior to dispensing the unit-of-use package, the dispenser shall add label(s) that provide the following information:

1. the name of the patient;
2. the name and strength, the directions for use as prescribed by a doctor or health-care provider, and the name of the prescriber; and
3. any storage instruction, beyond-use date, and other information as deemed appropriate by federal and state laws.

In the pharmacy setting, pharmacists are encouraged to use bar codes, in conjunction with computerized prescription orders, to confirm that the right drug is being dispensed to the right patient. Bar coding would minimize errors and create opportunity for medication traceability and accountability.

##### Information to Patient

Patients must be given information that applies to the specific prescription being dispensed.

#### QUALITY CONTROL OF PACKAGING SYSTEM

The packaging system shall meet the general considerations for system suitability, protection, safety, and performance characteristics as described in FDA Guidance for Industry on Container Closure Systems for Packaging Human Drugs and Biologics, in the general test chapters [Containers—Glass](#) (660), [Containers—Plastics](#) (661), and [Containers—Performance Testing](#) (671), and in the general information chapter [Packaging Practice—Repackaging a Single Solid Oral Drug Product into a Unit-Dose Container](#) (1146).

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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#### 1146 PACKAGING PRACTICE—REPACKAGING A SINGLE SOLID ORAL DRUG PRODUCT INTO A UNIT-DOSE CONTAINER

#### INTRODUCTION

Repackaging of solid oral drug products, such as tablets and capsules, into unit-dose configurations is common practice both for the pharmacy that is dispensing drugs pursuant to a prescription and for the pharmaceutical repackaging firm. This general chapter contains minimum standards to be used as a guideline for repackaging practices. This guideline is not intended to replace or supplant the requirements of regulatory agencies.

Repackaging preparations into unit-dose configurations is an important aspect of pharmaceutical care and of optimization of patient compliance. For purposes of this chapter, there are two types of repackaging: the first involves pharmacies that dispense prescription drugs; the second concerns commercial pharmaceutical repackaging firms.

#### NOMENCLATURE AND DEFINITIONS

**Dispenser**—A dispenser is a licensed or registered practitioner who is legally responsible for providing a preparation for patient use, with a specific patient label, pursuant to a prescription or a medication order. In addition, dispensers may prepare limited quantities in anticipation of a prescription or medication order from a physician. Dispensers are governed by the board of pharmacy of the individual state.

**Package**—The term “package” is synonymous with the term “container.” See Containers under Preservation, Packaging, Storage, and Labeling in the General Notices and Requirements.

**Pharmacy**—A pharmacy is an establishment that is legally responsible for providing the drug preparation for patient use, with a specific patient label, pursuant to a prescription or a medication order. The terms dispenser and pharmacy are used interchangeably.

**Rewrap**—Rewrap is the act of removing a preparation from its original primary container and placing it into another primary container, usually of smaller size.

**Rpackager**—A repackager is an establishment that repackages drugs and sends them to a second location in anticipation of a need. Repackaging firms repack preparations for distribution (e.g., for resale to distributors, hospitals, or other pharmacies), a function that is beyond the regular practice of a pharmacy. Distribution is not patient specific in that there are no prescriptions. Unlike dispensers, repackaging firms are required to register with the FDA and to comply with the Current Good Manufacturing Practice regulations in 21 CFR 210 and 211.

#### MATERIALS

Blister packages offer a wide array of designs both in functionality and in appearance. Various packaging materials are used to create blisters that are tailored to provide optimum performance. The blister container consists of two components: the blister, which is the formed cavity that holds the product, and the lid stock, which is the material that seals to the blister, as shown below.



Schematic Presentation of a Typical Blister Pack

Because of the variety of blister films available, film selection should be based upon the degree of protection required. The choice of lid stock depends on how the blister is to be used, but generally the lid stock is made of aluminum foil. The material used to form the cavity is typically a plastic, which can be designed to protect the dosage form from moisture. There

the widely varying degrees of moisture protection now available. For purposes of this general chapter, they are referred to as nominal, medium, high, and extreme moisture barrier properties.

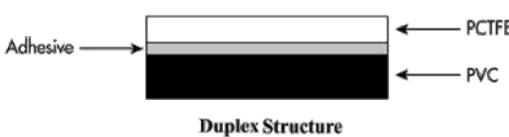
Polyvinyl Chloride— The most commonly used blister material is polyvinyl chloride (PVC). This material, which provides a nominal or zero barrier to moisture, is used when the product does not require effective moisture protection. PVC is available in a range of gauges and can be made opaque or can be tinted with pigments to block out specific light wavelengths.

The thickness of the PVC used is determined by the depth and size of the cavity to be formed. Because the plastic thins during the blister-forming process, care should be taken to ensure that the finished blister provides sufficient protection from light (if required) and that it is strong enough to adequately protect the dosage form. Common gauges of PVC used in the pharmaceutical industry range from 7.5 to 15 mil (0.0075 to 0.015 inch).

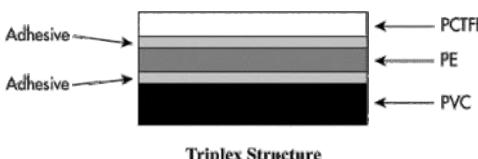
Barrier Films— Many drug preparations are extremely sensitive to moisture and therefore require high barrier films. Several materials may be used to provide moisture protection.

Barrier films commonly used in the pharmaceutical industry are described below.

PVC/PCTFE Laminations— Polychlorotrifluoroethylene (PCTFE) film<sup>1</sup> is a thermoplastic film made from polychlorotrifluoroethylene fluoropolymer. The PCTFE film is laminated to the PVC by an adhesive layer between the PVC and the PCTFE film (duplex structure)

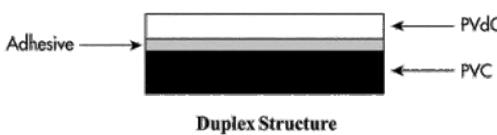


or by a layer of polyethylene (PE) between the PVC-adhesive and the PCTFE-adhesive layers (triplex structure).

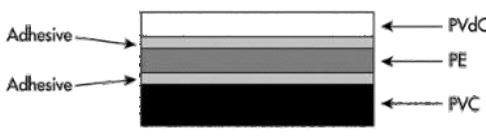


By using various gauges of the PCTFE film, medium to extreme moisture barriers can be obtained.

PVC/PVdC Laminations— PVC/PVdC is a film in which the PVC is coated with an emulsion of polyvinylidene chloride (PVdC).



The PVdC layer is specified in g per m<sup>2</sup> and can be constructed to provide medium to high barrier protection. The coating weights commonly used in the pharmaceutical industry are 40, 60, and 90 g per m<sup>2</sup>, and the film is offered with or without a middle layer of polyethylene (PE). The polyethylene is used with heavier coating weights, such as 60 and 90 g per m<sup>2</sup>, to improve the thermoforming characteristics of the blister cavity.



**Triplex Structure**

Polypropylene— Because of its morphology, polypropylene (PP) serves as a good moisture barrier, its spherulitic structure creating an arduous path for water molecules to traverse. Although not commonly used as a pharmaceutical blister film in the U.S., PP provides an economical alternative to medium barrier materials and is used in Europe as an alternative to PVC.

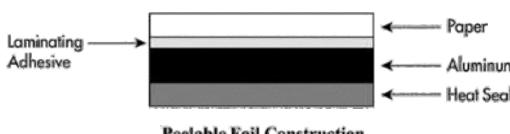
Cold Form Foil— This material is used for products that are extremely hygroscopic or light sensitive. It is an extreme moisture barrier and consists of three layers: PVC, aluminum foil, and nylon.



**Cold Form Foil**

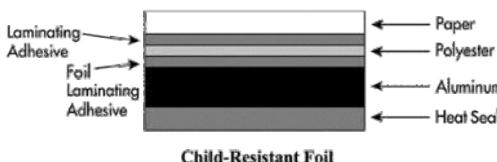
Lid Stock— Lid stock is sealed to the molded blister as described above. Different designs of lid stocks are available, and selection of a particular design depends on how the package will be used. Standard designs—peelable, child-resistant peelable, and push-through—are described below. The primary component of lid stock is typically aluminum, and its gauge varies from 18 to 25 µm (0.00078 to 0.001 inch). The side of the aluminum foil laminate in contact with the product provides the heat-sealable layer that forms the seal to the blister material. The heat-seal coating should be capable of forming an adequate seal with the blister film to which it is intended to seal. The materials used in the makeup of the heat-seal layer meet 21 CFR 175 and 177.

Peelable— Peelable foil, commonly used in an institutional setting, consists of several layers, as shown below, and can be peeled away from the blister. [note—For child-resistant peelable foil, a layer of polyester with the appropriate adhesives would be added.] With the peelable foil lid stock, which is used in conjunction with blister tooling, a three-step process is required to open the blister.

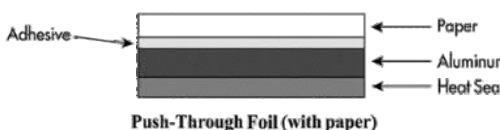


**Peelable Foil Construction**

First, the blister cavity must be separated from the rest of the blister card. Next, the paper and polyester layers are pulled back from an unsealed area. Finally, the product is pushed through the remaining aluminum foil. It is important to note that use of this type of foil structure helps make the package more child resistant. However, if child-resistant packaging is required, the package design should be tested in accordance with the protocol described in 16 CFR 1700, the Poison Prevention Packaging Act.



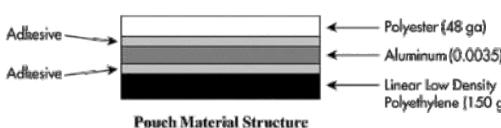
Push-Through— There are two commonly used types of push-through foil: one with a paper outer layer separated from the aluminum by a layer of adhesive and one without paper.



**Push-Through Foil (no paper)**

The paper outer layer serves as an aesthetic and makes it possible to print on the back of the blister.

Other Package Styles— Other types of packages used for unit-dose packaging of solid dosage forms are strip packs, pouches, and sachets.



## PROCESS

Unit-dose packages can be formed and sealed in a variety of ways. Larger scale repackagers may use thermoformers that accomplish these functions in-line, while smaller repackagers may purchase preformed blister material. This section begins with an overview of the process involved in thermoforming a blister, the fundamental process that also applies to other unit-dose package types such as pouches. The overview is not intended to be all-encompassing, but it highlights the major operations along with their critical parameters.

**Thermoforming a Blister Unit-Dose Package**— The complete thermoforming process consists of four basic stations where the following operations occur: forming, filling, sealing, and finishing. Thermoforming requires the use of heat and air in forming the blister. The lid stock material is sealed to the blister cavity material for a defined time (the stroke of the machine) at the point where the heat plate closes on the two materials.

**Forming Station**— Prior to entering the forming station, the blister material passes through a heating unit where the blister material is heated uniformly in stages to ensure proper formation. Because different plastics have different softening points, careful attention must be paid to determining the proper temperature of the heating station, which often has multiple temperature zones. The temperature, based on the blister material used and on the speed at which that material travels through the heating station, is a critical parameter for optimal performance. At the forming station the blister material is heated to the point where the plastic softens sufficiently to allow the cavity to be formed. The blister material is drawn from a reel-mounted roll (referred to as the web) and pulled through the machine. A splicing table is located at the reel unwind to provide room for a second roll of blister material to be readily available for splicing and resumption of the packaging process. An unwind device may be installed to aid in moving the blister material from the roll as adjusted for a specific index.

Once the blister material is properly heated, compressed air is generally used to form the blister cavity. Upper and lower forming dies close on the blister material as air is introduced, forming a blister that corresponds to the size of the cavity. A plug assist may be necessary, depending on the material and size of the cavity. The plug assist ensures a uniform thinning of the blister material to optimize the protective characteristics of the formed material. Once the blister material is formed into the desired blister configuration, it is advanced to the filling station.

**Filling Station**— The product is loaded into the blister cavity at this station. An automated filling device may be used, or the cavities may be hand filled. The critical parameter at this station is proper filling of the formed blisters.

**Sealing Station**— At this station, the lid stock is sealed to the filled blister cavity, using heat and pressure for a defined dwell time. The critical parameters to be considered at this station are temperature, pressure, and dwell time.

The lid stock material is staged on a roll above the blister cavity and may be preprinted or printed on-line. Lot numbers and expiration dates may be applied at this point. Preprinted lid stock materials will require a print registration system to control the position of the printing relative to the blister cavity. The critical parameters at this part of the station include legible and correct labeling.

**Finishing Station**— The finishing station encompasses all other steps in the packaging process, including embossing, perforation, and cutting. Embossing involves application of a lot number and expiration date to the package. Steel type is used to emboss information on the edges of the blister package. One of the critical parameters at this station is package integrity. It is important that the embossing, perforation, and cutting processes do not compromise the blister, lid, or seal. The quality of the embossing is another critical parameter in the process. The embossing must be legible, and correct and must include all required information.

**Pouch Unit-Dose Packages**— The pouch process is also a form, fill, and seal operation, but it does not provide a defined, formed cavity as does the thermoforming process. Although the equipment used to form pouch unit-dose packages may function differently from that described for thermoforming a blister, the main operations (form, fill, and seal) and critical parameters at those stations are quite similar. [note—See the aforementioned critical parameters defined in the section on thermoforming.]

The strip-pack process involves the drug product being dosed into a three-sided, formed pouch. Once filled with the drug, the machine seals the pouch, forming a strip of sealed unit-dose pouches. The basic flow of the process begins with the drug situated above the pouch material. One roll of strip-pack material is used to form the pouch. This is accomplished by moving the material over a device that forces the material to fold into two equal sides. The sides and bottom are sealed prior to dosing. The strip pack may be cut later during the equipment processing or roll continuously and be manually cut. Temperature and dwell time are the main critical factors for this equipment.

**Preformed Unit-Dose Packages**— Preformed containers are sealed either by heat or by adhesion. Heat sealers may be manual units requiring hand pressure application or automated units that provide a more controlled pressure for sealing.

Heat sealing may be accomplished through the use of manual tabletop equipment. This equipment is generally operated at a set pressure. Critical parameters with these devices are pressure and temperature control because undesirable variation in these parameters may yield inadequate seals.

**Critical Parameters**— In order to ensure that the finished container performs as intended, qualification of critical parameters should be determined. Typically, validation of a packaging

line consists of qualification of the installation, operation, and performance of a packaging system.

Installation Qualification— Equipment should be installed and found to be in proper working condition prior to use.

Operational Qualification— Operational qualification should be performed to establish that the equipment operates within the manufacturer's specified ranges. Incoming utilities for the equipment, such as air, electricity, etc., should be monitored and checked periodically.

Performance Qualification— Performance qualification should be done to ensure that the equipment is performing properly with the required materials to produce a container that functions as intended. The critical parameters include forming temperature and pressure, sealing temperature and pressure, and dwell time at the seal station. Qualified ranges should be readily available in a reference source for the setup of equipment. Re-evaluation may be necessary with changes to equipment, materials, or the process.

In-Process Inspections— Strict controls covering the packaging and labeling processes should be in place. The final container should be evaluated for performance in each of the stations described above. Specifically, the formed container should be inspected visually to ensure that it is properly formed. Evaluation of the filling station should include a check to ensure that the unit dose is properly filled (i.e., that the correct product is present). The sealing station should be evaluated to ensure that a proper seal has been made and that the moisture permeation specifications of the sealed container have been met. A visual examination of the package should be performed to ensure that the final steps of the packaging process are acceptable.

Repackagers and dispensers should use a standard inspection plan to verify the adequacy of the package. A visual inspection should be performed to verify that the correct product is in the proper packaging materials with correct labeling. Seal integrity should be evaluated, using vacuum testing,<sup>2</sup> helium testing, tear testing, and other testing methods suitable to establish whether seal integrity is maintained.

## PERFORMANCE

The primary purpose of the unit-dose package used in the packaging of a drug preparation is to ensure that until its intended expiration date there is adequate protection from the environment as the dosage form is distributed and stored. It is also essential that the materials used do not interact with the dosage form.

When determining what type of package to use in the repackaging operation, consideration must be given to the dosage form's sensitivities (if any) to the storage and distribution environments (e.g., temperature, light, and moisture).

The materials used in constructing the unit-dose container as well as the process of forming and sealing the container all together define the properties of the finished container. As discussed in Materials, there is a wide variety of commercially available film structures that provide unit-dose containers with a range of moisture and light protection. Suppliers of these materials typically provide quantitative data, obtained from well-established test methods, to highlight the protective properties of their material. These data are based on flat sheets of the film, not on the formed container.

It is critical to understand that once the film is formed, protective properties change because the overall thickness of the film decreases as the blister cavity is formed. Usually the change is a decrease, especially in the case of barrier properties. However, the extent of change will vary with the type of film structure used and is also highly dependent on the container-forming process used (see Process). Further, a suboptimal seal on the formed container will decrease the protective properties of the container. Insufficient temperature, time, or pressure during a heat-seal operation may enable the passage of moisture or oxygen through the seal area over time, which may have an effect on the dosage form. In addition, if the seal area is designed with insufficient surface area, the same problem may occur. To ensure a good seal, a minimum sealing distance of 3 mm from the edge of the blister cavity to the nearest edge or perforation is recommended. Therefore, it is important to measure the performance of the formed and sealed container rather than the performance of the flat sheet.

Moisture is a critical factor in preparation integrity. [Containers—Performance Testing \(671\)](#) describes how to determine and classify moisture permeation rates. If the manufacturer's labeling includes "Protect From Moisture," the repackager shall utilize a high barrier film.

If light protection is required for a drug preparation, the repackager should follow the requirements for light transmission established under [Containers—Performance Testing \(671\)](#). Again, this testing should be conducted on the formed container, because the light protective properties of the film are compromised once the film is thinned during the forming process. It is recommended that these tests, in conjunction with any guidance provided by the manufacturer, be considered appropriate for any container-closure system used in repackaging a drug preparation.

## BEYOND-USE DATE

In the absence of stability data for the drug product in the repackaged container, the beyond-use dating period is one year or the time remaining of the expiration date, whichever is shorter. If current stability data are available for the drug product in the repackaged container, the length of time established by the stability study may be used to establish the beyond-use date but must not exceed the manufacturer's expiration date.

As stated in the General Notices and Requirements, the dispenser must maintain the facility where the dosage forms are packaged and stored at a temperature such that the mean kinetic temperature is not greater than 25°. The plastic material used in packaging the dosage forms must afford better protection than polyvinyl chloride, which does not provide adequate protection against moisture permeation. Records must be kept of the temperature of the facility where the dosage forms are stored, and of the plastic materials used in packaging.

## MINIMUM REQUIREMENTS

The previous sections serve as a general introduction to repackaging by providing a basic understanding of materials selection, the form-fill-seal process, and the importance of performance of the sealed container. In this section, certain minimum requirements for repackaging, which must be met, are described in more detail.

Personnel— Each person with responsibility for the repackaging of a preparation shall have the education, training, and experience, or any combination thereof, to perform assigned functions in a manner such that the safety, identity, strength, quality, purity, potency, and pharmaceutical elegance of the drug dosage form are retained. Training should be documented.

Personnel engaged in the repackaging of a preparation shall wear clean clothing appropriate for the duties or processes performed.

Facility— The repackaging facility may require areas of low relative humidity, and temperature conditions should meet controlled room temperature requirements specified in the General Notices.

Equipment— Equipment used in the repackaging of a preparation shall be of appropriate design and suitably located to facilitate operations for its intended use. Its design should allow for cleaning to preclude cross-contamination as well as for maintenance to be performed. Equipment shall be constructed so that those surfaces that contact components or a preparation are not reactive, additive, or absorptive.

Any substances required for operation, such as lubricants or coolants, shall not come into contact with components or a preparation.

Equipment and utensils shall be cleaned, maintained, and sanitized at appropriate intervals to prevent malfunctions or contamination. Preventive maintenance should be performed at appropriate intervals in accordance with the equipment manufacturer's recommendation. Any instruments used to monitor critical parameters should be calibrated on a defined schedule.

Process— Steps should be taken to determine the critical process parameters (e.g., seal temperature, dwell time) in operating the equipment. Set points for these parameters should be documented and procedures established to ensure that they are adhered to each time the equipment is operated.

Labeling— The labeling requirements for a commercial repackager and a pharmacist are different. For example, the commercial repackager must comply with 21 CFR 201.1, but the pharmacist or dispenser does not have to comply with this requirement. If stability data are unavailable, the dispenser shall repack only an amount of stock sufficient for a limited time and shall include product name and strength, lot number, manufacturer, and appropriate beyond-use date on the label. When quantities are repackaged in advance of immediate



needs, each preparation must bear an identifying label, and the dispenser is required to maintain suitable repackaging records showing the name of the manufacturer, lot number, expiration date, date of repackaging, and designation of persons responsible for repackaging and for checking. The repackager or dispenser will use documented controls to prevent labeling errors.

Materials— The repackager or dispenser shall place an appropriate beyond-use date on the label and package in appropriate materials. Materials used by the repackager shall not be reactive, additive, or absorptive, and must meet the requirements described in 21 CFR 175 and 177.

Storage— The dispenser shall rotate and monitor stock closely to ensure that the dispensing of preparations is on a first-in-first-out (FIFO) basis. The repackager or dispenser shall store preparations under required environmental conditions (e.g., controlled room temperature with a mean kinetic temperature not higher than 25°).

Drug Product— The repackager or dispenser shall examine preparations for evidence of instability such as change in color or odor, and shall exercise professional judgment as to the acceptability of a package.

Complaints— The repackager or dispenser will maintain written procedures describing the handling of written and oral complaints regarding a drug product and will ensure that complaints are investigated and appropriately resolved.

Returned Goods— Policies and procedures relating to returned goods should be developed to ensure proper handling.

Reprocessing— Reprocessing of repackaged unit-dose containers (i.e., removing medication from one unit-dose container and placing it into another unit-dose container) shall not be done. However, reprocessing of the secondary package (e.g., removing the blister card from the cardboard carrier and placing the blister card into another cardboard carrier) is allowed provided the original beyond-use date is maintained, and provided the integrity of the blister is ensured.

Special Considerations— If a product is known to be oxygen sensitive or if it exhibits extreme moisture or light sensitivity (e.g., cold form foil), it shall not be repackaged. If a product is refrigerated, it shall not be repackaged unless proper environmental conditions and suitable materials are available. Certain drug products (such as oncologic agents, hormones, or penicillin derivatives) require special handling because they are considered very potent or toxic, and because transfer of any portion of these products to another product could have deleterious effects.

1 PCTFE film is available from Allied Signal (as Aclar) and from other sources.

2 Vacuum testing consists of placing samples from the packaging operation into a jar filled with water. A lid is placed over the samples to fully immerse them in the water. A container lid is applied to create a seal effective enough to create approximately 25 cm of vacuum. The vacuum pump is set, and the samples are tested for approximately 1 minute, removed from the water, wiped down, and opened to determine whether the inside of the unit-dose cavity or pouch is wet. This process should be adjusted until it is under control, and additional testing may be performed to ensure that the seal integrity is consistently acceptable. Wetness indicates a defective seal and therefore the potential for the drug to degrade when exposed to the atmosphere. Defective packages must be removed from further use.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Desmond G. Hunt, Ph.D.</a> Scientist 1-301-816-8341	(PS05) Packaging and Storage 05

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#### 1150 PHARMACEUTICAL STABILITY

The term "stability," with respect to a drug dosage form, refers to the chemical and physical integrity of the dosage unit and, when appropriate, the ability of the dosage unit to maintain protection against microbiological contamination. The shelf life of the dosage form is the time lapse from initial preparation to the specified expiration date. The monograph specifications of identity, strength, quality, and purity apply throughout the shelf life of the product.

The stability parameters of a drug dosage form can be influenced by environmental conditions of storage (temperature, light, air, and humidity), as well as the package components. Pharmacopeial articles should include required storage conditions on their labeling. These are the conditions under which the expiration date shall apply. The storage requirements specified in the labeling for the article must be observed throughout the distribution of the article (i.e., beyond the time it leaves the manufacturer up to and including its handling by the dispenser or seller of the article to the consumer). Although labeling for the consumer should indicate proper storage conditions, it is recognized that control beyond the dispenser or seller is difficult. The beyond-use date shall be placed on the container label.

#### Stability Protocols

Stability of manufactured dosage forms must be demonstrated by the manufacturer, using methods adequate for the purpose. Monograph assays may be used for stability testing if they are stability-indicating (i.e., if they accurately differentiate between the intact drug molecules and their degradation products). Stability considerations should include not only the specific compendial requirements, but also changes in physical appearance of the product that would warn users that the product's continued integrity is questionable.

Stability studies on active substances and packaged dosage forms are conducted by means of "real-time," long-term tests at specific temperatures and relative humidities representing storage conditions experienced in the distribution chain of the climatic zone(s) of the country or region of the world concerned. Labeling of the packaged active substance or dosage form should reflect the effects of temperature, relative humidity, air, and light on its stability. Label temperature storage warnings will both reflect the results of the real-time storage tests and allow for expected seasonal excursions of temperature.

#### Controlled Room Temperature

Controlled room temperature (see Storage Temperature and Humidity in Preservation, Packaging, Storage, and Labeling under General Notices and Requirements) delineates the allowable tolerance in storage circumstances at any location in the chain of distribution (e.g., pharmacies, hospitals, and warehouses). This terminology also allows patients or consumers to be counseled as to appropriate storage for the product. Products may be labeled either to store at "Controlled room temperature" or to store at temperatures "up to 25°" where labeling is supported by long-term stability studies at the designated storage condition of 25°. Controlled room temperature limits the permissible excursions to those consistent with the maintenance of a mean kinetic temperature calculated to be not more than 25°. See Mean Kinetic Temperature. The common international guideline for long-term stability studies specifies  $25 \pm 2^\circ$  at  $60 \pm 5\%$  relative humidity. Accelerated studies are specified at  $40 \pm 2^\circ$  and at  $75 \pm 5\%$  relative humidity. Accelerated studies also allow the interpretation of data and information on short-term spikes in storage conditions in addition to the excursions allowed by controlled room temperature.

The term "room temperature" is used in different ways in different countries, and for products to be shipped outside the continental U.S. it is usually preferable for product labeling to refer to a maximum storage temperature or temperature range in degrees Celsius.

#### Mean Kinetic Temperature

Mean Kinetic Temperature (MKT) is defined as the single calculated temperature at which the total amount of degradation over a particular period is equal to the sum of the individual degradations that would occur at various temperatures. Thus, MKT may be considered as an isothermal storage temperature that simulates the nonisothermal effects of storage temperature variation. It is not a simple arithmetic mean. MKT is calculated from temperatures in a storage facility. The temperatures for calculating MKT can be conveniently collected using electronic devices that measure temperatures at frequent intervals (e.g., every 15 minutes). MKT can be calculated directly or the data can be downloaded to a computer for



processing. For dispensing sites, such as pharmacies and hospitals, where the use of such instruments may not be feasible, devices such as high-low thermometers capable of indicating weekly high and low temperatures over a 52-week period may be employed. The arithmetic mean of the weekly high and low temperatures is then used in the calculation of MKT. MKT is calculated by the following equation (derived from the Arrhenius equation):

$$T_k = \frac{\Delta H/R}{-\ln\left(\frac{e^{-\Delta H/RT_1} + e^{-\Delta H/RT_2} + \dots + e^{-\Delta H/RT_n}}{n}\right)}$$

in which  $T_k$  is the mean kinetic temperature;  $\Delta H$  is the heat of activation, 83.144 kJ·mole<sup>-1</sup> (unless more accurate information is available from experimental studies);  $R$  is the universal gas constant,  $8.3144 \times 10^{-3}$  kJ·mole<sup>-1</sup>·degree<sup>-1</sup>;  $T_1$  is the value for the temperature recorded during the first time period, e.g., the first week;  $T_2$  is the value for the temperature recorded during the second time period, e.g., second week; and  $T_n$  is the value for the temperature recorded during the nth time period, e.g., nth week,  $n$  being the total number of storage temperatures recorded (minimum of 52 weekly entries) during the annual observation period. [note—All temperatures,  $T$ , are absolute temperatures in degrees Kelvin (K).]

The following is an example of a typical storage and distribution temperature range in Kelvin degrees and the conversion factors used to convert this range into degrees Fahrenheit and Celsius.

Kelvin (K)	Fahrenheit (°F)	Celsius (°C)
288.1–303.1	59–86	15–30

Conversion Factors:

$$\text{Fahrenheit to Kelvin} = \{[(°F - 32) \times 5/9] + 273.1\}$$

$$\text{Celsius to Kelvin} = 273.1 + °C$$

$$\text{Fahrenheit to Celsius} = [(°F - 32) \times 5/9]$$

#### Climatic Zones

For convenience in planning for packaging and storage, and for stability studies, international practice identifies four climatic zones, which are described in [Table 1](#). The United States, Europe, and Japan are characterized by zones I and II. The values in [Table 1](#) are based on observed temperatures and relative humidities, both outside and in rooms, from which mean kinetic temperatures and average humidity values are calculated.<sup>1</sup> Derived values are based on inspection of data from individual cities and on allowances for a margin of safety in assignment of these specified conditions.

Table 1. International Climatic Zones

Climatic Zone	Calculated Data				Derived Data		
	°C*	°C MKT**	% RH	mbar***	°C	% RH	mbar
I. Temperate	20.0	20.0	42	9.9	21	45	11.2
Japan							
United Kingdom							
Northern Europe							
Canada							
Russia							
United States							
II. Mediterranean, Subtropical	21.6	22.0	52	13.5	25	60	19.0
United States							
Japan							
Southern Europe (Portugal-Greece)							
III. Hot, Dry	26.4	27.9	35	11.9	30	35	15.0
Iran							
Iraq							
Sudan							
IV. Hot, Humid	26.7	27.4	76	26.6	30	70	30.0
Brazil							
Ghana							
Indonesia							
Nicaragua							
Philippines							

\* Data recorded as <19° calculated as 19°.

\*\* Calculated mean kinetic temperature.

\*\*\* Partial pressure of water vapor.

A discussion of aspects of drug product stability that are of primary concern to the pharmacist in the dispensing of medications may be found under [Stability Considerations in Dispensing Practice](#) (1191).

Inasmuch as this chapter is for purposes of general information only, no statement herein is intended to modify or supplant any of the specific requirements pertinent to pharmaceutical preparations, which are given elsewhere in this Pharmacopeia.

1 The source of the data and information in [Table 1](#) is the International Conference on Harmonization sponsored by the International Federation of Pharmaceutical Manufacturers Associations.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Desmond G. Hunt, Ph.D.</a> Scientist 1-301-816-8341	(PS05) Packaging and Storage 05



Dosage forms are provided for most of the Pharmacopeial drug substances, but the processes for the preparation of many of them are, in general, beyond the scope of the Pharmacopeia. In addition to defining the dosage forms, this section presents the general principles involved in the manufacture of some of them, particularly on a small scale. Other information that is given bears on the use of the Pharmacopeial substances in extemporaneous compounding of dosage forms.

## BIOAVAILABILITY

Bioavailability, or the extent to which the therapeutic constituent of a pharmaceutical dosage form intended for oral or topical use is available for absorption, is influenced by a variety of factors. Among the inherent factors known to affect absorption are the method of manufacture or method of compounding; the particle size and crystal form or polymorph of the drug substance; and the diluents and excipients used in formulating the dosage form, including fillers, binders, disintegrating agents, lubricants, coatings, solvents, suspending agents, and dyes. Lubricants and coatings are foremost among these. The maintenance of a demonstrably high degree of bioavailability requires particular attention to all aspects of production and quality control that may affect the nature of the finished dosage form.

## TERMINOLOGY

Occasionally it is necessary to add solvent to the contents of a container just prior to use, usually because of instability of some drugs in the diluted form. Thus, a solid diluted to yield a suspension is called [DRUG] for Suspension; a solid dissolved and diluted to yield a solution is called [DRUG] for Solution; and a solution or suspension diluted to yield a more dilute form of the drug is called [DRUG] Oral Concentrate. After dilution, it is important that the drug be homogeneously dispersed before administration.

## AEROSOLS

Pharmaceutical aerosols are products that are packaged under pressure and contain therapeutically active ingredients that are released upon activation of an appropriate valve system. They are intended for topical application to the skin as well as local application into the nose (nasal aerosols), mouth (lingual aerosols), or lungs (inhalation aerosols). These products may be fitted with valves enabling either continuous or metered-dose delivery; hence, the terms “[DRUG] Metered Topical Aerosols,” “[DRUG] Metered Nasal Aerosols,” etc.

The term “aerosol” refers to the fine mist of spray that results from most pressurized systems. However, the term has been broadly misapplied to all self-contained pressurized products, some of which deliver foams or semisolid fluids. In the case of Inhalation Aerosols, the particle size of the delivered medication must be carefully controlled, and the average size of the particles should be under 5  $\mu\text{m}$ . These products are also known as metered-dose inhalers (MDIs). Other aerosol sprays may contain particles up to several hundred micrometers in diameter.

The basic components of an aerosol system are the container, the propellant, the concentrate containing the active ingredient(s), the valve, and the actuator. The nature of these components determines such characteristics as particle size distribution, uniformity of dose for metered valves, delivery rate, wetness and temperature of the spray, spray pattern and velocity or plume geometry, foam density, and fluid viscosity.

### Types of Aerosols

Aerosols consist of two-phase (gas and liquid) or three-phase (gas, liquid, and solid or liquid) systems. The two-phase aerosol consists of a solution of active ingredients in liquefied propellant and the vaporized propellant. The solvent is composed of the propellant or a mixture of the propellant and cosolvents such as alcohol, propylene glycol, and polyethylene glycols, which are often used to enhance the solubility of the active ingredients.

Three-phase systems consist of a suspension or emulsion of the active ingredient(s) in addition to the vaporized propellants. A suspension consists of the active ingredient(s) that may be dispersed in the propellant system with the aid of suitable excipients such as wetting agents and/or solid carriers such as talc or colloidal silicas.

A foam aerosol is an emulsion containing one or more active ingredients, surfactants, aqueous or nonaqueous liquids, and the propellants. If the propellant is in the internal (discontinuous) phase (i.e., of the oil-in-water type), a stable foam is discharged; and if the propellant is in the external (continuous) phase (i.e., of the water-in-oil type), a spray or a quick-breaking foam is discharged.

### Propellants

The propellant supplies the necessary pressure within an aerosol system to expel material from the container and, in combination with other components, to convert the material into the desired physical form. Propellants may be broadly classified as liquefied or compressed gases having vapor pressures generally exceeding atmospheric pressure. Propellants within this definition include various hydrocarbons, especially halogenated derivatives of methane, ethane, and propane, low molecular weight hydrocarbons such as the butanes and pentanes, and compressed gases such as carbon dioxide, nitrogen, and nitrous oxide. Mixtures of propellants are frequently used to obtain desirable pressure, delivery, and spray characteristics. A good propellant system should have the proper vapor pressure characteristics consistent with the other aerosol components.

### Valves

The primary function of the valve is to regulate the flow of the therapeutic agent and propellant from the container. The spray characteristics of the aerosol are influenced by orifice dimension, number, and location. Most aerosol valves provide for continuous spray operation and are used on most topical products. However, pharmaceutical products for oral or nasal inhalation often utilize metered-dose valves that must deliver a uniform quantity of spray upon each valve activation. The accuracy and reproducibility of the doses delivered from metering valves are generally good, comparing favorably to the uniformity of solid dosage forms such as tablets and capsules. However, when aerosol packages are stored improperly, or when they have not been used for long periods of time, valves must be primed before use. Materials used for the manufacture of valves should be inert to the formulations used.

Plastic, rubber, aluminum, and stainless steel valve components are commonly used. Metered-dose valves must deliver an accurate dose within specified tolerances.

### Actuators

An actuator is the fitting attached to an aerosol valve stem, which when depressed or moved, opens the valve, and directs the spray containing the drug preparation to the desired area. The actuator usually indicates the direction in which the preparation is dispensed and protects the hand or finger from the refrigerant effects of the propellant. Actuators incorporate an orifice that may vary widely in size and shape. The size of this orifice, the expansion chamber design, and the nature of the propellant and formulation influence the delivered dose as well as the physical characteristics of the spray, foam, or stream of solid particles dispensed. For inhalation aerosols, an actuator capable of delivering the medication in the proper particle size range and with the appropriate spray pattern and plume geometry is utilized.

### Containers

Aerosol containers usually are made of glass, plastic, or metal, or a combination of these materials. Glass containers must be precisely engineered to provide the maximum in pressure safety and impact resistance. Plastics may be employed to coat glass containers for improved safety characteristics, or to coat metal containers to improve corrosion resistance and enhance stability of the formulation. Suitable metals include stainless steel, aluminum, and tin-plated steel. Extractables or leachables (e.g., drawing oils, cleaning agents, etc.) and particulates on the internal surfaces of containers should be controlled.

### Manufacture

Aerosols are usually prepared by one of two general processes. In the “cold-fill” process, the concentrate (generally cooled to a temperature below 0 $^{\circ}\text{C}$ ) and the refrigerated propellant are measured into open containers (usually chilled). The valve-actuator assembly is then crimped onto the container to form a pressure-tight seal. During the interval between propellant addition and crimping, sufficient volatilization of propellant occurs to displace air from the container. In the “pressure-fill” method, the concentrate is placed in the container, and either the propellant is forced under pressure through the valve orifice after the valve is sealed, or the propellant is allowed to flow under the valve cap and then the valve assembly is sealed (“under-the-cap” filling). In both cases of the “pressure-fill” method, provision must be made for evacuation of air by means of vacuum or displacement with a small amount of propellant vapor. Manufacturing process controls usually include monitoring of proper formulation and propellant fill weight and pressure testing, leak testing, and valve function testing.



of the finished aerosol. Microbiological attributes should also be controlled.

#### Extractable Substances

Since pressurized inhalers and aerosols are normally formulated with organic solvents as the propellant or the vehicle, leaching of extractables from the elastomeric and plastic components into the formulation is a potentially serious problem. Thus, the composition and the quality of materials used in the manufacture of the valve components (e.g., stem, gaskets, housing, etc.) must be carefully selected and controlled. Their compatibility with formulation components should be well established so as to prevent distortion of the valve components and to minimize changes in the medication delivery, leak rate, and impurity profile of the drug product over time. The extractable profiles of a representative sample of each of the elastomeric and plastic components of the valve should be established under specified conditions and should be correlated to the extractable profile of the aged drug product or placebo, to ensure reproducible quality and purity of the drug product. Extractables, which may include polynuclear aromatics, nitrosamines, vulcanization accelerators, antioxidants, plasticizers, monomers, etc., should be identified and minimized wherever possible.

Specifications and limits for individual and total extractables from different valve components may require the use of different analytical methods. In addition, the standard USP biological testing (see the general test chapters *Biological Reactivity Tests, In Vitro* 87 and *Biological Reactivity Tests, In Vivo* 88) as well as other safety data may be needed.

#### Labeling

Medicinal aerosols should contain at least the following warning information on the label as in accordance with appropriate regulations.

Warning— Avoid inhaling. Avoid spraying into eyes or onto other mucous membranes.

note—The statement "Avoid inhaling" is not necessary for preparations specifically designed for use by inhalation. The phrase "or other mucous membranes" is not necessary for preparations specifically designed for use on mucous membranes.

Warning— Contents under pressure. Do not puncture or incinerate container. Do not expose to heat or store at temperatures above 120° F (49° C). Keep out of reach of children.

In addition to the aforementioned warnings, the label of a drug packaged in an aerosol container in which the propellant consists in whole or in part of a halocarbon or hydrocarbon shall, where required under regulations of the FDA, bear either of the following warnings:

Warning— Do not inhale directly; deliberate inhalation of contents can cause death.

Warning— Use only as directed; intentional misuse by deliberately concentrating and inhaling the contents can be harmful or fatal.

#### BOLUSES

Boluses are large elongated tablets intended for administration to animals (see Tablets).

#### CAPSULES

Capsules are solid dosage forms in which the drug is enclosed within either a hard or soft soluble container or "shell." The shells are usually formed from gelatin; however, they also may be made from starch or other suitable substances. Hard-shell capsule sizes range from No. 5, the smallest, to No. 000, which is the largest, except for veterinary sizes. However, size No. 00 generally is the largest size acceptable to patients. Size 0 hard gelatin capsules having an elongated body (known as size OE) also are available, which provide greater fill capacity without an increase in diameter. Hard gelatin capsules consist of two, telescoping cap and body pieces. Generally, there are unique grooves or indentations molded into the cap and body portions to provide a positive closure when fully engaged, which helps prevent the accidental separation of the filled capsules during shipping and handling. Positive closure also may be affected by spot fusion ("welding") of the cap and body pieces together through direct thermal means or by application of ultrasonic energy. Factory-filled hard gelatin capsules may be completely sealed by banding, a process in which one or more layers of gelatin are applied over the seam of the cap and body, or by a liquid fusion process wherein the filled capsules are wetted with a hydroalcoholic solution that penetrates into the space where the cap overlaps the body, and then dried. Hard-shell capsules made from starch consist of two, fitted cap and body pieces. Since the two pieces do not telescope or interlock positively, they are sealed together at the time of filling to prevent their separation.

Starch capsules are sealed by the application of a hydroalcoholic solution to the recessed section of the cap immediately prior to its being placed onto the body.

The banding of hard-shell gelatin capsules or the liquid sealing of hard-shell starch capsules enhances consumer safety by making the capsules difficult to open without causing visible, obvious damage, and may improve the stability of contents by limiting O2 penetration. Industrially filled hard-shell capsules also are often of distinctive color and shape or are otherwise marked to identify them with the manufacturer. Additionally, such capsules may be printed axially or radially with strengths, product codes, etc. Pharmaceutical-grade printing inks are usually based on shellac and employ FDA-approved pigments and lake dyes.

In extemporaneous prescription practice, hard-shell capsules may be hand-filled; this permits the prescriber a latitude of choice in selecting either a single drug or a combination of drugs at the exact dosage level considered best for the individual patient. This flexibility gives hard-shell capsules an advantage over compressed tablets and soft-shell capsules as a dosage form. Hard-shell capsules are usually formed from gelatins having relatively high gel strength. Either type may be used, but blends of pork skin and bone gelatin are often used to optimize shell clarity and toughness. Hard-shell capsules also may be formed from starch or other suitable substances. Hard-shell capsules may also contain colorants, such as D&C and FD&C dyes or the various iron oxides, opaques such as titanium dioxide, dispersing agents, hardening agents such as sucrose, and preservatives. They normally contain between 10% and 15% water.

Hard gelatin capsules are made by a process that involves dipping shaped pins into gelatin solutions, after which the gelatin films are dried, trimmed, and removed from the pins, and the body and cap pieces are joined. Starch capsules are made by injection molding a mixture of starch and water, after which the capsules are dried. A separate mold is used for caps and bodies, and the two parts are supplied separately. The empty capsules should be stored in tight containers until they are filled. Since gelatin is of animal origin and starch is of vegetable origin, capsules made with these materials should be protected from potential sources of microbial contamination.

Hard-shell capsules typically are filled with powder, beads, or granules. Inert sugar beads (nonpareils) may be coated with active ingredients and coating compositions that provide extended-release profiles or enteric properties. Alternatively, larger-dose active ingredients themselves may be suitably formed into pellets and then coated. Semisolids or liquids also may be filled into hard-shell capsules; however, when the latter are encapsulated, one of the sealing techniques must be employed to prevent leakage.

In hard gelatin capsule filling operations, the body and cap of the shell are separated prior to dosing. In hard starch shell filling operations, the bodies and caps are supplied separately and are fed into separate hoppers of the filling machine. Machines employing various dosing principles may be employed to fill powders into hard-shell capsules; however, most fully automatic machines form powder plugs by compression and eject them into empty capsule bodies. Accessories to these machines generally are available for the other types of fills. Powder formulations often require adding fillers, lubricants, and glidants to the active ingredients to facilitate encapsulation. The formulation, as well as the method of filling, particularly the degree of compaction, may influence the rate of drug release. The addition of wetting agents to the powder mass is common where the active ingredient is hydrophobic.

Disintegrants also may be included in powder formulations to facilitate disaggregation and dispersal of capsule plugs in the gut. Powder formulations often may be produced by dry blending; however, bulky formulations may require densification by roll compaction or other suitable granulation techniques.

Powder mixtures that tend to liquefy may be dispensed in hard-shell capsules if an absorbent such as magnesium carbonate, colloidal silicon dioxide, or other suitable substance is used. Potent drugs are often mixed with an inert diluent before being filled into capsules. Where two mutually incompatible drugs are prescribed together, it is sometimes possible to place one in a small capsule and then enclose it with the second drug in a larger capsule. Incompatible drugs also can be separated by placing coated pellets or tablets, or soft-shell capsules of one drug into the capsule shell before adding the second drug.

Thixotropic semisolids may be formed by gelling liquid drugs or vehicles with colloidal silicas or powdered high molecular weight polyethylene glycols. Various waxy or fatty compounds may be used to prepare semisolid matrices by fusion.

Soft-shell capsules made from gelatin (sometimes called softgels) or other suitable material require large-scale production methods. The soft gelatin shell is somewhat thicker than that of hard-shell capsules and may be plasticized by the addition of a polyol such as sorbitol or glycerin. The ratio of dry plasticizer to dry gelatin determines the "hardness" of the shell and may be varied to accommodate environmental conditions as well as the nature of the contents. Like hard shells, the shell composition may include approved dyes and pigments, opaques such as titanium dioxide, and preservatives. Flavors may be added and up to 5% sucrose may be included for its sweetness and to produce a chewable shell. Soft

Gelatin shells normally contain 6% to 13% water. Soft-shell capsules also may be printed with a product code, strength, etc. In most cases, soft-shell capsules are filled with liquid contents. Typically, active ingredients are dissolved or suspended in a liquid vehicle. Classically, an oleaginous vehicle such as a vegetable oil was used; however, nonaqueous, water-miscible liquid vehicles such as the lower-molecular-weight polyethylene glycols are more common today due to fewer bioavailability problems.

Available in a wide variety of sizes and shapes, soft-shell capsules are both formed, filled, and sealed in the same machine; typically, this is a rotary die process, although a plate process or reciprocating die process also may be employed. Soft-shell capsules also may be manufactured in a bubble process that forms seamless spherical capsules. With suitable equipment, powders and other dry solids also may be filled into soft-shell capsules.

Liquid-filled capsules of either type involve similar formulation technology and offer similar advantages and limitations. For instance, both may offer advantages over dry-filled capsules and tablets in content uniformity and drug dissolution. Greater homogeneity is possible in liquid systems, and liquids can be metered more accurately. Drug dissolution may benefit because the drug may already be in solution or at least suspended in a hydrophilic vehicle. However, the contact between the hard or soft shell and its liquid content is more intimate than exists with dry-filled capsules, and this may enhance the chances for undesired interactions. The liquid nature of capsule contents presents different technological problems than dry-filled capsules in regard to disintegration and dissolution testing. From formulation, technological, and biopharmaceutical points of view, liquid-filled capsules of either type have more in common than liquid-filled and dry-filled capsules having the same shell composition. Thus, for compendial purposes, standards and methods should be established based on capsule contents rather than on whether the contents are filled into hard- or soft-shell capsules.

#### delayed-release capsules

Capsules may be coated, or, more commonly, encapsulated granules may be coated to resist releasing the drug in the gastric fluid of the stomach where a delay is important to alleviate potential problems of drug inactivation or gastric mucosal irritation. The term "delayed-release" is used for Pharmacopeial monographs on enteric coated capsules that are intended to delay the release of medicament until the capsule has passed through the stomach, and the individual monographs include tests and specifications for Drug release (see [Drug Release \(724\)](#)) or Disintegration (see [Disintegration \(701\)](#)).

#### extended-release capsules

Extended-release capsules are formulated in such manner as to make the contained medicament available over an extended period of time following ingestion. Expressions such as "prolonged-action," "repeat-action," and "sustained-release" have also been used to describe such dosage forms. However, the term "extended-release" is used for Pharmacopeial purposes and requirements for Drug release (see [Drug Release \(724\)](#)) typically are specified in the individual monographs.

#### CONCENTRATE FOR DIP

Concentrate for Dip is a preparation containing one or more active ingredients usually in the form of a paste or solution. It is used to prepare a diluted suspension, emulsion, or solution of the active ingredient(s) for the prevention and treatment of ectoparasitic infestations of animals. The diluted preparation (Dip) is applied by complete immersion of the animal or, where appropriate, by spraying. Concentrate for Dip may contain suitable antimicrobial preservatives.

#### CREAMS

Creams are semisolid dosage forms containing one or more drug substances dissolved or dispersed in a suitable base. This term has traditionally been applied to semisolids that possess a relatively fluid consistency formulated as either water-in-oil (e.g., Cold Cream) or oil-in-water (e.g., Fluocinolone Acetonide Cream) emulsions. However, more recently the term has been restricted to products consisting of oil-in-water emulsions or aqueous microcrystalline dispersions of long-chain fatty acids or alcohols that are water washable and more cosmetically and aesthetically acceptable. Creams can be used for administering drugs via the vaginal route (e.g., Triple Sulfa Vaginal Cream).

#### ELIXIRS

See Solutions.

#### EMULSIONS

Emulsions are two-phase systems in which one liquid is dispersed throughout another liquid in the form of small droplets. Where oil is the dispersed phase and an aqueous solution is the continuous phase, the system is designated as an oil-in-water emulsion. Conversely, where water or an aqueous solution is the dispersed phase and oil or oleaginous material is the continuous phase, the system is designated as a water-in-oil emulsion. Emulsions are stabilized by emulsifying agents that prevent coalescence, the merging of small droplets into larger droplets and, ultimately, into a single separated phase. Emulsifying agents (surfactants) do this by concentrating in the interface between the droplet and external phase and by providing a physical barrier around the particle to coalescence. Surfactants also reduce the interfacial tension between the phases, thus increasing the ease of emulsification upon mixing.

Natural, semisynthetic, and synthetic hydrophilic polymers may be used in conjunction with surfactants in oil-in-water emulsions as they accumulate at interfaces and also increase the viscosity of the aqueous phase, thereby decreasing the rate of formation of aggregates of droplets. Aggregation is generally accompanied by a relatively rapid separation of an emulsion into a droplet-rich and droplet-poor phase. Normally the density of an oil is lower than that of water, in which case the oil droplets and droplet aggregates rise, a process referred to as creaming. The greater the rate of aggregation, the greater the droplet size and the greater the rate of creaming. The water droplets in a water-in-oil emulsion generally sediment because of their greater density.

The consistency of emulsions varies widely, ranging from easily pourable liquids to semisolid creams. Generally oil-in-water creams are prepared at high temperature, where they are fluid, and cooled to room temperature, whereupon they solidify as a result of solidification of the internal phase. When this is the case, a high internal-phase volume to external-phase volume ratio is not necessary for semisolid character, and, for example, stearic acid creams or vanishing creams are semisolid with as little as 15% internal phase. Any semisolid character with water-in-oil emulsions generally is attributable to a semisolid external phase.

All emulsions require an antimicrobial agent because the aqueous phase is favorable to the growth of microorganisms. The presence of a preservative is particularly critical in oil-in-water emulsions where contamination of the external phase occurs readily. Since fungi and yeasts are found with greater frequency than bacteria, fungistatic as well as bacteriostatic properties are desirable. Bacteria have been shown to degrade nonionic and anionic emulsifying agents, glycerin, and many natural stabilizers such as tragacanth and guar gum.

Complications arise in preserving emulsion systems, as a result of partitioning of the antimicrobial agent out of the aqueous phase where it is most needed, or of complexation with emulsion ingredients that reduce effectiveness. Therefore, the effectiveness of the preservative system should always be tested in the final product. Preservatives commonly used in emulsions include methyl-, ethyl-, propyl-, and butyl-parabens, benzoic acid, and quaternary ammonium compounds.

See also Creams and Ointments.

#### EXTRACTS AND FLUIDEXTRACTS

Extracts are concentrated preparations of vegetable or animal drugs obtained by removal of the active constituents of the respective drugs with suitable menstrua, by evaporation of all or nearly all of the solvent, and by adjustment of the residual masses or powders to the prescribed standards.

In the manufacture of most extracts, the drugs are extracted by percolation. The entire percolates are concentrated, generally by distillation under reduced pressure in order to subject the drug principles to as little heat as possible.

Fluidextracts are liquid preparations of vegetable drugs, containing alcohol as a solvent or as a preservative, or both, and so made that, unless otherwise specified in an individual monograph, each mL contains the therapeutic constituents of 1 g of the standard drug that it represents.

A fluidextract that tends to deposit sediment may be aged and filtered or the clear portion decanted, provided the resulting clear liquid conforms to the Pharmacopeial standards.



Fluidextracts may be prepared from suitable extracts.

## GELS

Gels (sometimes called Jellies) are semisolid systems consisting of either suspensions made up of small inorganic particles or large organic molecules interpenetrated by a liquid. Where the gel mass consists of a network of small discrete particles, the gel is classified as a two-phase system (e.g., Aluminum Hydroxide Gel). In a two-phase system, if the particle size of the dispersed phase is relatively large, the gel mass is sometimes referred to as a magma (e.g., Bentonite Magma). Both gels and magmas may be thixotropic, forming semisolids on standing and becoming liquid on agitation. They should be shaken before use to ensure homogeneity and should be labeled to that effect. (See Suspensions.)

Single-phase gels consist of organic macromolecules uniformly distributed throughout a liquid in such a manner that no apparent boundaries exist between the dispersed macromolecules and the liquid. Single-phase gels may be made from synthetic macromolecules (e.g., Carbomer) or from natural gums (e.g., Tragacanth). The latter preparations are also called mucilages. Although these gels are commonly aqueous, alcohols and oils may be used as the continuous phase. For example, mineral oil can be combined with a polyethylene resin to form an oleaginous ointment base.

Gels can be used to administer drugs topically or into body cavities (e.g., Phenylephrine Hydrochloride Nasal Jelly).

## IMPLANTS (PELLETS)

Implants or pellets are small sterile solid masses consisting of a highly purified drug (with or without excipients) made by compression or molding. They are intended for implantation in the body (usually subcutaneously) for the purpose of providing continuous release of the drug over long periods of time. Implants are administered by means of a suitable special injector or surgical incision. This dosage form has been used to administer hormones such as testosterone or estradiol. They are packaged individually in sterile vials or foil strips.

## INFUSIONS, INTRAMAMMARY

Intramammary infusions are suspensions of drugs in suitable oil vehicles. These preparations are intended for veterinary use only, and are administered by instillation via the teat canals into the udders of milk-producing animals.

## INHALATIONS

Inhalations are drugs or solutions or suspensions of one or more drug substances administered by the nasal or oral respiratory route for local or systemic effect.

Solutions of drug substances in sterile water for inhalation or in sodium chloride inhalation solution may be nebulized by use of inert gases. Nebulizers are suitable for the administration of inhalation solutions only if they give droplets sufficiently fine and uniform in size so that the mist reaches the bronchioles. Nebulized solutions may be breathed directly from the nebulizer or the nebulizer may be attached to a plastic face mask, tent, or intermittent positive pressure breathing (IPPB) machine.

Another group of products, also known as metered-dose inhalers (MDIs) are propellant-driven drug suspensions or solutions in liquified gas propellant with or without a cosolvent and are intended for delivering metered doses of the drug to the respiratory tract. An MDI contains multiple doses, often exceeding several hundred. The most common single-dose volumes delivered are from 25 to 100  $\mu$ L (also expressed as mg) per actuation.

Examples of MDIs containing drug solutions and suspensions in this pharmacopeia are Epinephrine Inhalation Aerosol and Isoproterenol Hydrochloride and Phenylephrine Bitartrate Inhalation Aerosol, respectively.

Powders may also be administered by mechanical devices that require manually produced pressure or a deep inhalation by the patient (e.g., Cromolyn Sodium for Inhalation).

A special class of inhalations termed inhalants consists of drugs or combination of drugs, that by virtue of their high vapor pressure, can be carried by an air current into the nasal passage where they exert their effect. The container from which the inhalant generally is administered is known as an inhaler.

## INJECTIONS

An Injection is a preparation intended for parenteral administration or for constituting or diluting a parenteral article prior to administration (see [Injections](#)).

Each container of an Injection is filled with a volume in slight excess of the labeled "size" or that volume that is to be withdrawn. The excess volumes recommended in the accompanying table are usually sufficient to permit withdrawal and administration of the labeled volumes.

Labeled Size	Recommended Excess Volume	
	For Mobile Liquids	For Viscous Liquids
0.5 mL	0.10 mL	0.12 mL
1.0 mL	0.10 mL	0.15 mL
2.0 mL	0.15 mL	0.25 mL
5.0 mL	0.30 mL	0.50 mL
10.0 mL	0.50 mL	0.70 mL
20.0 mL	0.60 mL	0.90 mL
30.0 mL	0.80 mL	1.20 mL
50.0 mL or more	2%	3%

## IRRIGATIONS

Irrigations are sterile solutions intended to bathe or flush open wounds or body cavities. They are used topically, never parenterally. They are labeled to indicate that they are not intended for injection.

## LOTIONS

See Solutions or Suspensions.

## LOZENGES

Lozenges are solid preparations, that are intended to dissolve or disintegrate slowly in the mouth. They contain one or more medicaments, usually in a flavored, sweetened base. They can be prepared by molding (gelatin and/or fused sucrose or sorbitol base) or by compression of sugar-based tablets. Molded lozenges are sometimes referred to as pastilles while compressed lozenges are often referred to as troches. They are usually intended for treatment of local irritation or infections of the mouth or throat but may contain active ingredients intended for systemic absorption after swallowing.



## OINTMENTS

Ointments are semisolid preparations intended for external application to the skin or mucous membranes.

Ointment bases recognized for use as vehicles fall into four general classes: the hydrocarbon bases, the absorption bases, the water-removable bases, and the water-soluble bases.

Each therapeutic ointment possesses as its base a representative of one of these four general classes.

### Hydrocarbon Bases

These bases, which are known also as "oleaginous ointment bases," are represented by White Petrolatum and White Ointment. Only small amounts of an aqueous component can be incorporated into them. They serve to keep medicaments in prolonged contact with the skin and act as occlusive dressings. Hydrocarbon bases are used chiefly for their emollient effects, and are difficult to wash off. They do not "dry out" or change noticeably on aging.

### Absorption Bases

This class of bases may be divided into two groups: the first group consisting of bases that permit the incorporation of aqueous solutions with the formation of a water-in-oil emulsion (Hydrophilic Petrolatum and Lanolin), and the second group consisting of water-in-oil emulsions that permit the incorporation of additional quantities of aqueous solutions (Lanolin).

Absorption bases are useful also as emollients.

### Water-Removable Bases

Such bases are oil-in-water emulsions, e.g., Hydrophilic Ointment, and are more correctly called "creams." (See Creams.) They are also described as "water-washable," since they may be readily washed from the skin or clothing with water, an attribute that makes them more acceptable for cosmetic reasons. Some medicaments may be more effective in these bases than in hydrocarbon bases. Other advantages of the water-removable bases are that they may be diluted with water and that they favor the absorption of serous discharges in dermatological conditions.

### Water-Soluble Bases

This group of so-called "greaseless ointment bases" comprises water-soluble constituents. Polyethylene Glycol Ointment is the only Pharmacopeial preparation in this group. Bases of this type offer many of the advantages of the water-removable bases and, in addition, contain no water-insoluble substances such as petrolatum, anhydrous lanolin, or waxes. They are more correctly called "Gels." (See Gels.)

**Choice of Base**— The choice of an ointment base depends upon many factors, such as the action desired, the nature of the medicament to be incorporated and its bioavailability and stability, and the requisite shelf-life of the finished product. In some cases, it is necessary to use a base that is less than ideal in order to achieve the stability required. Drugs that hydrolyze rapidly, for example, are more stable in hydrocarbon bases than in bases containing water, even though they may be more effective in the latter.

## OPHTHALMIC PREPARATIONS

Drugs are administered to the eyes in a wide variety of dosage forms, some of which require special consideration. They are discussed in the following paragraphs.

### Ointments

Ophthalmic ointments are ointments for application to the eye. Special precautions must be taken in the preparation of ophthalmic ointments. They are manufactured from sterilized ingredients under rigidly aseptic conditions and meet the requirements under [Sterility Tests \(71\)](#). If the specific ingredients used in the formulation do not lend themselves to routine sterilization techniques, ingredients that meet the sterility requirements described under [Sterility Tests \(71\)](#), along with aseptic manufacture, may be employed. Ophthalmic ointments must contain a suitable substance or mixture of substances to prevent growth of, or to destroy, microorganisms accidentally introduced when the container is opened during use, unless otherwise directed in the individual monograph, or unless the formula itself is bacteriostatic (see Added Substances under [Ophthalmic Ointments \(771\)](#)). The medicinal agent is added to the ointment base either as a solution or as a micronized powder. The finished ointment must be free from large particles and must meet the requirements for Leakage and for Metal Particles under [Ophthalmic Ointments \(771\)](#). The immediate containers for ophthalmic ointments shall be sterile at the time of filling and closing. It is mandatory that the immediate containers for ophthalmic ointments be sealed and tamper-proof so that sterility is assured at time of first use.

The ointment base that is selected must be nonirritating to the eye, permit diffusion of the drug throughout the secretions bathing the eye, and retain the activity of the medicament for a reasonable period under proper storage conditions.

Petrolatum is mainly used as a base for ophthalmic drugs. Some absorption bases, water-removable bases, and water-soluble bases may be desirable for water-soluble drugs. Such bases allow for better dispersion of water-soluble medicaments, but they must be nonirritating to the eye.

### Solutions

Ophthalmic solutions are sterile solutions, essentially free from foreign particles, suitably compounded and packaged for instillation into the eye. Preparation of an ophthalmic solution requires careful consideration of such factors as the inherent toxicity of the drug itself, isotonicity value, the need for buffering agents, the need for a preservative (and, if needed, its selection), sterilization, and proper packaging. Similar considerations are also made for nasal and otic products.

### isotonicity value

Lacrimal fluid is isotonic with blood, having an isotonicity value corresponding to that of a 0.9% sodium chloride solution. Ideally, an ophthalmic solution should have this isotonicity value; but the eye can tolerate isotonicity values as low as that of a 0.6% sodium chloride solution and as high as that of a 2.0% sodium chloride solution without marked discomfort.

Some ophthalmic solutions are necessarily hypertonic in order to enhance absorption and provide a concentration of the active ingredient(s) strong enough to exert a prompt and effective action. Where the amount of such solutions used is small, dilution with lacrimal fluid takes place rapidly so that discomfort from the hypertonicity is only temporary. However, any adjustment toward isotonicity by dilution with tears is negligible where large volumes of hypertonic solutions are used as collyria to wash the eyes; it is, therefore, important that solutions used for this purpose be approximately isotonic.

### buffering

Many drugs, notably alkaloidal salts, are most effective at pH levels that favor the undissociated free bases. At such pH levels, however, the drug may be unstable so that compromise levels must be found and held by means of buffers. One purpose of buffering some ophthalmic solutions is to prevent an increase in pH caused by the slow release of hydroxyl ions by glass. Such a rise in pH can affect both the solubility and the stability of the drug. The decision whether or not buffering agents should be added in preparing an ophthalmic solution must be based on several considerations. Normal tears have a pH of about 7.4 and possess some buffer capacity. The application of a solution to the eye stimulates the flow of tears and the rapid neutralization of any excess hydrogen or hydroxyl ions within the buffer capacity of the tears. Many ophthalmic drugs, such as alkaloidal salts, are weakly acidic and have only weak buffer capacity. Where only 1 or 2 drops of a solution containing them are added to the eye, the buffering action of the tears is usually adequate to raise the pH and prevent marked discomfort. In some cases pH may vary between 3.5 and 8.5. Some drugs, notably pilocarpine hydrochloride and epinephrine bitartrate, are more acid and overtax the buffer capacity of the lacrimal fluid. Ideally, an ophthalmic solution should have the same pH, as well as the same isotonicity value, as lacrimal fluid. This is not usually possible since, at pH 7.4, many drugs are not appreciably soluble in water. Most alkaloidal salts precipitate as the free alkaloid at this pH. Additionally, many drugs are chemically unstable at pH levels approaching 7.4. This instability is more marked at the high temperatures employed in heat sterilization. For this reason, the buffer system should be selected that is nearest to the physiological pH of 7.4 and does not cause precipitation of the drug or its rapid deterioration.

An ophthalmic preparation with a buffer system approaching the physiological pH can be obtained by mixing a sterile solution of the drug with a sterile buffer solution using aseptic technique. Even so, the possibility of a shorter shelf-life at the higher pH must be taken into consideration, and attention must be directed toward the attainment and maintenance of sterility throughout the manipulations.

Many drugs, when buffered to a therapeutically acceptable pH, would not be stable in solution for long periods of time. These products are lyophilized and are intended for reconstitution immediately before use (e.g., Acetylcholine Chloride for Ophthalmic Solution).

#### sterilization

The sterility of solutions applied to an injured eye is of the greatest importance. Sterile preparations in special containers for individual use on one patient should be available in every hospital, office, or other installation where accidentally or surgically traumatized eyes are treated. The method of attaining sterility is determined primarily by the character of the particular product (see [Sterilization and Sterility Assurance of Compendial Articles](#) (1211)).

Whenever possible, sterile membrane filtration under aseptic conditions is the preferred method. If it can be shown that product stability is not adversely affected, sterilization by autoclaving in the final container is also a preferred method.

Buffering certain drugs near the physiological pH range makes them quite unstable at high temperature.

Avoiding the use of heat by employing a bacteria-retaining filter is a valuable technique, provided caution is exercised in the selection, assembly, and use of the equipment. Single-filtration, presterilized disposable units are available and should be utilized wherever possible.

#### preservation

Ophthalmic solutions may be packaged in multiple-dose containers when intended for the individual use of one patient and where the ocular surfaces are intact. It is mandatory that the immediate containers for ophthalmic solutions be sealed and tamper-proof so that sterility is assured at time of first use. Each solution must contain a suitable substance or mixture of substances to prevent the growth of, or to destroy, microorganisms accidentally introduced when the container is opened during use.

Where intended for use in surgical procedures, ophthalmic solutions, although they must be sterile, should not contain antibacterial agents, since they may be irritating to the ocular tissues.

#### thickening agent

A pharmaceutical grade of methylcellulose (e.g., 1% if the viscosity is 25 centipoises, or 0.25% if 4000 centipoises) or other suitable thickening agents such as hydroxypropyl methylcellulose or polyvinyl alcohol occasionally are added to ophthalmic solutions to increase the viscosity and prolong contact of the drug with the tissue. The thickened ophthalmic solution must be free from visible particles.

#### Suspensions

Ophthalmic suspensions are sterile liquid preparations containing solid particles dispersed in a liquid vehicle intended for application to the eye (see [Suspensions](#)). It is imperative that such suspensions contain the drug in a micronized form to prevent irritation and/or scratching of the cornea. Ophthalmic suspensions should never be dispensed if there is evidence of caking or aggregation.

#### Strips

Fluorescein sodium solution should be dispensed in a sterile, single-use container or in the form of a sterile, impregnated paper strip. The strip releases a sufficient amount of the drug for diagnostic purposes when touched to the eye being examined for a foreign body or a corneal abrasion. Contact of the paper with the eye may be avoided by leaching the drug from the strip onto the eye with the aid of sterile water or sterile sodium chloride solution.

#### PASTES

Pastes are semisolid dosage forms that contain one or more drug substances intended for topical application. One class is made from a single-phase aqueous gel (e.g., Carboxymethylcellulose Sodium Paste). The other class, the fatty pastes (e.g., Zinc Oxide Paste), consists of thick, stiff ointments that do not ordinarily flow at body temperature, and therefore serve as protective coatings over the areas to which they are applied.

The fatty pastes appear less greasy and more absorptive than ointments by reason of a high proportion of drug substance(s) having an affinity for water. These pastes tend to absorb serous secretions, and are less penetrating and less macerating than ointments, so that they are preferred for acute lesions that have a tendency towards crusting, vesiculation, or oozing.

A dental paste is intended for adhesion to the mucous membrane for local effect (e.g., Triamcinolone Acetonide Dental Paste). Some paste preparations intended for administration to animals are applied orally. The paste is squeezed into the mouth of the animal, generally at the back of the tongue, or is spread inside the mouth.

#### PELLETS

See [Implants](#).

#### POWDERS

Powders are intimate mixtures of dry, finely divided drugs and/or chemicals that may be intended for internal (Oral Powders) or external (Topical Powders) use. Because of their greater specific surface area, powders disperse and dissolve more readily than compacted dosage forms. Children and those adults who experience difficulty in swallowing tablets or capsules may find powders more acceptable. Drugs that are too bulky to be formed into tablets or capsules of convenient size may be administered as powders. Immediately prior to use, oral powders are mixed in a beverage or apple sauce.

Often, stability problems encountered in liquid dosage forms are avoided in powdered dosage forms. Drugs that are unstable in aqueous suspensions or solutions may be prepared in the form of granules or powders. These are intended to be constituted by the pharmacist by the addition of a specified quantity of water just prior to dispensing. Because these constituted products have limited stability, they are required to have a specified expiration date after constitution and may require storage in a refrigerator.

Oral powders may be dispensed in doses premeasured by the pharmacist, i.e., divided powders, or in bulk. Traditionally, divided powders have been wrapped in materials such as bond paper and parchment. However, the pharmacist may provide greater protection from the environment by sealing individual doses in small cellophane or polyethylene envelopes.

Granules for veterinary use may be administered by sprinkling the dry powder on animal feed or by mixing it with animal food.

Bulk oral powders are limited to relatively nonpotent drugs such as laxatives, antacids, dietary supplements, and certain analgesics that the patient may safely measure by the teaspoonful or capful. Other bulky powders include douche powders, tooth powders, and dusting powders. Bulk powders are best dispensed in tight, wide-mouth glass containers to afford maximum protection from the atmosphere and to prevent the loss of volatile constituents.

Dusting powders are impalpable powders intended for topical application. They may be dispensed in sifter-top containers to facilitate dusting onto the skin. In general, dusting powders should be passed through at least a 100-mesh sieve to assure freedom from grit that could irritate traumatized areas (see [Powder Fineness](#) (811)).

#### PREMIXES

Premixes are mixtures of one or more drug substances with suitable vehicles. Premixes are intended for admixture to animal feedstuffs before administration. They are used to facilitate dilution of the active drug components with animal feed. Premixes should be as homogeneous as possible. It is essential that materials of suitable fineness be used and that thorough mixing be achieved at all stages of premix preparation. Premixes may be prepared as powder, pellets, or in granulated form. The granulated form is free-flowing and free from aggregates.



## SOLUTIONS

Solutions are liquid preparations that contain one or more chemical substances dissolved, i.e., molecularly dispersed, in a suitable solvent or mixture of mutually miscible solvents. Since molecules in solutions are uniformly dispersed, the use of solutions as dosage forms generally provides for the assurance of uniform dosage upon administration, and good accuracy when diluting or otherwise mixing solutions.

Substances in solutions, however, are more susceptible to chemical instability than the solid state and dose for dose, generally require more bulk and weight in packaging relative to solid dosage forms. For all solutions, but particularly those containing volatile solvents, tight containers, stored away from excessive heat, should be used. Consideration should also be given to the use of light-resistant containers when photolytic chemical degradation is a potential stability problem. Dosage forms categorized as "Solutions" are classified according to route of administration, such as "Oral Solutions" and "Topical Solutions," or by their solute and solvent systems, such as "Spirits," "Tinctures," and "Waters." Solutions intended for parenteral administration are officially entitled "Injections" (see [Injections](#) 1).

### Oral Solutions

Oral Solutions are liquid preparations, intended for oral administration, that contain one or more substances with or without flavoring, sweetening, or coloring agents dissolved in water or cosolvent-water mixtures. Oral Solutions may be formulated for direct oral administration to the patient or they may be dispensed in a more concentrated form that must be diluted prior to administration. It is important to recognize that dilution with water of Oral Solutions containing cosolvents, such as alcohol, could lead to precipitation of some ingredients. Hence, great care must be taken in diluting concentrated solutions when cosolvents are present. Preparations dispensed as soluble solids or soluble mixtures of solids, with the intent of dissolving them in a solvent and administering them orally, are designated "for Oral Solution" (e.g., Potassium Chloride for Oral Solution).

Oral Solutions containing high concentrations of sucrose or other sugars traditionally have been designated as Syrups. A near-saturated solution of sucrose in purified water, for example, is known as Syrup or "Simple Syrup." Through common usage the term, syrup, also has been used to include any other liquid dosage form prepared in a sweet and viscous vehicle, including oral suspensions.

In addition to sucrose and other sugars, certain polyols such as sorbitol or glycerin may be present in Oral Solutions to inhibit crystallization and to modify solubility, taste, mouth-feel, and other vehicle properties. Antimicrobial agents to prevent the growth of bacteria, yeasts, and molds are generally also present. Some sugarless Oral Solutions contain sweetening agents such as sorbitol or aspartame, as well as thickening agents such as the cellulose gums. Such viscous sweetened solutions, containing no sugars, are occasionally prepared as vehicles for administration of drugs to diabetic patients.

Many oral solutions, that contain alcohol as a cosolvent, have been traditionally designated as Elixirs. However, many others designated as Oral Solutions also contain significant amounts of alcohol. Since high concentrations of alcohol can produce a pharmacologic effect when administered orally, other cosolvents, such as glycerin and propylene glycol, should be used to minimize the amount of alcohol required. To be designated as an Elixir, however, the solution must contain alcohol.

### Topical Solutions

Topical Solutions are solutions, usually aqueous but often containing other solvents, such as alcohol and polyols, intended for topical application to the skin, or as in the case of Lidocaine Oral Topical Solution, to the oral mucosal surface. The term "lotion" is applied to solutions or suspensions applied topically.

### Otic Solutions

Otic Solutions, intended for instillation in the outer ear, are aqueous, or they are solutions prepared with glycerin or other solvents and dispersing agents (e.g., Antipyrine and Benzocaine Otic Solution and Neomycin and Polymyxin B Sulfates and Hydrocortisone Otic Solution).

### Ophthalmic Solutions

See Ophthalmic Preparations.

### Spirits

Spirits are alcoholic or hydroalcoholic solutions of volatile substances prepared usually by simple solution or by admixture of the ingredients. Some spirits serve as flavoring agents while others have medicinal value. Reduction of the high alcoholic content of spirits by admixture with aqueous preparations often causes turbidity.

Spirits require storage in tight, light-resistant containers to prevent loss by evaporation and to limit oxidative changes.

### Tinctures

Tinctures are alcoholic or hydroalcoholic solutions prepared from vegetable materials or from chemical substances.

The proportion of drug represented in the different chemical tinctures is not uniform but varies according to the established standards for each. Traditionally, tinctures of potent vegetable drugs essentially represent the activity of 10 g of the drug in each 100 mL of tincture, the potency being adjusted following assay. Most other vegetable tinctures represent 20 g of the respective vegetable material in each 100 mL of tincture.

### process p

Carefully mix the ground drug or mixture of drugs with a sufficient quantity of the prescribed solvent or solvent mixture to render it evenly and distinctly damp, allow it to stand for 15 minutes, transfer it to a suitable percolator, and pack the drug firmly. Pour on enough of the prescribed solvent or solvent mixture to saturate the drug, cover the top of the percolator, and, when the liquid is about to drip from the percolator, close the lower orifice and allow the drug to macerate for 24 hours or for the time specified in the monograph. If no assay is directed, allow the percolation to proceed slowly, or at the specified rate, gradually adding sufficient solvent or solvent mixture to produce 1000 mL of tincture, and mix (for definitions of flow rates, see under Extracts and Fluidextracts). If an assay is directed, collect only 950 mL of percolate, mix this, and assay a portion of it as directed. Dilute the remainder with such quantity of the prescribed solvent or solvent mixture as calculation from the assay indicates is necessary to produce a tincture that conforms to the prescribed standard, and mix.

### process m

Macerate the drug with 750 mL of the prescribed solvent or solvent mixture in a container that can be closed, and put in a warm place. Agitate it frequently during 3 days or until the soluble matter is dissolved. Transfer the mixture to a filter, and when most of the liquid has drained away, wash the residue on the filter with a sufficient quantity of the prescribed solvent or solvent mixture, combining the filtrates, to produce 1000 mL of tincture, and mix.

Tinctures require storage in tight, light-resistant containers, away from direct sunlight and excessive heat.

### Waters, Aromatic

Aromatic waters are clear, saturated aqueous solutions (unless otherwise specified) of volatile oils or other aromatic or volatile substances. Their odors and tastes are similar, respectively, to those of the drugs or volatile substances from which they are prepared, and they are free from empyreumatic and other foreign odors. Aromatic waters may be prepared by distillation or solution of the aromatic substance, with or without the use of a dispersing agent.

Aromatic waters require protection from intense light and excessive heat.

## SUPPOSITORIES

Suppositories are solid bodies of various weights and shapes, adapted for introduction into the rectal, vaginal, or urethral orifice of the human body. They usually melt, soften, or dissolve at body temperature. A suppository may act as a protectant or palliative to the local tissues at the point of introduction or as a carrier of therapeutic agents for systemic or local action. Suppository bases usually employed are cocoa butter, glycerinated gelatin, hydrogenated vegetable oils, mixtures of polyethylene glycols of various molecular weights, and fatty acid esters of polyethylene glycol.



The suppository base employed has a marked influence on the release of the active ingredient incorporated in it. While cocoa butter melts quickly at body temperature, it is immiscible with body fluids and this inhibits the diffusion of fat-soluble drugs to the affected sites. Polyethylene glycol is a suitable base for some antiseptics. In cases where systemic action is expected, it is preferable to incorporate the ionized rather than the nonionized form of the drug, in order to maximize bioavailability. Although nonionized drugs partition more readily out of water-miscible bases such as glycerinated gelatin and polyethylene glycol, the bases themselves tend to dissolve very slowly and thus retard release in this manner. Oleaginous vehicles such as cocoa butter are seldom used in vaginal preparations because of the nonabsorbable residue formed, while glycerinated gelatin is seldom used rectally because of its slow dissolution. Cocoa butter and its substitutes (Hard Fat) are superior for allaying irritation, as in preparations intended for treating internal hemorrhoids.

#### Cocoa Butter Suppositories

Suppositories having cocoa butter as the base may be made by means of incorporating the finely divided medicinal substance into the solid oil at room temperature and suitably shaping the resulting mass, or by working with the oil in the melted state and allowing the resulting suspension to cool in molds. A suitable quantity of hardening agents may be added to counteract the tendency of some medicaments such as chloral hydrate and phenol to soften the base. It is important that the finished suppository melt at body temperature.

The approximate weights of suppositories prepared with cocoa butter are given below. Suppositories prepared from other bases vary in weight and generally are heavier than the weights indicated here.

Rectal Suppositories for adults are tapered at one or both ends and usually weigh about 2 g each.

Vaginal Suppositories are usually globular or oviform and weigh about 5 g each. They are made from water-soluble or water-miscible vehicles such as polyethylene glycol or glycerinated gelatin.

Suppositories with cocoa butter base require storage in well-closed containers, preferably at a temperature below 30° (controlled room temperature).

#### Cocoa Butter Substitutes

Fat-type suppository bases can be produced from a variety of vegetable oils, such as coconut or palm kernel, which are modified by esterification, hydrogenation, and fractionation to obtain products of varying composition and melting temperatures (e.g., Hydrogenated Vegetable Oil and Hard Fat). These products can be so designed as to reduce rancidity. At the same time, desired characteristics such as narrow intervals between melting and solidification temperatures, and melting ranges to accommodate various formulation and climatic conditions, can be built in.

#### Glycerinated Gelatin Suppositories

Medicinal substances may be incorporated into glycerinated gelatin bases by addition of the prescribed quantities to a vehicle consisting of about 70 parts of glycerin, 20 parts of gelatin, and 10 parts of water.

Glycerinated gelatin suppositories require storage in tight containers, preferably at a temperature below 35°.

#### Polyethylene Glycol-Base Suppositories

Several combinations of polyethylene glycols having melting temperatures that are above body temperature have been used as suppository bases. Inasmuch as release from these bases depends on dissolution rather than on melting, there are significantly fewer problems in preparation and storage than exist with melting-type vehicles. However, high concentrations of higher-molecular-weight polyethylene glycols may lengthen dissolution time, resulting in problems with retention. Labels on polyethylene glycol suppositories should contain directions that they be moistened with water before inserting. Although they can be stored without refrigeration, they should be packaged in tightly closed containers.

#### Surfactant Suppository Bases

Several nonionic surface-active agents closely related chemically to the polyethylene glycols can be used as suppository vehicles. Examples of such surfactants are polyoxyethylene sorbitan fatty acid esters and the polyoxyethylene stearates. These surfactants are used alone or in combination with other suppository vehicles to yield a wide range of melting temperatures and consistencies. One of the major advantages of such vehicles is their water-dispersibility. However, care must be taken with the use of surfactants, because they may either increase the rate of drug absorption or interact with drug molecules, causing a decrease in therapeutic activity.

#### Tabled Suppositories or Inserts

Vaginal suppositories occasionally are prepared by the compression of powdered materials into a suitable shape. They are prepared also by encapsulation in soft gelatin.

### SUSPENSIONS

Suspensions are liquid preparations that consist of solid particles dispersed throughout a liquid phase in which the particles are not soluble. Dosage forms officially categorized as "Suspensions" are designated as such if they are not included in other more specific categories of suspensions, such as Oral Suspensions, Topical Suspensions, etc. (see these other categories). Some suspensions are prepared and ready for use, while others are prepared as solid mixtures intended for constitution just before use with an appropriate vehicle. Such products are designated "for Oral Suspension", etc. The term "Milk" is sometimes used for suspensions in aqueous vehicles intended for oral administration (e.g., Milk of Magnesia). The term "Magma" is often used to describe suspensions of inorganic solids such as clays in water, where there is a tendency for strong hydration and aggregation of the solid, giving rise to gel-like consistency and thixotropic rheological behavior (e.g., Bentonite Magma). The term "Lotion" has been used to categorize many topical suspensions and emulsions intended for application to the skin (e.g., Calamine Lotion). Some suspensions are prepared in sterile form and are used as Injectables, as well as for ophthalmic and otic administration. These may be of two types, ready to use or intended for constitution with a prescribed amount of Water for Injection or other suitable diluent before use by the designated route. Suspensions should not be injected intravenously or intrathecally.

Suspensions intended for any route of administration should contain suitable antimicrobial agents to protect against bacteria, yeast, and mold contamination (see Emulsions for some consideration of antimicrobial preservative properties that apply also to Suspensions). By its very nature, the particular matter in a suspension may settle or sediment to the bottom of the container upon standing. Such sedimentation may also lead to caking and solidification of the sediment with a resulting difficulty in redispersing the suspension upon agitation. To prevent such problems, suitable ingredients that increase viscosity and the gel state of the suspension, such as clays, surfactants, polyols, polymers, or sugars, should be added. It is important that suspensions always be shaken well before use to ensure uniform distribution of the solid in the vehicle, thereby ensuring uniform and proper dosage. Suspensions require storage in tight containers.

#### Oral Suspensions

Oral Suspensions are liquid preparations containing solid particles dispersed in a liquid vehicle, with suitable flavoring agents, intended for oral administration. Some suspensions labeled as "Milks" or "Magmas" fall into this category.

#### Topical Suspensions

Topical Suspensions are liquid preparations containing solid particles dispersed in a liquid vehicle, intended for application to the skin. Some suspensions labeled as "Lotions" fall into this category.

#### Otic Suspensions

Otic Suspensions are liquid preparations containing micronized particles intended for instillation in the outer ear.

#### Ophthalmic Suspensions

See Ophthalmic Preparations.

### SYRUPS



See Oral Solutions.

## SYSTEMS

In recent years, a number of dosage forms have been developed using modern technology that allows for the uniform release or targeting of drugs to the body. These products are commonly called delivery systems. The most widely used of these are Transdermal Systems.

### Transdermal Systems

Transdermal drug delivery systems are self-contained, discrete dosage forms that, when applied to intact skin, are designed to deliver the drug(s) through the skin to the systemic circulation. Systems typically comprise an outer covering (barrier), a drug reservoir, which may have a rate-controlling membrane, a contact adhesive applied to some or all parts of the system and the system/skin interface, and a protective liner that is removed before applying the system. The activity of these systems is defined in terms of the release rate of the drug (s) from the system. The total duration of drug release from the system and the system surface area may also be stated.

Transdermal drug delivery systems work by diffusion: the drug diffuses from the drug reservoir, directly or through the rate-controlling membrane and/or contact adhesive if present, and then through the skin into the general circulation. Typically, modified-release systems are designed to provide drug delivery at a constant rate, such that a true steady-state blood concentration is achieved and maintained until the system is removed. At that time, blood concentration declines at a rate consistent with the pharmacokinetics of the drug.

Transdermal drug delivery systems are applied to body areas consistent with the labeling for the product(s). As long as drug concentration at the system/skin interface remains constant, the amount of drug in the dosage form does not influence plasma concentrations. The functional lifetime of the system is defined by the initial amount of drug in the reservoir and the release rate from the reservoir.

note—Drugs for local rather than systemic effect are commonly applied to the skin embedded in glue on a cloth or plastic backing. These products are defined traditionally as plasters or tapes.

### Ocular System

Another type of system is the ocular system, which is intended for placement in the lower conjunctival fornix from which the drug diffuses through a membrane at a constant rate (e.g., Pilocarpine Ocular System).

### Intrauterine System

An intrauterine system, based on a similar principle but intended for release of drug over a much longer period of time, e.g., one year, is also available (e.g., Progesterone Intrauterine Contraceptive System).

## TABLETS

Tablets are solid dosage forms containing medicinal substances with or without suitable diluents. They may be classed, according to the method of manufacture, as compressed tablets or molded tablets.

The vast majority of all tablets manufactured are made by compression, and compressed tablets are the most widely used dosage form in this country. Compressed tablets are prepared by the application of high pressures, utilizing steel punches and dies, to powders or granulations. Tablets can be produced in a wide variety of sizes, shapes, and surface markings, depending upon the design of the punches and dies. Capsule-shaped tablets are commonly referred to as caplets. Boluses are large tablets intended for veterinary use, usually for large animals.

Molded tablets are prepared by forcing dampened powders under low pressure into die cavities. Solidification depends upon crystal bridges built up during the subsequent drying process, and not upon the compaction force.

Tablet triturates are small, usually cylindrical, molded or compressed tablets. Tablet triturates were traditionally used as dispensing tablets in order to provide a convenient, measured quantity of a potent drug for compounding purposes. Such tablets are rarely used today. Hypodermic tablets are molded tablets made from completely and readily water-soluble ingredients and formerly were intended for use in making preparations for hypodermic injection. They are employed orally, or where rapid drug availability is required such as in the case of Nitroglycerin Tablets, sublingually.

Buccal tablets are intended to be inserted in the buccal pouch, and sublingual tablets are intended to be inserted beneath the tongue, where the active ingredient is absorbed directly through the oral mucosa. Few drugs are readily absorbed in this way, but for those that are (such as nitroglycerin and certain steroid hormones), a number of advantages may result.

Soluble, effervescent tablets are prepared by compression and contain, in addition to active ingredients, mixtures of acids (citric acid, tartaric acid) and sodium bicarbonate, which release carbon dioxide when dissolved in water. They are intended to be dissolved or dispersed in water before administration. Effervescent tablets should be stored in tightly closed containers or moisture-proof packs and labeled to indicate that they are not to be swallowed directly.

### Chewable Tablets

Chewable tablets are formulated and manufactured so that they may be chewed, producing a pleasant tasting residue in the oral cavity that is easily swallowed and does not leave a bitter or unpleasant aftertaste. These tablets have been used in tablet formulations for children, especially multivitamin formulations, and for the administration of antacids and selected antibiotics. Chewable tablets are prepared by compression, usually utilizing mannitol, sorbitol, or sucrose as binders and fillers, and containing colors and flavors to enhance their appearance and taste.

### Preparation of Molded Tablets

Molded tablets are prepared from mixtures of medicinal substances and a diluent usually consisting of lactose and powdered sucrose in varying proportions. The powders are dampened with solutions containing high percentages of alcohol. The concentration of alcohol depends upon the solubility of the active ingredients and fillers in the solvent system and the desired degree of hardness of the finished tablets. The dampened powders are pressed into molds, removed, and allowed to dry. Molded tablets are quite friable and care must be taken in packaging and dispensing.

### Formulation of Compressed Tablets

Most compressed tablets consist of the active ingredient and a diluent (filler), binder, disintegrating agent, and lubricant. Approved FD&C and D&C dyes or lakes (dyes adsorbed onto insoluble aluminum hydroxide), flavors, and sweetening agents may also be present. Diluents are added where the quantity of active ingredient is small or difficult to compress. Common tablet fillers include lactose, starch, dibasic calcium phosphate, and microcrystalline cellulose. Chewable tablets often contain sucrose, mannitol, or sorbitol as a filler. Where the amount of active ingredient is small, the overall tabletting properties are in large measure determined by the filler. Because of problems encountered with bioavailability of hydrophobic drugs of low water-solubility, water-soluble diluents are used as fillers for these tablets.

Binders give adhesiveness to the powder during the preliminary granulation and to the compressed tablet. They add to the cohesive strength already available in the diluent. While binders may be added dry, they are more effective when added out of solution. Common binders include acacia, gelatin, sucrose, povidone, methylcellulose, carboxymethylcellulose, and hydrolyzed starch pastes. The most effective dry binder is microcrystalline cellulose, which is commonly used for this purpose in tablets prepared by direct compression.

A disintegrating agent serves to assist in the fragmentation of the tablet after administration. The most widely used tablet disintegrating agent is starch. Chemically modified starches and cellulose, alginic acid, microcrystalline cellulose, and cross-linked povidone, are also used for this purpose. Effervescent mixtures are used in soluble tablet systems as disintegrating agents. The concentration of the disintegrating agent, method of addition, and degree of compaction play a role in effectiveness.

Lubricants reduce friction during the compression and ejection cycle. In addition, they aid in preventing adherence of tablet material to the dies and punches. Metallic stearates, stearic acid, hydrogenated vegetable oils, and talc are used as lubricants. Because of the nature of this function, most lubricants are hydrophobic, and as such tend to reduce the rates of



...tablet disintegration and dissolution. Consequently, excessive concentrations of lubricant should be avoided. Polyethylene glycols and some lauryl sulfate salts have been used as soluble lubricants, but such agents generally do not possess optimal lubricating properties, and comparatively high concentrations are usually required.

Glidants are agents that improve powder fluidity, and they are commonly employed in direct compression where no granulation step is involved. The most effective glidants are the colloidal pyrogenic silicas.

Colorants are often added to tablet formulations for esthetic value or for product identification. Both D&C and FD&C dyes and lakes are used. Most dyes are photosensitive and they fade when exposed to light. The federal Food and Drug Administration regulates the colorants employed in drugs.

#### Manufacturing Methods

Tablets are prepared by three general methods: wet granulation, dry granulation (roll compaction or slugging), and direct compression. The purpose of both wet and dry granulation is to improve flow of the mixture and/or to enhance its compressibility.

Dry granulation (slugging) involves the compaction of powders at high pressures into large, often poorly formed tablet compacts. These compacts are then milled and screened to form a granulation of the desired particle size. The advantage of dry granulation is the elimination of both heat and moisture in the processing. Dry granulations can be produced also by extruding powders between hydraulically operated rollers to produce thin cakes which are subsequently screened or milled to give the desired granule size.

Excipients are available that allow production of tablets at high speeds without prior granulation steps. These directly compressible excipients consist of special physical forms of substances such as lactose, sucrose, dextrose, or cellulose, which possess the desirable properties of fluidity and compressibility. The most widely used direct-compaction fillers are microcrystalline cellulose, anhydrous lactose, spray-dried lactose, compressible sucrose, and some forms of modified starches. Direct compression avoids many of the problems associated with wet and dry granulations. However, the inherent physical properties of the individual filler materials are highly critical, and minor variations can alter flow and compression characteristics so as to make them unsuitable for direct compression.

Physical evidence of poor tablet quality is discussed under [Stability Considerations in Dispensing Practice \(1191\)](#).

#### weight variation and content uniformity

Tablets are required to meet a weight variation test (see [Uniformity of Dosage Units \(905\)](#)) where the active ingredient comprises a major portion of the tablet and where control of weight may be presumed to be an adequate control of drug content uniformity. Weight variation is not an adequate indication of content uniformity where the drug substance comprises a relatively minor portion of the tablet, or where the tablet is sugar-coated. Thus, the Pharmacopeia generally requires that coated tablets and tablets containing 50 mg or less of active ingredient, comprising less than 50% by weight of the dosage-form unit, pass a content uniformity test (see [Uniformity of Dosage Units \(905\)](#)), wherein individual tablets are assayed for actual drug content.

#### disintegration and dissolution

Disintegration is an essential attribute of tablets intended for administration by mouth, except for those intended to be chewed before being swallowed and for some types of extended-release tablets. A disintegration test is provided (see [Disintegration \(701\)](#)), and limits on the times in which disintegration is to take place, appropriate for the types of tablets concerned, are given in the individual monographs.

For drugs of limited water-solubility, dissolution may be a more meaningful quality attribute than disintegration. A dissolution test (see [Dissolution \(711\)](#)) is required in a number of monographs on tablets. In many cases, it is possible to correlate dissolution rates with biological availability of the active ingredient. However, such tests are useful mainly as a means of screening preliminary formulations and as a routine quality-control procedure.

#### Coatings

Tablets may be coated for a variety of reasons, including protection of the ingredients from air, moisture, or light, masking of unpleasant tastes and odors, improvement of appearance, and control of the site of drug release in the gastrointestinal tract.

#### plain coated tablets

Classically, tablets have been coated with sugar applied from aqueous suspensions containing insoluble powders such as starch, calcium carbonate, talc, or titanium dioxide, suspended by means of acacia or gelatin. For purposes of identification and esthetic value, the outside coatings may be colored. The finished coated tablets are polished by application of dilute solutions of wax in solvents such as chloroform or powdered mix. Water-protective coatings consisting of substances such as shellac or cellulose acetate phthalate are often applied out of nonaqueous solvents prior to application of sugar coats. Excessive quantities should be avoided. Drawbacks of sugar coating include the lengthy time necessary for application, the need for waterproofing, which also adversely affects dissolution, and the increased bulk of the finished tablet. These factors have resulted in increased acceptance of film coatings. Film coatings consist of water-soluble or dispersible materials such as hydroxypropyl methylcellulose, methylcellulose, hydroxypropylcellulose, carboxymethylcellulose sodium, and mixtures of cellulose acetate phthalate and polyethylene glycols applied out of nonaqueous or aqueous solvents. Evaporation of the solvents leaves a thin film that adheres directly to the tablet and allows it to retain the original shape, including grooves or identification codes.

#### delayed-release tablets

Where the drug may be destroyed or inactivated by the gastric juice or where it may irritate the gastric mucosa, the use of "enteric" coatings is indicated. Such coatings are intended to delay the release of the medication until the tablet has passed through the stomach. The term "delayed-release" is used for Pharmacopeial purposes, and the individual monographs include tests and specifications for Drug release (see [Drug Release \(724\)](#)) or Disintegration (see [Disintegration \(701\)](#)).

#### extended-release tablets

Extended-release tablets are formulated in such manner as to make the contained medicament available over an extended period of time following ingestion. Expressions such as "prolonged-action," "repeat-action," and "sustained-release" have also been used to describe such dosage forms. However, the term "extended-release" is used for Pharmacopeial purposes, and requirements for Drug release typically are specified in the individual monographs.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">William E. Brown</a> Senior Scientist 1-301-816-8380	(PDF05) Pharmaceutical Dosage Forms 05

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#### 1160 PHARMACEUTICAL CALCULATIONS IN PRESCRIPTION COMPOUNDING

#### INTRODUCTION

The purpose of this chapter is to provide general information to guide and assist pharmacists in performing the necessary calculations when preparing or compounding any pharmaceutical article (see [Pharmaceutical Compounding—Nonsterile Preparations \(795\)](#), [Pharmaceutical Compounding—Sterile Preparations \(797\)](#), and [Good Compounding Practices \(1075\)](#)) or when simply dispensing prescriptions (see [Stability Considerations in Dispensing Practice \(1191\)](#)).

Correct pharmaceutical calculations can be accomplished by using, for example, proper conversions from one measurement system to another and properly placed decimal points, by

Understanding the arithmetical concepts, and by paying close attention to the details of the calculations. Before proceeding with any calculation, pharmacists should do the following: (a) read the entire formula or prescription carefully; (b) determine which materials are needed; and then (c) select the appropriate methods of preparation and the appropriate calculation.

There are often several ways to solve a given problem. Logical methods that require as few steps as possible should be selected in order to ensure that calculations are done correctly. The best approach is the one that yields results that are accurate and free of error. The pharmacist must double-check each calculation before proceeding with the preparation of the article or prescription order. One way of double-checking is by estimation. This involves rounding off the quantities involved in the calculation, and comparing the estimated result with the calculated value.

Finally, the following steps should be taken: the dosage of each active ingredient in the prescription should be checked; all calculations should be doubly checked, preferably by another pharmacist; and where instruments are used in compounding, they should be carefully checked to ascertain that they will function properly. See USP general chapters [Aerosols, Nasal Sprays, Metered-Dose Inhalers, and Dry Powder Inhalers](#) (601), [Deliverable Volume](#) (698), [Density of Solids](#) (699), [Osmolality and Osmolarity](#) (785), [pH](#) (791), [Pharmaceutical Compounding—Nonsterile Preparations](#) (795), [Pharmaceutical Compounding—Sterile Preparations](#) (797), [Viscosity](#) (911), [Specific Gravity](#) (841), [Cleaning Glass Apparatus](#) (1051), [Medicine Dropper](#) (1101), [Prescription Balances and Volumetric Apparatus](#) (1176), [Teaspoon](#) (1221), [Weighing on an Analytical Balance](#) (1251), and [Good Compounding Practices](#) (1075) for information on specific instruments.

#### BASIC MATHEMATICAL CONCEPTS

##### significant figures

Expressed values are considered significant to the last digit shown (see Significant Figures and Tolerances in the General Notices). Significant figures are digits with practical meaning. The accuracy of the determination is implied by the number of figures used in its expression. In some calculations zeros may not be significant. For example, for a measured weight of 0.0298 g, the zeros are not significant; they are used merely to locate the decimal point. In the example, 2980 g, the zero may also be used to indicate the decimal point, in which case the zero is not significant. Alternately, however, the zero may indicate that the weight is closer to 2981 g or 2979 g, in which case the zero is significant. In such a case, knowledge of the method of measurement would be required in order to indicate whether the zero is or is not significant. In the case of a volume measurement of 298 mL, all of the digits are significant. In a given result, the last significant figure written is approximate but all preceding figures are accurate. For example, a volume of 29.8 mL implies that 8 is approximate. The true volume falls between 29.75 and 29.85. Thus, 29.8 mL is accurate to the nearest 0.1 mL, which means that the measurement has been made within  $\pm 0.05$  mL. Likewise, a value of 298 mL is accurate to the nearest 1 mL and implies a measurement falling between 297.5 and 298.5, which means that the measurement has been made within  $\pm 0.5$  mL and is subject to a maximum error calculated as follows:

$$\frac{0.5\text{mL}}{298\text{mL}} \times 100\% = 0.17\%$$

A zero in a quantity such as 298.0 mL is a significant figure and implies that the measurement has been made within the limits of 297.95 and 298.05 with a possible error calculated as follows:

$$\frac{0.05\text{mL}}{298.0\text{mL}} \times 100\% = 0.017\%$$

##### examples—

1.  $29.8\text{ mL} = 29.8 \pm 0.05\text{ mL}$  (accurate to the nearest 0.1 mL)
2.  $29.80\text{ mL} = 29.80 \pm 0.005\text{ mL}$  (accurate to the nearest 0.01 mL)
3.  $29.800\text{ mL} = 29.800 \pm 0.0005\text{ mL}$  (accurate to the nearest 0.001 mL)

The degree of accuracy in the last example is greatest. Thus, the number of significant figures provides an estimate both of true value and of accuracy.

##### examples of significant figures—

Measurement	Number of Significant
	Figures
2.98	3
2.980	4
0.0298	3
0.0029	2

Calculations— All figures should be retained until the calculations have been completed. Only the appropriate number of significant figures, however, should be retained in the final result.

##### Determining the number of significant figures—

Sums and Differences— When adding or subtracting, the number of decimal places in the result shall be the same as the number of decimal places in the component with the fewest decimal places.

##### example—

$$11.5 + 11.65 + 9.90 = 33.1$$

Products and Quotients— When multiplying or dividing, the result shall have no more significant figures than the measurement with the smallest number of significant figures entering into the calculation.

##### example—

$$4.266 \times 21 = 90$$

Rounding Off— For rules on rounding off measurements or calculated results, see Interpretation of Requirements under Significant Figures and Tolerances in the General Notices. Note, however, that in the example above, if 21 is an absolute number (e.g., the number of doses), then the answer, 89.586, is rounded off to 89.59 which has 4 significant figures.

##### logarithms

The logarithm of a number is the exponent or the power to which a given base must be raised in order to equal that number.

##### Definitions—

$$\text{pH} = -\log [\text{H}^+], \text{ and}$$



$$pK_a = -\log K_a$$

$pH = -\log [H^+]$ , and  $pK_a = -\log K_a$ , where  $[H^+]$  is the hydrogen ion concentration in an aqueous solution and  $K_a$  is the ionization constant of the acid in an aqueous solution. The  $[H^+] =$  the antilogarithm of  $(-\text{pH})$ , and the  $K_a =$  the antilogarithm of  $(-\text{p}K_a)$ .

The pH of an aqueous solution containing a weak acid may be calculated using the Henderson-Hasselbalch equation:

$$pH = pK_a + \log [salt]/[acid]$$

example—

A solution contains 0.020 moles per L of sodium acetate and 0.010 mole per L of acetic acid, which has a  $pK_a$  value of 4.76. Calculate the pH and the  $[H^+]$  of the solution. Substituting into the above equation,  $pH = 4.76 + \log (0.020/0.010) = 5.06$ , and the  $[H^+] =$  antilogarithm of  $(-5.06) = 8.69 \times 10^{-6}$ .

#### BASIC PHARMACEUTICAL CALCULATIONS

The remainder of this chapter will focus on basic pharmaceutical calculations. It is important to recognize the rules involved when adding, subtracting, dividing, and multiplying values. The interrelationships between various units within the different weighing and measuring systems are also important and have to be understood.

calculations in compounding

The pharmacist must be able to calculate the amount or concentration of drug substances in each unit or dosage portion of a compounded preparation at the time it is dispensed. Pharmacists must perform calculations and measurements to obtain, theoretically, 100% of the amount of each ingredient in compounded formulations. Calculations must account for the active ingredient, or active moiety, and water content of drug substances, which includes that in the chemical formulas of hydrates. Official drug substances and added substances must meet the requirements under [Loss on Drying \(731\)](#), which must be included in the calculations of amounts and concentrations of ingredients. The pharmacist should consider the effect of ambient humidity on the gain or loss of water from drugs and added substances in containers subjected to intermittent opening over prolonged storage. Each container should be opened for the shortest duration necessary and then closed tightly immediately after use.

The nature of the drug substance that is to be weighed and used in compounding a prescription must be known exactly. If the substance is a hydrate, its anhydrous equivalent weight may need to be calculated. On the other hand, if there is adsorbed moisture present that is either specified on a certificate of analysis or that is determined in the pharmacy immediately before the drug substance is used by the procedure under [Loss on Drying \(731\)](#), this information must be used when calculating the amount of drug substance that is to be weighed in order to determine the exact amount of anhydrous drug substance required.

There are cases in which the required amount of a dose is specified in terms of a cation [e.g.,  $Li^+$ , netilmicin ( $n^+$ )], an anion [e.g.,  $F^-$ ], or a molecule (e.g., theophylline in aminophylline). In these instances, the drug substance weighed is a salt or complex, a portion of which represents the pharmacologically active moiety. Thus, the exact amount of such substances weighed must be calculated on the basis of the required quantity of the pharmacological moiety.

The following formula may be used to calculate the exact theoretical weight of an ingredient in a compounded preparation:

$$W = ab/de$$

in which  $W$  is the actual weighed amount;  $a$  is the prescribed or pharmacist-determined weight of the active or functional moiety of drug or added substance;  $b$  is the chemical formula weight of the ingredient, including waters of hydration for hydrous ingredients;  $d$  is the fraction of dry weight when the percent by weight of adsorbed moisture content is known from the loss on drying procedure (see [Loss on Drying \(731\)](#)); and  $e$  is the formula weight of the active or functional moiety of a drug or added substance that is provided in the formula weight of the weighed ingredient.

Example 1: Triturate Morphine Sulfate USP and Lactose NF to obtain 10 g in which there are 30 mg of Morphine Sulfate USP for each 200 mg of the morphine-lactose mixture. [note—Clinical dosages of morphine mean Morphine Sulfate USP, which is the pentahydrate.]

Equation Factor	Numerical Value
$W$	weight, in g, of Morphine Sulfate USP
$a$	1.5 g of morphine sulfate pentahydrate in the prescription
$b$	759 g/mole
$d$	1.0
$e$	759 g/mole

$$W = \frac{1.5\text{g}(759\text{g/mole})}{1.0(759\text{g/mole})} = 1.5\text{g}$$

Example 2: Accurately weigh an amount of Aminophylline USP to obtain 250 mg of anhydrous theophylline. [note—The powdered aminophylline dihydrate weighed contains 0.4% w/w adsorbed moisture as stated in the Certificate of Analysis.]

Equation Factor	Numerical Value
$W$	weight, in mg, of Aminophylline USP (dihydrate)
$a$	250 mg of theophylline
$b$	456 g/mole
$d$	0.996
$e$	360 g/mole

$$W = \frac{250\text{mg}(456\text{g/mole})}{0.996(360\text{g/mole})} = 318\text{mg}$$

Example 3: Accurately weigh an amount of Lithium Citrate USP (containing 2.5% moisture as stated in the Certificate of Analysis) to obtain 200 mEq of lithium ( $Li^+$ ). [note—One mEq of  $Li^+$  is equivalent to 0.00694 g of  $Li^+$ .]

Equation Factor	Numerical Value
$W$	weight, in g, of Lithium Citrate USP (tetrahydrate)
$a$	200 mEq of $Li^+$ or 1.39 g of $Li^+$
$b$	282 g/mole
$d$	0.975
$e$	3 $\times$ 6.94 g/mole or 20.8 g/mole



$$W = \frac{1.39 \text{ g}(282 \text{ g/mole})}{0.975(20.8 \text{ g/mole})} = 19.3 \text{ g}$$

Example 4: Accurately weigh an amount of Netilmicin Sulfate USP, equivalent to 2.5 g of netilmicin. [note—Using the procedure under [Loss on Drying \(731\)](#), the Netilmicin Sulfate USP that was weighed lost 12% of its weight.]

Equation Factor	Numerical Value
W	weight, in g, of Netilmicin Sulfate USP
a	2.5 g
b	1442 g/mole
d	0.88
e	951 g/mole

$$W = \frac{2.5 \text{ g}(1442 \text{ g/mole})}{0.88(951 \text{ g/mole})} = 4.31 \text{ g}$$

#### buffer solutions

Definition— A buffer solution is an aqueous solution that resists a change in pH when small quantities of acid or base are added, when diluted with the solvent, or when the temperature changes. Most buffer solutions are mixtures of a weak acid and one of its salts or mixtures of a weak base and one of its salts. Water and solutions of a neutral salt such as sodium chloride have very little ability to resist the change of pH and are not capable of effective buffer action.

Preparation, Use, and Storage of Buffer Solutions— Buffer solutions for Pharmacopeial tests should be prepared using freshly boiled and cooled water (see Standard Buffer Solutions under Buffer Solutions in [Reagents, Indicators, and Solutions](#)). They should be stored in containers such as Type I glass bottles and used within 3 months of preparation.

Buffers used in physiological systems are carefully chosen so as not to interfere with the pharmacological activity of the medicament or the normal function of the organism. Commonly used buffers in parenteral products, for example, are acetic, citric, glutamic, and phosphoric acids and their salts. Buffer solutions should be freshly prepared.

The Henderson-Hasselbalch equation, noted above, allows the pH of a buffer solution of a weak acid and its salt to be calculated. Appropriately modified, this equation may be applied to buffer solutions composed of a weak base and its salt.

Buffer Capacity— The buffer capacity of a solution is the measurement of the ability of that solution to resist a change in pH upon addition of small quantities of a strong acid or base. An aqueous solution has a buffer capacity of 1 when 1 L of the buffer solution requires 1 gram equivalent of strong acid or base to change the pH by 1 unit. Therefore, the smaller the pH change upon the addition of a specified amount of acid or base, the greater the buffer capacity of the buffer solution. Usually, in analysis, much smaller volumes of buffer are used in order to determine the buffer capacity. An approximate formula for calculating the buffer capacity is gram equivalents of strong acid or base added per L of buffer solution per unit of pH change, i.e.,  $(\text{Eq/L})/(\text{pH change})$ .

#### example—

The addition of 0.01 g equivalents of sodium hydroxide to 0.25 L of a buffer solution produced a pH change of 0.50. The buffer capacity of the buffer solution is calculated as follows:

$$(0.01/0.25)/0.50 = 0.08(\text{Eq/L})/(\text{pH change})$$

#### dosage calculations

Special Dosage Regimens— Geriatric and pediatric patients require special consideration when designing dosage regimens. In geriatric patients, the organs are often not functioning efficiently as a result of age-related pharmacokinetic changes or disease. For these patients, modifications in dosing regimens are available in references such as USP Drug Information.

For pediatric patients, where organs are often not fully developed and functioning, careful consideration must be applied during dosing. Modifications in dosing regimens for pediatric patients are also available in references such as USP Drug Information. General rules for calculating doses for infants and children are available in pharmacy calculation textbooks. These rules are not drug-specific and should be used only in the absence of more complete information.

The usual method for calculating a dose for children is to use the information provided for children for the specific drug. The dose is frequently expressed as mg of drug per kg of body weight for a 24-hour period, and is then usually given in divided portions.

The calculation may be made using the following equation:

$$(\text{mg of drug per kg of body weight}) \times (\text{kg of body weight}) = \text{dose for an individual for a 24-hour period}$$

A less frequently used method of calculating the dose is based on the surface area of the individual's body. The dose is expressed as amount of drug per body surface area in m<sup>2</sup>, as shown in the equation below:

$$(\text{amount of drug per m}^2 \text{ of body surface area}) \times (\text{body surface area in m}^2) = \text{dose for an individual for a 24-hour period}$$

The body surface area (BSA) may be determined from nomograms relating height and weight in dosage handbooks. The BSA for adult and pediatric patients may also be determined using the following equations:

$$\text{BSA (m}^2\text{)} = \text{square root of } \{[\text{Height (in)} \times \text{Weight (lb)}] / 3131\}$$

or

$$\text{BSA (m}^2\text{)} = \text{square root of } \{[\text{Height (cm)} \times \text{Weight (kg)}] / 3600\}$$

#### example—

Rx for Spironolactone Suspension 25 mg/tsp. Sig: 9 mg BID for an 18 month-old child who weighs 22 lbs.

The USP DI 2002, 22nd ed., states that the normal pediatric dosing regimen for Spironolactone is 1 to 3 mg per kg per day. In this case, the weight of the child is 22 lbs, which equals  $22 \text{ lbs} / (2.2 \text{ lbs/kg}) = 10 \text{ kg}$ . Therefore the normal dose for this child is 10 to 30 mg per day and the dose ordered is 18 mg per day as a single dose or divided into 2 to 4 doses. The dose is acceptable based on published dosing guidelines.

#### percentage concentrations

Percentage concentrations of solutions are usually expressed in one of three common forms:

$$\text{Volume percent (v/v)} = \frac{\text{Volume of solute}}{\text{Volume of solution}} \times 100\%$$



$$\text{Weight percent (w/w)} = \frac{\text{Weight of solute} \times 100\%}{\text{Weight of solution}}$$
$$\text{Weight in volume percent (w/v)} = \frac{\text{Weight of solute (in g)}}{\text{Volume of solution (in mL)}} \times 100\%$$

See also Percentage Measurements under Concentrations in the General Notices. The above three equations may be used to calculate any one of the three values (i.e., weights, volumes, or percentages) in a given equation if the other two values are known.

Note that weights are always additive, i.e., 50 g plus 25 g = 75 g. Volumes of two different solvents or volumes of solvent plus a solid solute are not strictly additive. Thus 50 mL of water + 50 mL of pure alcohol do not produce a volume of 100 mL. Nevertheless, it is assumed that in some pharmaceutical calculations, volumes are additive, as discussed below under Reconstitution of Drugs Using Volumes Other than Those on the Label.

examples—

1. Calculate the percentage concentrations (w/w) of the constituents of the solution prepared by dissolving 2.50 g of phenol in 10.00 g of glycerin. Using the weight percent equation above, the calculation is as follows.

$$\text{Total weight of the solution} = 10.00 \text{ g} + 2.50 \text{ g} = 12.50 \text{ g}$$

$$\text{Weight percent of phenol} = (2.50 \text{ g} \times 100\%) / 12.50 \text{ g} = 20.0\% \text{ of phenol}$$

$$\text{Weight percent of glycerin} = (10.00 \text{ g} \times 100\%) / 12.50 \text{ g} = 80.0\% \text{ of glycerin}$$

2. A prescription order reads as follows:

Eucalyptus Oil 3% (v/v) in Mineral Oil.

Dispense 30.0 mL.

What quantities should be used for this prescription? Using the volume percent equation above, the calculation is as follows.

Amount of Eucalyptus Oil:

$$3\% = (\text{Volume of oil in mL} / 30.0 \text{ mL}) \times 100\%$$

Solving the equation, the volume of oil = 0.90 mL

Amount of Mineral Oil:

To 0.90 mL of Eucalyptus Oil add sufficient Mineral Oil to prepare 30.0 mL.

3. A prescription order reads as follows:

Zinc oxide	7.5 g
Calamine	7.5 g
Starch	15 g
White petrolatum	30 g

Calculate the percentage concentration for each of the four components. Using the weight percent equation above, the calculation is as follows.

$$\text{Total weight} = 7.5 \text{ g} + 7.5 \text{ g} + 15 \text{ g} + 30 \text{ g} = 60.0 \text{ g}$$

$$\text{Weight percent of zinc oxide} = (7.5 \text{ g zinc oxide} / 60.0 \text{ g ointment}) \times 100\% = 12.5\%$$

$$\text{Weight percent of calamine} = (7.5 \text{ g calamine} / 60.0 \text{ g ointment}) \times 100\% = 12.5\%$$

$$\text{Weight percent of starch} = (15 \text{ g starch} / 60.0 \text{ g ointment}) \times 100\% = 25\%$$

$$\text{Weight percent of white petrolatum} = (30 \text{ g white petrolatum} / 60.0 \text{ g ointment}) \times 100\% = 50\%$$

specific gravity

The definition of specific gravity is usually based on the ratio of weight of a substance in air at 25° to that of the weight of an equal volume of water at the same temperature. The weight of 1 mL of water at 25° is approximately 1 g. The following equation may be used for calculations.

$$\text{Specific Gravity} = (\text{Weight of the substance}) / (\text{Weight of an equal volume of water})$$

examples—

1. A liquid weighs 125 g and has a volume of 110 mL. What is the specific gravity?

The weight of an equal volume of water is 110 g.

Using the above equation, specific gravity = 125 g / 110 g = 1.14.

2. Hydrochloric Acid NF is approximately a 37% (w/w) solution of hydrochloric acid (HCl) in water. How many grams of HCl are contained in 75.0 mL of HCl NF? (Specific gravity of Hydrochloric Acid NF is 1.18.)

Calculate the weight of HCl NF using the above equation.

The weight of an equal volume of water is 75 g.

$$\text{Specific Gravity} 1.18 = \text{weight of the HCl NF g} / 75.0 \text{ g}$$

Solving the equation, the weight of HCl NF is 88.5 g.

Now calculate the weight of HCl using the weight percent equation.

$$37.0\% \text{ (w/w)} = (\text{weight of solute g} / 88.5 \text{ g}) \times 100$$

Solving the equation, the weight of the HCl is 32.7 g.

dilution and concentration

A concentrated solution can be diluted. Powders and other solid mixtures can be triturated or diluted to yield less concentrated forms. Because the amount of solute in the diluted solution or mixture is the same as the amount in the concentrated solution or mixture, the following relationship applies to dilution problems.

The quantity of Solution 1 (Q1) × concentration of Solution 1 (C1) = the quantity of Solution 2 (Q2) × concentration of Solution 2 (C2), or



(Q1)(C1) = (Q2)(C2)

Almost any quantity and concentration terms may be used. However, the units of the terms must be the same on both sides of the equation.

examples—

1. Calculate the quantity (Q2), in g, of diluent that must be added to 60 g of a 10% (w/w) ointment to make a 5% (w/w) ointment.

Let (Q1) = 60 g, (C1) = 10%, and (C2) = 5%.

Using the above equation,

$$60 \text{ g} \times 10\% = (Q2) \times 5\% \text{ (w/w)}$$

Solving the above equation, the quantity of product needed, Q2, is 120 g. The initial quantity of product added was 60 g, and therefore an additional 60 g of diluent must be added to the initial quantity to give a total of 120 g.

2. How much diluent should be added to 10 g of a trituration (1 in 100) to make a mixture that contains 1 mg of drug in each 10 g of final mixture?

Determine the final concentration by first converting mg to g. One mg of drug in 10 g of mixture is the same as 0.001 g in 10 g.

Let (Q1) = 10 g, (C1) = (1 in 100), and (C2) = (0.001 in 10).

Using the equation for dilution,  $10 \text{ g} \times (1/100) = (Q2) \text{ g} \times (0.001/10)$ .

Solving the above equation, (Q2) = 1000 g.

Because 10 g of the final mixture contains all of the drug and some diluent, (1000 g - 10 g) or 990 g of diluent is required to prepare the mixture at a concentration of 0.001 g of drug in 10 g of final mixture.

3. Calculate the percentage strength of a solution obtained by diluting 400 mL of a 5.0% solution to 800 mL.

Let (Q1) = 400 mL, (C1) = 5%, and (Q2) = 800 mL.

Using the equation for dilution,  $400 \text{ mL} \times 5\% = 800 \text{ mL} \times (C2)\%$ .

Solving the above equation, (C2) = 2.5% (w/v).

use of potency units

See Units of Potency in the General Notices.

Because some substances may not be able to be defined by chemical and physical means, it may be necessary to express quantities of activity in biological units of potency.

examples—

1. One mg of Pancreatin contains not less than 25 USP Units of amylase activity, 2.0 USP Units of lipase activity, and 25 USP Units of protease activity. If the patient takes 0.1 g (100 mg) per day, what is the daily amylase activity ingested?

1 mg of Pancreatin corresponds to 25 USP Units of amylase activity.

100 mg of Pancreatin corresponds to  $100 \times (25 \text{ USP Units of amylase activity}) = 2500 \text{ Units}$ .

2. A dose of penicillin G benzathine for streptococcal infection is 1.2 million units intramuscularly. If a specific product contains 1180 units per mg, how many milligrams would be in the dose?

1180 units of penicillin G benzathine are contained in 1 mg.

1 unit is contained in  $1/1180 \text{ mg}$ .

$1,200,000 \text{ units are contained in } (1,200,000 \times 1)/1180 \text{ units} = 1017 \text{ mg}$ .

base vs salt or ester forms of drugs

Frequently, for stability or other reasons such as taste or solubility, the base form of a drug is administered in an altered form such as an ester or salt. This altered form of the drug usually has a different molecular weight (MW), and at times it may be useful to determine the amount of the base form of the drug in the altered form.

examples—

1. Four hundred milligrams of erythromycin ethylsuccinate (molecular weight, 862.1) is administered. Determine the amount of erythromycin (molecular weight, 733.9) in this dose.

862.1 g of erythromycin ethylsuccinate corresponds to 733.9 g of erythromycin.

1 g of erythromycin ethylsuccinate corresponds to  $(733.9/862.1) \text{ g}$  of erythromycin.

0.400 g of erythromycin ethylsuccinate corresponds to  $(733.9/862.1) \times 0.400 \text{ g}$  or 0.3405 g of erythromycin.

2. The molecular weight of testosterone cypionate is 412.6 and that of testosterone is 288.4. What is the dose of testosterone cypionate that would be equivalent to 60.0 mg of testosterone?

288.4 g of testosterone corresponds to 412.6 g of testosterone cypionate.

1 g of testosterone corresponds to  $412.6/288.4 \text{ g}$  of testosterone cypionate.

60.0 mg or 0.0600 g of testosterone corresponds to  $(412.6/288.4) \times 0.0600 = 0.0858 \text{ g}$  or 85.8 mg of testosterone cypionate.

reconstitution of drugs using volumes other than those on the label

Occasionally it may be necessary to reconstitute a powder in order to provide a suitable drug concentration in the final product. This may be accomplished by estimating the volume of the powder and liquid medium required.

examples—

1. If the volume of 250 mg of ceftriaxone sodium is 0.1 mL, how much diluent should be added to 500 mg of ceftriaxone sodium powder to make a suspension having a concentration of 250 mg per mL?



$$500 \text{ mg} \times \frac{1 \text{ mL}}{250 \text{ mg}} = 2 \text{ mL}$$

Volume of 500 mg of ceftriaxone sodium =

$$500 \text{ mg} \times \frac{0.1 \text{ mL}}{250 \text{ mg}} = 0.2 \text{ mL}$$

Volume of the diluent required = (2 mL of suspension) - (0.2 mL of Ceftriaxone Sodium) = 1.8 mL.

2. What is the volume of dry powder cefonicid, if 2.50 mL of diluent is added to 1 g of powder to make a solution having a concentration of 325 mg per mL?

Volume of solution containing 1 g of the powder =

$$1 \text{ g of cefonicid} \times \frac{1000 \text{ mg}}{1 \text{ g}} \times \frac{1 \text{ mL of solution}}{325 \text{ mg of cefonicid}} = 3.08 \text{ mL}$$

Volume of dry powder cefonicid = 3.08 mL of solution - 2.50 mL of diluent = 0.58 mL.

#### alligation alternate and algebra

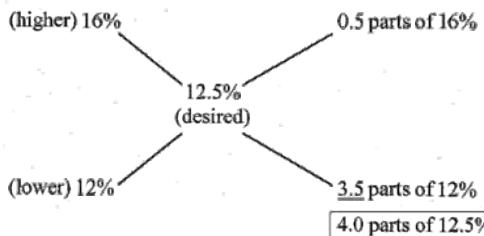
Alligation— Alligation is a rapid method of determining the proportions in which substances of different strengths are mixed to yield a desired strength or concentration. Once the proportion is found, the calculation may be performed to find the exact amounts of substances required. Set up the problem as follows.

1. Place the desired percentage or concentration in the center.
2. Place the percentage of the substance with the lower strength on the lower left-hand side.
3. Place the percentage of the substance with the higher strength on the upper left-hand side.
4. Subtract the desired percentage from the lower percentage, and place the obtained difference on the upper right-hand side.
5. Subtract the higher percentage from the desired percentage, and place the obtained difference on the lower right-hand side.

The results obtained will determine how many parts of the two different percentage strengths should be mixed to produce the desired percentage strength of a drug mixture.

examples—

1. How much ointment having a 12% drug concentration and how much ointment having a 16% drug concentration must be used to make 1 kg of a preparation containing a 12.5% drug concentration?



In a total of 4.0 parts of 12.5% product, 3.5 parts of 12% ointment and 0.5 parts of 16% ointment are needed.

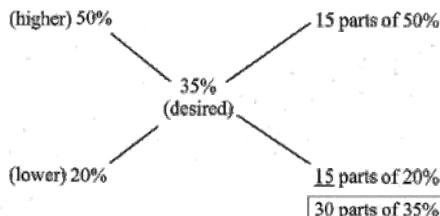
4 parts correspond to 1 kg or 1000 g.

1 part corresponds to 250 g.

3.5 parts correspond to  $3.5 \times 250$  g or 875 g.

0.5 parts correspond to  $0.5 \times 250$  g or 125 g.

2. How many mL of 20% dextrose in water and 50% dextrose in water are needed to make 750 mL of 35% dextrose in water?



In a total of 30 parts of 35% dextrose in water, 15 parts of 50% dextrose in water and 15 parts of 20% dextrose in water are required.

30 parts correspond to 750 mL.

15 parts correspond to 375 mL.

Thus use 375 mL of the 20% solution and 375 mL of the 50% solution to prepare the product.

Algebra— Instead of using alligation to solve the above problems, algebra may be used, following the scheme outlined below.

In order to represent the total quantity (weights, parts, or volumes) of the final mixture or solution, 1 or a specified quantity is used.

Let  $x$  be the quantity of one portion and  $[1 (or the specified amount) - x]$  be the remaining portion. Set up the equation according to the statement below, and solve.

The amount of drug in one part plus the amount of drug in the other part equals the total amount in the final mixture or solution.

examples—



1. How much ointment having a 12% drug concentration and how much ointment having a 16% drug concentration must be used to make 1 kg of a preparation containing 12.5% drug concentration?

Let 1 kg be the total quantity of ointment to be prepared, let  $x$  be the quantity, in kg, of the 12% ointment, and let  $(1 - x)$  be the quantity in kg of the 16% ointment. The equation is as follows:

$$(12/100)x + (16/100)(1 - x) = (12.5/100)(1)$$

Solving the equation,  $x$  equals 0.875 kg of the 12% ointment and  $(1 - x)$  equals  $(1 - 0.875)$  or 0.125 kg of the 16% ointment.

2. How many mL of 20% dextrose in water and 50% dextrose in water are needed to make 750 mL of 35% dextrose in water?

Let  $x$  be the volume, in mL, of the 20% solution, and let  $(750 - x)$  be the volume in mL of the 50% solution. The equation is as follows:

$$(20/100)x + (50/100)(750 - x) = (35/100)(750)$$

Solving the equation,  $x$  equals 375 mL of the 20% solution and  $(750 - x)$  equals  $(750 - 375)$  or 375 mL of the 50% solution.

molar, molal, and normal concentrations

See Concentrations in the General Notices.

Molarity— The molar concentration,  $M$ , of the solution is the number of moles of the solute contained in one L of solution.

Molality— The molal concentration,  $m$ , is the number of moles of the solute contained in one kilogram of solvent.

Normality— The normal concentration,  $N$ , of a solution expresses the number of milliequivalents (mEq) of solute contained in 1 mL of solution or the number of equivalents (Eq, gram-equivalent weight) of solute contained in 1 L of solution. When using normality, the pharmacist must apply quantitative chemical analysis principles using molecular weight (MW). Normality depends on the reaction capacity of a chemical compound and therefore the reaction capacity must be known. For acids and bases, reaction capacity is the number of accessible protons available from, or the number of proton binding sites available on, each molecular aggregate. For electron transfer reactions, reaction capacity is the number of electrons gained or lost per molecular aggregate.

examples—

1. How much sodium bicarbonate powder is needed to prepare 50.0 mL of a 0.07 N solution of sodium bicarbonate ( $\text{NaHCO}_3$ )? (MW of  $\text{NaHCO}_3$  is 84.0 g per mol.)

In an acid or base reaction, because  $\text{NaHCO}_3$  may act as an acid by giving up one proton, or as a base by accepting one proton, one Eq of  $\text{NaHCO}_3$  is contained in each mole of  $\text{NaHCO}_3$ . Thus the equivalent weight of  $\text{NaHCO}_3$  is 84 g. [note—The volume, in L,  $\times$  normality of a solution equals the number of equivalents in the solution.]

The number of equivalents of  $\text{NaHCO}_3$  required =  $(0.07 \text{ Eq/L})(50.0 \text{ mL}/1000 \text{ mL/L}) = 0.0035 \text{ equivalents}$ .

1 equivalent weight is 84.0 g.

0.0035 equivalents equals  $84.0 \text{ g/Eq} \times 0.0035 \text{ Eq} = 0.294 \text{ g}$ .

2. A prescription calls for 250 mL of a 0.1 N hydrochloric acid (HCl) solution. How many mL of concentrated hydrochloric acid are needed to make this solution? [note—The specific gravity of concentrated hydrochloric acid is 1.18, the molecular weight is 36.46 and the concentration is 37.5% (w/w). Because hydrochloric acid functions as an acid and reacts by giving up one proton in a chemical reaction, 1 Eq is contained in each mole of the compound. Thus the equivalent weight is 36.46 g.]

The number of equivalents of HCl required is  $0.250 \text{ L} \times 0.1 \text{ N} = 0.025 \text{ equivalents}$ .

1 equivalent is 36.46 g.

0.025 equivalents correspond to  $0.025 \text{ Eq} \times 36.46 \text{ g/Eq} = 0.9115 \text{ g}$ .

37.5 g of pure HCl are contained in 100 g of concentrated HCl.

Thus 1 g of pure HCl is contained in  $(100/37.5) \text{ g} = 2.666 \text{ g}$  of concentrated acid, and  $0.9115 \text{ g}$  is contained in  $(0.9115 \times 2.666) \text{ g} = 2.43 \text{ g}$  of concentrated acid.

In order to determine the volume of the supplied acid required, use the definition for specific gravity as shown below.

Specific gravity = (weight of the substance)/(weight of an equal volume of water).

$1.18 = 2.43 \text{ g}/(\text{weight of an equal volume of water})$ .

The weight of an equal volume of water is 2.056 g or 2.06 g, which measures 2.06 mL. Thus, 2.06 mL of concentrated acid is required.

milliequivalents and millimoles

note—This section addresses milliequivalents (mEq) and millimoles (mmol) as they apply to electrolytes for dosage calculations.

The quantities of electrolytes administered to patients are usually expressed in terms of mEq. This term must not be confused with a similar term used in quantitative chemical analysis as discussed above. Weight units such as mg or g are not often used for electrolytes because the electrical properties of ions are best expressed as mEq. An equivalent is the weight of a substance (equivalent weight) that supplies one unit of charge. An equivalent weight is the weight, in g, of an atom or radical divided by the valence of the atom or radical. A milliequivalent is one-thousandth of an equivalent (Eq). Because the ionization of phosphate depends on several factors, the concentration is usually expressed in millimoles, moles, or milliosmoles, which are described below. [note—Equivalent weight (Eq.wt) = wt. of an atom or radical (ion) in g/valence (or charge) of the atom or radical. Milliequivalent weight (mEq.wt) = Eq.wt. (g)/1000.]

examples—

1. Potassium ( $\text{K}^+$ ) has a gram-atomic weight of 39.10. The valence of  $\text{K}^+$  is 1+. Calculate its milliequivalent weight (mEq wt).

$$\text{Eq wt} = 39.10 \text{ g}/1 = 39.10 \text{ g}$$

$$\text{mEq wt} = 39.10 \text{ g}/1000 = 0.03910 \text{ g} = 39.10 \text{ mg}$$

2. Calcium ( $\text{Ca}^{2+}$ ) has a gram-atomic weight of 40.08. Calculate its milliequivalent weight (mEq wt).

$$\text{Eq wt} = 40.08 \text{ g}/2 = 20.04 \text{ g}$$

$$\text{mEq wt} = 20.04 \text{ g}/1000 = 0.02004 \text{ g} = 20.04 \text{ mg}$$

note—The equivalent weight of a compound may be determined by dividing the molecular weight in g by the product of the valence of either relevant ion and the number of times this ion occurs in one molecule of the compound.

3. How many milliequivalents of potassium ion ( $\text{K}^+$ ) are there in a 250-mg Penicillin V Potassium Tablet? [note—Molecular weight of penicillin V potassium is 388.48 g per mol; there is one potassium atom in the molecule; and the valence of  $\text{K}^+$  is 1.]

$$\text{Eq wt} = 388.48 \text{ g}/[1(\text{valence}) \times 1(\text{number of charges})] = 388.48 \text{ g}$$

$$\text{mEq wt} = 388.48 \text{ g}/1000 = 0.38848 \text{ g} = 388.48 \text{ mg}$$



(250 mg per Tablet)/(388.48 mg per mEq) = 0.644 mEq of K+ per Tablet.

4. How many equivalents of magnesium ion and sulfate ion are contained in 2 mL of a 50% Magnesium Sulfate Injection? (Molecular weight of MgSO4·7H2O is 246.48 g per mol.)

Amount of magnesium sulfate in 2 mL of 50% Magnesium Sulfate Injection

$$2\text{mL of Injection} \times \frac{50\text{g of magnesiumsulfate}}{100\text{mL of Injection}} = 1\text{g}$$

Eq wt of MgSO4·7H2O = MW (g)/(valence of specified ion × number of specified ions in one mole of salt).

For the magnesium ion:

The number of equivalents is calculated as follows:

$$246.48/[2(valence) \times 1 (\text{number of ions in the compound})] = 123.24 \text{ g/Eq of magnesium ion.}$$

$$\text{The number of equivalents in 1 g is } 1\text{g} / 123.24 \text{ g/Eq} = 0.008114 \text{ Eq.}$$

The number of mEq may be calculated as follows:

$$\text{The mEq wt} = \text{Eq wt (g)} / 1000 = (123.24 \text{ g/Eq}) / 1000 = 0.12324 \text{ g.}$$

The number of milliequivalents of magnesium ion in 1 g is 1g/0.12324 g/mEq = 8.114 mEq.

For the sulfate ion:

The number of equivalents is calculated as follows:

$$246.48/[2(valence) \times 1 (\text{number of ions in the compound})] = 123.24 \text{ g/Eq of sulfate ion.}$$

$$\text{The number of equivalents in 1 g is } 1\text{g} / 123.24 \text{ g/Eq} = 0.008114 \text{ Eq.}$$

The number of mEq may be calculated as follows:

$$\text{The mEq wt} = \text{Eq wt (g)} / 1000 = (123.24 \text{ g/Eq}) / 1000 = 0.12324 \text{ g.}$$

The number of milliequivalents of sulfate ion in 1 g is 1g/0.12324 g/mEq = 8.114 mEq.

5. A vial of Sodium Chloride Injection contains 3 mEq of sodium chloride per mL. What is the percentage strength of this solution? (Molecular weight of sodium chloride is 58.44 g per mol.)

$$1 \text{ mEq} = 1 \text{ Eq}/1000 = 58.44 \text{ g}/1000 = 0.05844 \text{ g} = 58.44 \text{ mg.}$$

Amount of sodium chloride in 3 mEq per mL = 58.44 mg per mEq × 3 mEq per mL = 175.32 mg per mL.

$$\frac{175.32 \text{ mg}}{1 \text{ mL}} = \frac{17532 \text{ mg}}{100 \text{ mL}} = \frac{17.532 \text{ g}}{100 \text{ mL}} = 17.5\%$$

Using mols and mmols—

A number of countries have adopted the International System of Units and no longer calculate doses using mEq as described above, but instead use the terms moles (mol) and millimoles (mmol). In USP-NF or in the Pharmacists' Pharmacopeia the International System of Units is used except for the labeling of electrolytes.

Definitions—

A mole equals one gram atomic weight or gram molecular weight of a substance.

A millimole equals 1/1000 of a mole.

examples—

1. Potassium (K) has a gram-atomic weight of 39.10. Calculate its weight in millimoles (mmol).

The weight of one mole is 39.10 g and the weight in millimoles is 39.10 g/1000 = 0.0391 g or 39.1 mg.

2. How many millimoles of Penicillin V are in a tablet that contains 250 mg of Penicillin V Potassium? (Molecular weight of penicillin V potassium is 388.48 g per mol.)

The weight of one mole is 388.48 and the weight in millimoles is 388.48/1000 = 0.3848 g or 388.48 mg. Thus there are 250 mg/388.48 mg/mmol = 0.644 mmol of Penicillin V ion per tablet.

isoosmotic solutions

The following discussion and calculations have therapeutic implications in preparations of dosage forms intended for ophthalmic, subcutaneous, intravenous, intrathecal, and neonatal use.

Cells of the body, such as erythrocytes, will neither swell nor shrink when placed in a solution that is isotonic with the body fluids. However, the measurement of tonicity, a physiological property, is somewhat difficult. It is found that a 0.9% (w/v) solution of sodium chloride, which has a freezing point of  $-0.52^{\circ}\text{C}$ , is isotonic with body fluids and is said to be isoosmotic with body fluids. In contrast to isotonicity, the freezing point depression is a physical property. Thus many solutions that are isoosmotic with body fluids are not necessarily isotonic with body fluids, e.g., a solution of urea. Nevertheless many pharmaceutical products are prepared using freezing point data or related sodium chloride data to prepare solutions that are

isoosmotic with the body fluids. A closely related topic is osmolarity (see [Osmolality and Osmolarity \(785\)](#)).

Freezing point data or sodium chloride equivalents of pharmaceuticals and excipients (see [Table 1](#) below) may be used to prepare isoosmotic solutions, as shown in the examples below.

Table 1. Sodium Chloride Equivalents (E) and Freezing Point (FP) Depressions for a 1% Solution of the Drug or Excipient

Drug or Excipient	E	FP Depression
Atropine sulfate	0.13	0.075
Sodium chloride	1.00	0.576

example—

Determine the amount of sodium chloride required to prepare 60 mL of an isoosmotic solution of atropine sulfate 0.5% using the sodium chloride equivalent values and also the freezing point depression values.

**Using the sodium chloride equivalent values—**

The total amount of substances equivalent to sodium chloride (for a 0.9% solution) =  $(0.9 \text{ g}/100 \text{ mL}) \times 60 \text{ mL} = 0.54 \text{ g}$ .

The amount of atropine sulfate required =  $(0.5 \text{ g}/100 \text{ mL}) \times 60 \text{ mL} = 0.3 \text{ g}$ .

1 g of atropine sulfate is equivalent to 0.13 g of sodium chloride.

0.3 g atropine sulfate is equivalent to  $0.3 \times 0.13 \text{ g} = 0.039 \text{ g}$  of sodium chloride.

Thus the required amount of sodium chloride is  $0.54 - 0.039 = 0.501 \text{ g}$  or 0.50 g.

**Using freezing point depression values—**

The freezing point depression required is  $0.52^\circ$ .

A 1% solution of atropine sulfate causes a freezing point depression of  $0.075^\circ$ .

A 0.5% solution of atropine sulfate causes a freezing point depression of  $0.075^\circ \times 0.5 = 0.0375^\circ$ .

The additional freezing point depression required is  $0.52^\circ - 0.0375^\circ = 0.482^\circ$ .

A 1% solution of sodium chloride causes a freezing point depression of  $0.576^\circ$ .

A (1% / 0.576) solution of sodium chloride causes a freezing point depression of  $1^\circ$ .

A  $(1\% / 0.576) \times 0.482 = 0.836\%$  solution of sodium chloride causes a freezing point depression of  $0.482^\circ$ .

The required amount of sodium chloride is  $(0.836 \text{ g}/100 \text{ mL}) \times 60 \text{ mL} = 0.502 \text{ g}$  or 0.50 g.

**flow rates in intravenous sets**

Some calculations concerning flow rates in intravenous sets are provided below. [note—Examples below are not to be used for treatment purposes.]

**examples—**

1. Sodium Heparin 8,000 units in 250 mL Sodium Chloride Injection 0.9% solution are to be infused over 4 hours. The administration set delivers 20 drops per mL.

What is the flow rate in mL per hour?

In 4 hours, 250 mL are to be delivered.

In 1 hour,  $250 \text{ mL}/4 = 62.5 \text{ mL}$  are delivered.

What is the flow rate in drops per minute?

In 60 minutes, 62.5 mL are delivered.

In 1 minute,  $62.5 \text{ mL}/60 = 1.04 \text{ mL}$  are delivered.

1 mL = 20 drops.

$1.04 \text{ mL} = 1.04 \times 20 \text{ drops} = 20.8 \text{ drops}$ .

Thus in 1 minute, 20.8 or 21 drops are administered.

2. A 14.5 kg patient is to receive 50 mg of Sodium Nitroprusside in 250 mL of dextrose 5% in water (D5W) at the rate of 1.3 µg per kg per minute. The set delivers 50 drops per mL.

Calculate the flow rate in mL per hour.

The dose for 1 kg is 1.3 µg per minute.

The 14.5 kg patient should receive  $14.5 \times 1.3 \text{ µg} = 18.85 \text{ µg}$  per minute.

50 mg or 50,000 µg of drug are contained in 250 mL of D5W.

$18.85 \text{ µg}$  are contained in  $250 \text{ mL} \times 18.85/50,000 = 0.09425 \text{ mL}$  D5W, which is administered every minute.

In 1 minute, 0.09425 mL are administered.

In 1 hour or 60 minutes,  $60 \times 0.09425 \text{ mL} = 5.655$  or 5.7 mL are administered.

Calculate the flow rate in drops per minute.

1 mL corresponds to 50 drops per minute.

$0.09425 \text{ mL}$  corresponds to  $0.09425 \times 50 = 4.712$  or 4.7 drops per minute.

**temperature**

The relationship between Celsius degrees ( $^\circ\text{C}$ ) and Fahrenheit degrees ( $^\circ\text{F}$ ) is expressed by the following equation:

$$9(\text{ }^\circ\text{C}) = 5(\text{ }^\circ\text{F}) - 160$$

in which  $^\circ\text{C}$  and  $^\circ\text{F}$  are the numbers of Celsius degrees and Fahrenheit degrees, respectively.

**examples—**

1. Convert 77  $^\circ\text{F}$  to Celsius degrees.

$$9(\text{ }^\circ\text{C}) = 5(\text{ }^\circ\text{F}) - 160$$

$$^\circ\text{C} = [5(\text{ }^\circ\text{F}) - 160]/9 = [(5 \times 77) - 160]/9 = 25 \text{ }^\circ\text{C}$$

2. Convert 30  $^\circ\text{C}$  to Fahrenheit degrees.

$$9(\text{ }^\circ\text{C}) = 5(\text{ }^\circ\text{F}) - 160$$

$$^\circ\text{F} = [9(\text{ }^\circ\text{C}) + 160]/5 = [(9 \times 30) + 160]/5 = 86 \text{ }^\circ\text{F}$$

The relationship between the Kelvin and the Celsius scales is expressed by the equation:



$$K = {}^\circ C + 273.1$$

in which K and  ${}^\circ C$  are the numbers of Kelvin degrees and Celsius degrees, respectively.

application of mean kinetic temperature

See [Pharmaceutical Stability](#) (1150) for the definition of mean kinetic temperature (MKT). MKT is usually higher than the arithmetic mean temperature and is derived from the Arrhenius equation. MKT addresses temperature fluctuations during the storage period of the product. The mean kinetic temperature,  $T_K$ , is calculated by the following equation:

$$T_K = \frac{\frac{-\Delta H}{R}}{\ln\left(\frac{e^{-\Delta H/RT_1} + e^{-\Delta H/RT_2} + \dots + e^{-\Delta H/RT_n}}{n}\right)}$$

in which  $\Delta H$  is the heat of activation, which equals 83.144 kJ per mol (unless more accurate information is available from experimental studies); R is the universal gas constant, which equals  $8.3144 \times 10^{-3}$  kJ per degree per mol;  $T_1$  is the average temperature, in degrees Kelvin, during the first time period, e.g., the first week;  $T_2$  is the average temperature, in degrees Kelvin, during the second time period, e.g., second week; and  $T_n$  is the average temperature, in degrees Kelvin during the nth time period, e.g., nth week, n being the total number of temperatures recorded. The mean kinetic temperature is calculated from average storage temperatures recorded over a one-year period, with a minimum of twelve equally spaced average storage temperature observations being recorded (see [Pharmaceutical Stability](#) (1150)). This calculation can be performed manually with a pocket calculator or electronically with computer software.

examples—

1. The means of the highest and lowest temperatures for 52 weeks are  $25 {}^\circ C$  each. Calculate the MKT.

$$n = 52$$

$$\Delta H/R = 10,000 \text{ K}$$

$$T_1, T_2, \dots, T_n = 25 {}^\circ C = 273.1 + 25 = 298.1 \text{ K}$$

$$R = 0.0083144 \text{ kJ K}^{-1}\text{mol}^{-1}$$

$$\Delta H = 83.144 \text{ kJ per mol}$$

$$T_K = \frac{\frac{-\Delta H}{R}}{\ln\left(\frac{e^{-\Delta H/RT_1} + e^{-\Delta H/RT_2} + \dots + e^{-\Delta H/RT_n}}{n}\right)}$$

$$= \frac{-10,000 \text{ K}}{\ln\left(\frac{52 \times e^{-\Delta H/R \times 298.1}}{52}\right)}$$

$$= \frac{-10,000 \text{ K}}{\ln\left(\frac{52 \times e^{-33.5158}}{52}\right)}$$

$$= \frac{-10,000 \text{ K}}{-33.5458} = 298.1 \text{ K} = 25.0 {}^\circ C$$

The calculated MKT is  $25.0 {}^\circ C$ . Therefore the controlled room temperature requirement is met by this pharmacy. [note—If the averages of the highest and lowest weekly temperatures differed from each other and were in the allowed range of  $15 {}^\circ C$  to  $30 {}^\circ C$  (see Controlled Room Temperature under Preservation, Packaging, Storage, and Labeling in the General Notices), then each average would be substituted individually into the equation. The remaining two examples illustrate such calculations, except that the monthly averages are used.]

2. A pharmacy recorded a yearly MKT on a monthly basis, starting in January and ending in December. Each month, the pharmacy recorded the monthly highest temperature and the monthly lowest temperature, and the average of the two was calculated and recorded for the MKT calculation at the end of the year (see [Table 2](#)). From these data the MKT may be estimated or it may be calculated. If more than half of the observed temperatures are lower than  $25 {}^\circ C$  and a mean lower than  $23 {}^\circ C$  is obtained, the MKT may be estimated without performing the actual calculation.

Table 2. Data for Calculation of MKT

n	Month	Lowest Temperature (in ${}^\circ C$ )	Highest Temperature (in ${}^\circ C$ )	Average Temperature (in ${}^\circ C$ )	Average Temperature (in K)	$\Delta H/RT$	$e^{-\Delta H/RT}$
1	Jan.	15	27	21	294.1	34.002	$1.710 \times 10^{-15}$
2	Feb.	20	25	22.5	295.6	33.830	$2.033 \times 10^{-15}$
3	Mar.	17	25	21	294.1	34.002	$1.710 \times 10^{-15}$
4	Apr.	20	25	22.5	295.6	33.830	$2.033 \times 10^{-15}$



5	May	22	27	24.5	297.6	33.602	$2.551 \times 10^{-15}$
6	June	15	25	20	293.1	34.118	$1.523 \times 10^{-15}$
7	July	20	26	23	296.1	33.772	$2.152 \times 10^{-15}$
8	Aug.	22	26	24	297.1	33.659	$2.411 \times 10^{-15}$
9	Sept.	23	27	25	298.1	33.546	$2.699 \times 10^{-15}$
10	Oct.	20	28	24	297.1	33.659	$2.411 \times 10^{-15}$
11	Nov.	20	24	22	295.1	33.887	$1.919 \times 10^{-15}$
12	Dec.	22	28	25	298.1	33.546	$2.699 \times 10^{-15}$

- a. To estimate the MKT, the recorded temperatures are evaluated and the average is calculated. In this case, the calculated arithmetic mean is  $22.9^{\circ}\text{C}$ . Therefore, the above requirements are met and it can be concluded that the mean kinetic temperature is lower than  $25^{\circ}\text{C}$ . Therefore, the controlled room temperature requirement is met.

- b. The second approach is to perform the actual calculation.

$$n = 12$$

$$T_K = \frac{\frac{-\Delta H}{R}}{\ln\left(\frac{e^{-\Delta H/RT_1} + e^{-\Delta H/RT_2} + \dots + e^{-\Delta H/RT_{12}}}{12}\right)}$$

$$= \frac{-10,000\text{K}}{\ln\left(\frac{1.710 \times 10^{-15} + 2.033 \times 10^{-15} + 1.710 \times 10^{-15} + \dots + 2.699 \times 10^{-15}}{12}\right)}$$

$$= \frac{-10,000\text{K}}{\ln\left(\frac{2.585 \times 10^{-14}}{12}\right)}$$

$$= \frac{-10,000\text{K}}{-33.771} = 296.11\text{K} = 23.0^{\circ}\text{C}$$

The calculated MKT is  $23.0^{\circ}\text{C}$ , so the controlled room temperature requirement is met. [note—These data and calculations are used only as an example.]

3. An article was stored for one year in a pharmacy where the observed monthly average of the highest and lowest temperatures was  $25^{\circ}\text{C}$  (298.1 K), except for one month with an average of  $28^{\circ}\text{C}$  (301.1 K). Calculate the MKT of the pharmacy.

$$n = 12$$

$$T_K = \frac{\frac{-\Delta H}{R}}{\ln\left(\frac{e^{-\Delta H/RT_1} + e^{-\Delta H/RT_2} + \dots + e^{-\Delta H/RT_{12}}}{12}\right)}$$

$$= \frac{\frac{-\Delta H}{R}}{\ln\left(\frac{11 \times e^{-\Delta H/(R \times 298.1)} + 1 \times e^{-\Delta H/(R \times 301.1)}}{12}\right)}$$

$$= \frac{-10,000\text{K}}{\ln\left(\frac{11 \times e^{-33.546} + 1 \times e^{-33.212}}{12}\right)}$$

$$= \frac{-10,000\text{K}}{\ln\left(\frac{2.9692 \times 10^{-14} + 3.7705 \times 10^{-15}}{12}\right)}$$

$$= \frac{-10,000\text{K}}{\ln\left(\frac{3.3463 \times 10^{-14}}{12}\right)}$$

$$= \frac{-10,000\text{K}}{\ln(2.7886 \times 10^{-15})}$$
$$= \frac{-10,000\text{K}}{-33.513} = 298.39\text{K} = 25.29^\circ\text{C}$$

The controlled room temperature requirement is not met because the calculated MKT exceeds  $25^\circ\text{C}$ . (See Note in Example 2 above.)

4. Using the same calculation technique for controlled room temperature, the MKT for controlled cold temperatures can also be calculated.
- a. For example, if the mean of the highest and lowest temperatures for each week over a period of 52 weeks was  $8^\circ\text{C}$  (i.e., the same mean for each week), then the MKT can be calculated as follows:

$$T_K = \frac{-10,000}{\ln[52e^{-\Delta H/R(281.1)}]/52}$$
$$T_K = \frac{-10,000}{\ln[e^{-\Delta H/R(281.1)}]}$$
$$T_K = \frac{-10,000}{\ln[e^{-35.575}]}$$
$$= \frac{-10,000}{\ln[3.548 \times 10^{-16}]}$$
$$= \frac{-10,000}{-35.575}$$

$$T_K = 281.1\text{K}$$

$$C = 281.1 - 273.1$$

$$C = 8^\circ$$

- b. In another example, where a variety of average temperatures are used, as would be the case in reality, if the average of the highest and lowest temperatures ranges from  $0^\circ$  to  $15^\circ\text{C}$ , then these averages would be individually substituted into the equation. For simplification of the mathematical process, 10 intervals are shown in [Table 3](#) below. This illustration is intended for calculation of MKT at storage or in transit; i.e., during shipping or distribution of the critical drug product. These calculations can be performed manually or with a computer.

Table 3. Sample Data for MKT Calculations

Intervals	Low Temperature (in $^\circ\text{C}$ )	High Temperature (in $^\circ\text{C}$ )	Average Temperature (in $^\circ\text{C}$ )	Average Temperature (in K)	$\Delta H/RT$	$e^{-\Delta H/RT} \times 1016$
1	0	5	2.5	275.6	36.284	1.746



2	2	8	5	278.1	35.958	2.419
3	3	9	6	279.1	35.829	2.752
4	3	14	8.5	281.6	35.511	3.782
5	7	15	11	284.0	35.211	5.106
6	1	6	3.5	276.6	36.153	1.990
7	5	15	10	283.1	35.323	4.565
8	2	14	8	281.1	35.575	3.548
9	2	6	4	277.1	36.088	2.124
10	3	10	6.5	279.6	35.765	2.934

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Rick G. Schnatz</a> Manager, Compounding Pharmacy Expert Committee 1-301-816-8526	(CRX05) Compounding Pharmacy05

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## 1163 QUALITY ASSURANCE IN PHARMACEUTICAL COMPOUNDING

### INTRODUCTION

A quality assurance program is a system of steps and actions taken to ensure the maintenance of proper standards in compounded preparations. The need for a quality assurance system is well documented in USP chapters (see Compounding Controls under [Good Compounding Practices](#) (1075); Quality Control and Verification under [Pharmaceutical Compounding—Nonsterile Preparations](#) (795); and The Quality Assurance Program under [Pharmaceutical Compounding—Sterile Preparations](#) (797)). A quality assurance program for compounding should include at least four separate but integrated components: (1) Standard Operating Procedures, (2) Documentation, (3) Verification, and (4) Testing.

### STANDARD OPERATING PROCEDURES

Standard Operating Procedures (SOPs) for pharmaceutical compounding are documents that describe how to perform routine and expected tasks in the compounding environment, including formulation development, purchasing, compounding, testing, maintenance, training, materials handling and storage, quality assurance, labeling, beyond-use dating, cleaning, safety, and dispensing. SOPs are itemized instructions that describe how a task will be performed, who will do it, why it is done, any limits, and what to do if a deviation occurs.

SOPs must be reviewed regularly and updated as necessary. The SOP should be specific to each device, process, and decision used in compounding. Properly maintained and implemented SOPs should result in quality preparations and fewer compounding errors.

### DOCUMENTATION

The purpose of the documentation is to provide a permanent record of all aspects of each compounding operation. Two essential compounding documents, the formulation record and the compounding record, are described in [Pharmaceutical Compounding—Nonsterile Preparations](#) (795). The compounding record is completed and reviewed for accuracy during the compounding process for the preparation being made.

In addition, many SOPs require specific cross-referenced data collection forms (e.g., air temperature and humidity records and balance maintenance and calibration records). Data collection forms required by SOPs are completed during routine tasks directed by the SOPs and may provide fill-in-the-blank spaces for data, including logbook entries, data printouts, and reports.

### VERIFICATION

Verification involves assurance and documentation that a process, procedure, or piece of equipment is functioning properly and producing the expected results. [Pharmaceutical Compounding—Nonsterile Preparations](#) (795) states: "The act of verification of a compounding procedure involves checking to ensure that calculations, weighing and measuring, order of mixing, and compounding techniques were appropriate and accurately performed." Verification may require outside laboratory testing when in-house capabilities are not adequate. Equipment verification methods are sometimes available from manufacturers of the specific equipment or can be developed in-house. The responsibility for assuring that equipment performance is verified, including work completed by contractors, resides with the compounder.

### TESTING OF FINISHED COMPOUNDED PREPARATIONS

A quality assurance program should include testing of finished compounded preparations. It is important for the compounder to have a basic understanding of pharmaceutical analysis to ensure that valid results are obtained when tests are being conducted, whether they are done in-house or outsourced. While it is not practical to test every compounded preparation, it is incumbent on the compounder to know (1) the importance of testing in the overall quality program in the compounding facility, (2) when to test, (3) what to test, (4) what method(s) to use, (5) how to interpret the results, (6) the limits of the test, and (7) what to do if the preparations listed do not meet specifications. Investigative and corrective action should extend to other preparations that may have been associated with the specific failure or discrepancy.

The goal in testing is to produce results as accurately, efficiently, and quickly as possible. Any testing method used should have accuracy, speed, reproducibility, and specificity. No single testing method is suited for all drugs. There are a number of factors that determine the validity and reliability of results.

Compounding facilities have two options when testing is required. Some testing methods can easily be performed in-house, but some may need to be outsourced to a contract laboratory. Relatively basic testing methods that can be conducted in-house with proper training and a modest investment in instrumentation include weight and volumetric measurements, pH, density/specific gravity, refractive index, and UV and visible spectroscopy (see [Weights and Balances](#) (41), [Volumetric Apparatus](#) (31), [Prescription Balances and Volumetric Apparatus](#) (1176), [pH](#) (791), [Specific Gravity](#) (841), [Refractive Index](#) (831), and [Spectrophotometry and Light-Scattering](#) (851)). Testing methods often outsourced to a contract laboratory include chromatography (high-pressure liquid chromatography (HPLC) and gas chromatography (GC), see [Chromatography](#) (621)), mass spectroscopy (MS) (see [Mass Spectrometry](#) (736)), hyphenated methods (HPLC-MS and GC-MS), UV and visible spectroscopy (see [Spectrophotometry and Light-Scattering](#) (851)), and other sophisticated methods.

If testing is done in-house, appropriate equipment must be obtained, verified either by the manufacturer or by the compounder upon purchase, maintained, calibrated, and used properly. If testing is outsourced, the compounder needs to determine what to outsource and how to select a laboratory, and should develop ongoing relationships with the laboratories chosen. Contract laboratories should follow USP general chapter standards, as appropriate.

**Selection of a Testing Method**— One general consideration in testing method selection is the type of information that is needed, such as quantitative (potency, concentration), semiquantitative (where a tolerance level is involved, as in endotoxin levels), or qualitative (presence/absence type of testing, including substance identification, sterility). Another



Consideration involves the physical and chemical characteristics of the analyte, including solubility, partition coefficient, dissociation constant (pKa), volatility, binding, and the quantity present.

The degree of quantitative measurement and specificity must be considered in the validation process. The typical analytical characteristics used in method validation include accuracy,

precision, specificity, detection limit, quantitation limit, linearity, range, and ruggedness (see [Validation of Compendial Procedures](#) 1225). Generally, the greater the level of accuracy, precision, or specificity required, the more sophisticated and expensive the testing methods needed. The methods used are also governed by the types of instrumentation available and the standards available for comparison.

Pharmaceutical analysis decisions include not only method selection but also administrative and economic factors, obtaining a representative sample, storage/shipping of the sample, sample preparation for analysis, the actual analysis, data acquisition, data treatment, and interpretation.

Factors Involved in Method Selection— The testing method selected depends upon a number of factors, including sample requirements, sample handling/preparation/purification requirements, type of data needed, and levels of specificity and accuracy required.

Sampling Requirements— Prior to collecting samples for testing, the following factors should be considered: the number of samples needed, appropriate methods of obtaining representative samples, the physical state of the samples (solid, liquid, or gas), the type of container required for collection and storage, and possible shipping requirements or restrictions. Storage requirements for samples must be specified, such as type of container, temperature, humidity, and light protection (see General Notices and Requirements).

The effect of any substances in the formulation that may interfere or alter the results must be known beforehand. When sending a preparation to a contract laboratory, the compounder should provide the complete formulation so the laboratory can quickly determine if there may be any interfering substances.

Controlled drug substances, dangerous or hazardous chemicals, flammable or caustic substances, and refrigerated or frozen preparations require special handling during shipping.

Data Interpretation Requirements— The collection of raw data from the testing process must be completed accurately. One must ensure that appropriate and valid descriptive statistics are used to analyze the data, and that the operating parameters of the analytical instruments are well established. Reference values, if available, should be provided with the analytical results. A description of the analytical controls used by the laboratory is important for documentation, as well as the source of reference standards used to establish standard curves.

Personnel Requirements and Considerations— If testing is done in-house, personnel involved in this activity must be appropriately trained and evaluated with documentation of the training and evaluation. If testing is outsourced, the compounder must be assured of the credentials, proper training, and continuing competency activities of the personnel in the contract laboratory. It is preferable that the contract laboratory be registered with the Food and Drug Administration (FDA). Also, it may be advantageous if the contract laboratory performs testing for pharmaceutical companies.

Testing Methods— Testing methods can be generally divided into physical testing methods, methods that interact with electromagnetic radiation, conductometric techniques, immunoassay methods, separation techniques, and others.

#### Classification of Analytical Methods

##### Physical Testing Procedures

- Weight
- Volumetric
- Melting point
- Freezing point
- Boiling point
- Density
- Refractive index
- Optical rotation (Polarimetry)
- Thermal analysis
- Color change
- Precipitate formation
- Viscosity change
- Particle size
- Light scattering
- Zeta potential
- Light obscuration
- Microscopic examination

##### Interaction of Electromagnetic Radiation

- Ultraviolet/Visible spectroscopy
- Infrared spectroscopy
- Fluorescence/Phosphorescence spectroscopy
- Raman spectroscopy
- X-ray spectroscopy
- Flame emission and Atomic absorption spectroscopy

##### Polarimetry

- Refractometry
- Interferometry

##### Conductance Methods

- pH
- Ion selective electrodes
- Polarography

##### Immunoassay

- Radioimmunoassay
- Enzyme Multiplied Immunoassay Technique (EMIT)
- Enzyme Linked ImmunoSorbent Assay (ELISA)
- Fluorescent Immunoassay (FIA)

##### Separation Techniques

- High Performance Liquid Chromatography (HPLC)
- Gas Chromatography (GC)
- Thin-Layer Chromatography (TLC)
- Paper Chromatography (PC)



Column Chromatography (CC)  
 Others  
 Osmolality  
 Microbiological Methods  
 Sterility Testing  
 Microbial Limit Testing  
 Endotoxin Testing  
 Preservative Effectiveness Testing

Nonspecific methods include melting, freezing and boiling points, density, refractive index, UV/visible spectroscopy, and pH. Methods that are somewhat more specific include IR spectroscopy, mass spectroscopy, ion selective electrodes, immunoassay methods, and chromatographic methods (HPLC and GC). Suggested testing methods for different dosage forms are shown in [Table 1](#). It is the responsibility of the compounder to implement a program using selected methods for the preparations compounded in the facility.

Table 1. Suggested Analytical Methods for Various Dosage Forms, Depending Upon the Active Drug

Dosage Form	Analytical Method												
	Wt	Vol	pH	Osm	RI	Sp Gr	MP	UV/Vis	HPLC	GC	IR	Sterile	Endo-toxin
Bulk substances	—	—	+	—	+	—	+	+	+	+	+	—	—
Powders	+	—	—	—	—	—	—	—	+	+	—	—	—
Capsules	+	—	—	—	—	—	—	—	+	+	—	—	—
Tablets	+	—	—	—	—	—	—	—	+	+	—	—	—
Lozenges	+	—	—	—	—	—	—	—	+	+	—	—	—
Suppositories	+	—	—	—	—	+	+	—	+	+	—	—	—
Sticks	+	—	—	—	—	+	+	—	+	+	—	—	—
Solutions	+	+	+	+	+	+	—	+	+	+	—	—	—
Suspensions	+	+	+	—	—	+	—	—	+	+	—	—	—
Emulsions	+	+	+	—	—	+	—	—	+	+	—	—	—
Semisolids	+	—	—	—	—	+	+	—	+	+	—	—	—
Gels	+	+	+	—	+	+	—	—	+	+	—	—	—
Ophthalmics	+	+	+	+	+	+	—	+	+	+	—	+	—
Otics	+	+	+	+	+	+	—	+	+	+	—	—	—
Nasals	+	+	+	+	+	+	—	+	+	+	—	◊	—
Irrigations	+	+	+	+	+	+	—	+	+	+	—	+	+
Inhalations	+	+	+	+	+	+	—	+	+	+	—	+	+
Injections	+	+	+	+	+	+	—	+	+	+	—	+	+
Sterile implant gels	+	+	+	+	+	+	—	+	+	+	—	+	+
Sterile implant solids	+	+	—	—	—	—	+	+	+	+	—	+	+

+ test required; — test not required

◊ Microbial limits—(see [Microbial Enumeration Tests](#) (61) and [Tests for Specified Microorganisms](#) (62))

Methods that can be routinely used for testing incoming bulk materials, whether active or excipients, include melting, freezing and boiling points, density, refractive index, UV/Visible spectroscopy, IR spectroscopy, polarimetry, pH, and the separation methods. Final preparations may generally require a method such as HPLC or GC.

#### MICROBIOLOGICAL TESTING

Microbiological testing for pharmacy compounding includes sterility, endotoxin, and microbial limit testing. Preservative effectiveness may also be considered.

**Sterility Testing**— Sterility tests can be conducted using commercial kits or by developing and validating USP sterility testing protocols, which are more detailed than the commercial sterility-testing kits. Standards and procedures are explained in [Sterility Tests](#) (71).

**Endotoxin Testing**— Endotoxin tests can be conducted using commercially available kits or by purchasing the components separately. Endotoxin testing endpoints can be difficult to interpret and in-house testing should only be done after obtaining training and experience. See [Bacterial Endotoxins Test](#) (85).

**Microbial Limit Testing**— Microbial limit testing can be conducted to provide an estimate of the number of viable aerobic microorganisms and for freedom from designated microbial species. See [Microbial Enumeration Tests](#) (61) and [Tests for Specified Microorganisms](#) (62).

#### SUMMARY

A sound quality assurance program is important in a compounding pharmacy. It includes detailed SOPs, documentation, verification, and analytical and microbiological testing as appropriate. Analytical and microbiological testing will no doubt become a more important part of pharmaceutical compounding as the public and regulatory agencies demand more documentation on the quality of compounded preparations. Compounders must decide on the types of testing and degree of testing that will be a part of their quality assurance program. A decision must also be made on whether to do testing in-house or outsource it to a contract laboratory. It may be practical for larger compounding facilities to have their own analytical and/or microbiological testing laboratory, analytical chemist, or microbiologist to provide rapid turnaround of testing results.

Analytical and microbiological testing should only be performed by those who are trained and experienced, and who can demonstrate validated performance of their operations.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Rick G. Schnatz</a> Manager, Compounding Pharmacy Expert Committee 1-301-816-8526	(CRX05) Compounding Pharmacy05



Phase-solubility analysis is the quantitative determination of the purity of a substance through the application of precise solubility measurements. At a given temperature, a definite amount of a pure substance is soluble in a definite quantity of solvent. The resulting solution is saturated with respect to the particular substance, but the solution remains unsaturated with respect to other substances, even though such substances may be closely related in chemical structure and physical properties to the particular substance being tested. Constancy of solubility, like constancy of melting temperature or other physical properties, indicates that a material is pure or is free from foreign admixture except in the unique case in which the percentage composition of the substance under test is in direct ratio to solubilities of the respective components. Conversely, variability of solubility indicates the presence of an impurity or impurities.

Phase-solubility analysis is applicable to all species of compounds that are crystalline solids and that form stable solutions. It is not readily applicable to compounds that form solid solutions with impurities.

The standard solubility method consists of six distinct steps: (1) mixing, in a series of separate systems, increasing quantities of material with measured, fixed amounts of a solvent; (2) establishment of equilibrium for each system at identical constant temperature and pressure; (3) separation of the solid phase from the solutions; (4) determination of the concentration of the material dissolved in the various solutions; (5) plotting the concentration of the dissolved materials per unit of solvent (y-axis or solution composition) against the weight of material per unit of solvent (x-axis or system composition); and (6) extrapolation and calculation.

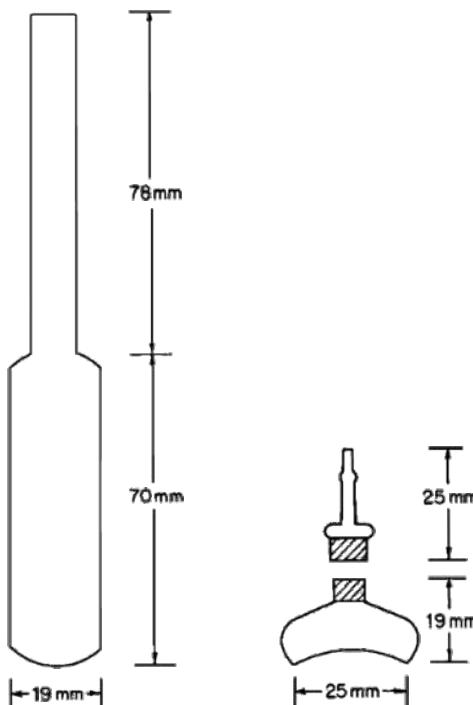
#### Solvents

A proper solvent for phase-solubility analysis meets the following criteria: (1) The solvent is of sufficient volatility that it can be evaporated under vacuum, but is not so volatile that difficulty is experienced in transferring and weighing the solvent and its solutions. Normally, solvents having boiling points between 60° and 150° are suitable. (2) The solvent does not adversely affect the substance being tested. Solvents that cause decomposition or react with the test substance are not to be used. Solvents that solvate or form salts are to be avoided, if possible. (3) The solvent is of known purity and composition. Carefully prepared mixed solvents are permissible. Trace impurities may affect solubility greatly. (4) A solubility of 10 mg to 20 mg per g is optimal, but a wider working range can be used.

#### Apparatus<sup>\*</sup>

Constant-Temperature Bath— Use a constant-temperature bath that is capable of maintaining the temperature within  $\pm 0.1^{\circ}$  and that is equipped with a horizontal shaft capable of rotating at approximately 25 rpm. The shaft is equipped with clamps to hold the Ampuls. Alternatively, the bath may contain a suitable vibrator, capable of agitating the ampuls at 100 to 120 vibrations per second, and equipped with a shaft and suitable clamps to hold the ampuls.

Ampuls— Use 15-mL ampuls of the type shown in the [accompanying illustration](#). Other containers may be used provided that they are leakproof and otherwise suitable.



Ampul (left) and Solubility Flask (right) Used in Phase-Solubility Analysis

Solubility Flasks— Use solubility flasks of the type shown in the [accompanying illustration](#).

#### Procedure

note—Make all weighings within  $\pm 10 \mu\text{g}$ .

System Composition— Weigh accurately, in g, not less than 7 scrupulously cleaned 15-mL ampuls. Weigh accurately, in g, increasingly larger amounts of the test substance into each of the ampuls. The weight of the test substance is selected so that the first ampul contains slightly less material than will go into solution in 5 mL of the selected solvent, the second ampul contains slightly more material, and each subsequent ampul contains increasingly more material than meets the indicated solubility. Transfer 5.0 mL of the solvent to each of the ampuls, cool in a dry ice-acetone mixture, and seal, using a double-jet air-gas burner and taking care to save all glass. Allow the ampuls and their contents to come to room temperature, and weigh the individual sealed ampuls with the corresponding glass fragments. Calculate the system composition, in mg per g, for each ampul by the formula:

$$1000(W_2 - W_1) / (W_3 - W_2)$$

in which  $W_2$  is the weight of the ampul plus test substance,  $W_1$  is the weight of the empty ampul, and  $W_3$  is the weight of ampul plus test substance, solvent, and separated glass.

Equilibration— The time required for equilibration varies with the substance, the method of mixing (rotation or vibration), and the temperature. Normally, equilibrium is obtained more rapidly by the vibration method (1 to 7 days) than by the rotational method (7 to 14 days). In order to determine whether equilibration has been effected, 1 ampul, i.e., the next to the last in the series, may be warmed to 40° to produce a supersaturated solution. Equilibration is ensured if the solubility obtained on the supersaturated solution falls in line with the test specimens that approach equilibrium from an undersaturated solution.

Solution Composition— After equilibration, place the ampuls vertically in a rack in the constant-temperature bath, with the necks of the ampuls above the water level, and allow the



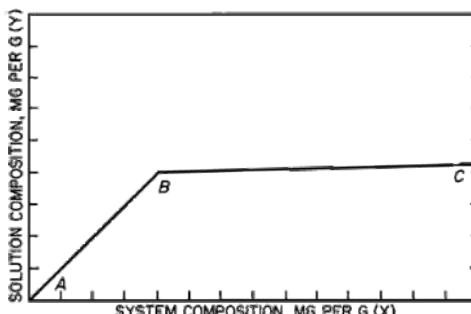
contents to settle. Open the ampuls, and remove a portion greater than 2 mL from each by means of a pipet equipped with a small pledget of cotton membrane or other suitable filter. Transfer a 2.0-mL aliquot of clear solution from each ampul to a marked, tared solubility flask, and weigh each flask plus its solution to obtain the weight of the solution. Cool the flasks in a dry ice-acetone bath, and then evaporate the solvent in vacuum. Gradually increase the temperature to a temperature consistent with the stability of the compound, and dry the residue to constant weight. Calculate the solution composition, in mg per g, by the formula:

$$1000(F_3 - F_1) / (F_2 - F_3)$$

in which  $F_3$  is the weight of the flask plus residue,  $F_1$  is the weight of the solubility flask, and  $F_2$  is the weight of the flask plus solution.

#### Calculation

For each portion of the test substance taken, plot the solution composition as the ordinate and the system composition as the abscissa. As shown in the [accompanying diagram](#).



Typical Phase-Solubility Diagram

the points for those containers, frequently only one, that represent a true solution fall on a straight line (AB) with a slope of 1, passing through the origin; the points corresponding to saturated solutions fall on another straight line (BC), the slope,  $S$ , of which represents the weight fraction of impurity or impurities present in the test substance. Failure of points to fall on a straight line indicates that equilibrium has not been achieved. A curve indicates that the material under test may be a solid solution. Calculate the percentage purity of the test substance by the formula:

$$100 - 100S.$$

The slope,  $S$ , may be calculated graphically or by least-squares treatment for best fit of the experimental values to a straight line.

The solubility of the main component is obtained by extending the solubility line (BC) through the y-axis. The point of interception on the y-axis is the extrapolated solubility, in mg per g, and is a constant for a given compound.

#### Purification Technique

Since the solvent phase in all combinations of solvent and solute that are used to construct segment BC of a phase-solubility diagram contains essentially all the impurities originally present in the substance under analysis, whereas the solid phase is essentially free from impurities, phase-solubility analysis can be used to prepare pure reference specimens of desired compounds as well as concentrates of impurities from substances otherwise considered pure. A simple modification of this technique can be used to accomplish these purposes with considerably less effort than is usually required for rigorous phase-solubility analysis.

In practice, a weighed amount of test specimen is suspended in a nonreactive solvent of suitable composition and amount so that about 10% of the material is dissolved at equilibrium. The suspension is sealed (a screw-cap vial is usually adequate) and shaken at room temperature until equilibrium is attained (usually 24 hours is sufficient for this purpose). The mother liquor is then drawn off and evaporated at or near room temperature to dryness. Since the mother liquor contained essentially all the impurities that were present in the specimen, the residue has been concentrated with respect to the impurities roughly in proportion to the ratio of the weight of specimen taken to the weight of solids dissolved in the volume of solvent used.

The undissolved crystals remaining after withdrawal of the mother liquor are usually sufficiently pure to be used as a reference standard after appropriate rinsing and drying.

\* Available from Hanson Research Corp., 19727 Bahama St., P. O. Box 35, Northridge, CA 91324.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Horacio N. Pappa, Ph.D.</a> Senior Scientist and Latin American Liaison 1-301-816-8319	(GC05) General Chapters 05

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#### 1174 POWDER FLOW

The widespread use of powders in the pharmaceutical industry has generated a variety of methods for characterizing powder flow. Not surprisingly, scores of references appear in the pharmaceutical literature, attempting to correlate the various measures of powder flow to manufacturing properties. The development of such a variety of test methods was inevitable; powder behavior is multifaceted and thus complicates the effort to characterize powder flow. The purpose of this chapter is to review the methods for characterizing powder flow that have appeared most frequently in the pharmaceutical literature. In addition, while it is clear that no single and simple test method can adequately characterize the flow properties of pharmaceutical powders, this chapter proposes the standardization of test methods that may be valuable during pharmaceutical development.

Four commonly reported methods for testing powder flow are (1) angle of repose, (2) compressibility index or Hausner ratio, (3) flow rate through an orifice, and (4) shear cell. In addition, numerous variations of each of these basic methods are available. Given the number of test methods and variations, standardizing the test methodology, where possible, would be advantageous.

With this goal in mind, the most frequently used methods are discussed below. Important experimental considerations are identified and recommendations are made regarding standardization of the methods. In general, any method of measuring powder flow should be practical, useful, reproducible, sensitive, and yield meaningful results. It bears repeating that no one simple powder flow method will adequately or completely characterize the wide range of flow properties experienced in the pharmaceutical industry. An appropriate strategy may well be the use of multiple standardized test methods to characterize the various aspects of powder flow as needed by the pharmaceutical scientist.

#### ANGLE OF REPOSE

The angle of repose has been used in several branches of science to characterize the flow properties of solids. Angle of repose is a characteristic related to interparticulate friction or resistance to movement between particles. Angle of repose test results are reported to be very dependent upon the method used. Experimental difficulties arise as a result of



segregation of material and consolidation or aeration of the powder as the cone is formed. Despite its difficulties, the method continues to be used in the pharmaceutical industry, and number of examples demonstrating its value in predicting manufacturing problems appear in the literature.

The angle of repose is the constant, three-dimensional angle (relative to the horizontal base) assumed by a cone-like pile of material formed by any of several different methods (described briefly below).

#### Basic Methods for Angle of Repose

A variety of angle of repose test methods are described in the literature. The most common methods for determining the static angle of repose can be classified on the basis of the following two important experimental variables:

1. The height of the "funnel" through which the powder passes may be fixed relative to the base, or the height may be varied as the pile forms.
2. The base upon which the pile forms may be of fixed diameter or the diameter of the powder cone may be allowed to vary as the pile forms.

#### Variations in Angle of Repose Methods

In addition to the above methods, the following variations have been used to some extent in the pharmaceutical literature:

- Drained angle of repose is determined by allowing an excess quantity of material positioned above a fixed diameter base to "drain" from the container. Formation of a cone of powder on the fixed diameter base allows determination of the drained angle of repose.
- Dynamic angle of repose is determined by filling a cylinder (with a clear, flat cover on one end) and rotating it at a specified speed. The dynamic angle of repose is the angle (relative to the horizontal) formed by the flowing powder. The internal angle of kinetic friction is defined by the plane separating those particles sliding down the top layer of the powder and those particles that are rotating with the drum (with roughened surface).

#### Angle of Repose General Scale of Flowability

Although there is some variation in the qualitative description of powder flow using the angle of repose, much of the pharmaceutical literature appears to be consistent with the classification by Carr\*, which is shown in [Table 1](#). There are examples in the literature of formulations with an angle of repose in the range of 40° to 50° that were manufactured satisfactorily. When the angle of repose exceeds 50°, the flow is rarely acceptable for manufacturing purposes.

Table 1. Flow Properties and Corresponding Angles of Repose\*

Flow Property	Angle of Repose (degrees)
Excellent	25–30
Good	31–35
Fair—aid not needed	36–40
Passable—may hang up	41–45
Poor—must agitate, vibrate	46–55
Very poor	56–65
Very, very poor	>66

#### Experimental Considerations for Angle of Repose

Angle of repose is not an intrinsic property of the powder; i.e., it is very much dependent upon the method used to form the cone of powder. The following important considerations are raised in the existing literature:

- The peak of the cone of powder can be distorted by the impact of powder from above. By carefully building the powder cone, the distortion caused by impact can be minimized.
- The nature of the base upon which the powder cone is formed influences the angle of repose. It is recommended that the powder cone be formed on a "common base," which can be achieved by forming the cone of powder on a layer of powder. This can be done by using a base of fixed diameter with a protruding outer edge to retain a layer of powder upon which the cone is formed.

#### Recommended Procedure for Angle of Repose

Form the angle of repose on a fixed base with a retaining lip to retain a layer of powder on the base. The base should be free of vibration. Vary the height of the funnel to carefully build up a symmetrical cone of powder. Care should be taken to prevent vibration as the funnel is moved. The funnel height should be maintained approximately 2–4 cm from the top of the powder pile as it is being formed in order to minimize the impact of falling powder on the tip of the cone. If a symmetrical cone of powder cannot be successfully or reproducibly prepared, this method is not appropriate. Determine the angle of repose by measuring the height of the cone of powder and calculating the angle of repose,  $\alpha$ , from the following equation:

$$\tan(\alpha) = \frac{\text{height}}{0.5 \text{ base}}$$

#### COMPRESSIBILITY INDEX AND HAUSNER RATIO

In recent years the compressibility index and the closely related Hausner ratio have become the simple, fast, and popular methods of predicting powder flow characteristics. The compressibility index has been proposed as an indirect measure of bulk density, size and shape, surface area, moisture content, and cohesiveness of materials because all of these can influence the observed compressibility index. The compressibility index and the Hausner ratio are determined by measuring both the bulk volume and the tapped volume of a powder.

#### Basic Methods for Compressibility Index and Hausner Ratio

Although there are some variations in the method of determining the compressibility index and Hausner ratio, the basic procedure is to measure (1) the unsettled apparent volume,  $V_0$ , and (2) the final tapped volume,  $V_f$ , of the powder after tapping the material until no further volume changes occur. The compressibility index and the Hausner ratio are calculated as follows:



$$\text{Compressibility Index} = 100 \times \left( \frac{V_o - V_f}{V_o} \right)$$

$$\text{Hausner Ratio} = \frac{V_o}{V_f}$$

Alternatively, the compressibility index and Hausner ratio may be calculated using measured values for bulk density ( $\rho_{\text{bulk}}$ ) and tapped density ( $\rho_{\text{tapped}}$ ) as follows:

$$\text{Compressibility Index} = 100 \times \left( \frac{\rho_{\text{tapped}} - \rho_{\text{bulk}}}{\rho_{\text{tapped}}} \right)$$

$$\text{Hausner Ratio} = \left( \frac{\rho_{\text{tapped}}}{\rho_{\text{bulk}}} \right)$$

In a variation of these methods, the rate of consolidation is sometimes measured rather than, or in addition to, the change in volume that occurs on tapping. For the compressibility index and the Hausner ratio, the generally accepted scale of flowability is given in [Table 2](#).

Table 2. Scale of Flowability\*

Compressibility Index (%)	Flow Character	Hausner Ratio
≤10	Excellent	1.00–1.11
11–15	Good	1.12–1.18
16–20	Fair	1.19–1.25
21–25	Passable	1.26–1.34
26–31	Poor	1.35–1.45
32–37	Very poor	1.46–1.59
>38	Very, very poor	>1.60

#### Experimental Considerations for the Compressibility Index and Hausner Ratio

Compressibility index and Hausner ratio are not intrinsic properties of the powder; i.e., they depend on the methodology used. In the existing literature, there are discussions of the following important considerations affecting the determination of (1) the unsettled apparent volume,  $V_o$ , (2) the final tapped volume,  $V_f$ , (3) the bulk density,  $\rho_{\text{bulk}}$ , and (4) the tapped density,  $\rho_{\text{tapped}}$ :

- The diameter of the cylinder used
- The number of times the powder is tapped to achieve the tapped density
- The mass of material used in the test
- Rotation of the sample during tapping

#### Recommended Procedure for Compressibility Index and Hausner Ratio

Use a 250-mL volumetric cylinder with a test sample weight of 100 g. Smaller weights and volumes may be used, but variations in the method should be described with the results. An average of three determinations is recommended.

#### FLOW THROUGH AN ORIFICE

The flow rate of a material depends upon many factors, some of which are particle-related and some related to the process. Monitoring the rate of flow of material through an orifice has been proposed as a better measure of powder flowability. Of particular significance is the utility of monitoring flow continuously because pulsating flow patterns have been observed even for free flowing materials. Changes in flow rate as the container empties can also be observed. Empirical equations relating flow rate to the diameter of the opening, particle size, and particle density have been determined. However, determining the flow rate through an orifice is useful only with free-flowing materials.

The flow rate through an orifice is generally measured as the mass per time flowing from any of a number of types of containers (cylinders, funnels, hoppers). Measurement of the flow rate can be in discrete increments or continuous.

#### Basic Methods for Flow Through an Orifice

There are a variety of methods described in the literature. The most common method for determining the flow rate through an orifice can be classified on the basis of three important experimental variables:

1. The type of container used to contain the powder. Common containers are cylinders, funnels, and hoppers from production equipment.
2. The size and shape of the orifice used. The orifice diameter and shape are critical factors in determining powder flow rate.
3. The method of measuring powder flow rate. Flow rate can be measured continuously using an electronic balance with some sort of recording device (strip chart recorder, computer). It can also be measured in discrete samples (for example, the time it takes for 100 g of powder to pass through the orifice to the nearest tenth of a second or the amount of powder passing through the orifice in 10 seconds to the nearest tenth of a gram).

#### Variations in Methods for Flow Through an Orifice

Either mass flow rate or volume flow rate can be determined. Mass flow rate is the easier of the methods, but it biases the results in favor of high-density materials. Because die fill is volumetric, determining volume flow rate may be preferable. A vibrator is occasionally attached to facilitate flow from the container; however, this appears to complicate interpretation of the results. A moving orifice device has been proposed to more closely simulate rotary press conditions. The minimum diameter orifice through which powder flows can also be identified.

#### General Scale of Flowability for Flow Through an Orifice

No general scale is available because flow rate is critically dependent on the method used to measure it. Comparison between published results is difficult.

#### Experimental Considerations for Flow Through an Orifice

Flow rate through an orifice is not an intrinsic property of the powder. It very much depends on the methodology used. Several important considerations affecting these methods are



discussed in the existing literature:

- The diameter and shape of the orifice
- The type of container material (metal, glass, plastic)
- The diameter and height of the powder bed.

#### Recommended Procedure for Flow Through an Orifice

Flow rate through an orifice can be used only for materials that have some capacity to flow. It is not useful for cohesive materials. Provided that the height of the powder bed (the "head" of the powder) is much greater than the diameter of the orifice, the flow rate is virtually independent of the powder head. Use a cylinder as the container because the cylinder material should have little effect on flow. This configuration results in flow rate being determined by the movement of powder over powder rather than powder along the wall of the container. Powder flow rate often increases when the height of the powder column is less than two times the diameter of the column. The orifice should be circular and the cylinder should be free of vibration. General guidelines for dimensions of the cylinder are as follows:

- Diameter of opening > 6 times the diameter of the particles
- Diameter of the cylinder > 2 times the diameter of the opening

Use of a hopper as the container may be appropriate and representative of flow in a production situation. It is not advisable to use a funnel, particularly one with a stem, because flow rate will be determined by the size and length of the stem as well as the friction between the stem and the powder. A truncated cone may be appropriate, but flow will be influenced by the powder-wall friction coefficient, making selection of an appropriate construction material an important consideration.

For the opening in the cylinder, use a flat-faced bottom plate with the option to vary orifice diameter to provide maximum flexibility and to better ensure a powder-over-powder flow pattern. Rate measurement can be either discrete or continuous. Continuous measurement using an electronic balance can more effectively detect momentary flow rate variations.

#### SHEAR CELL METHODS

In an effort to put powder flow studies and hopper design on a more fundamental basis, a variety of powder shear testers and methods that permit more thorough and precisely defined assessment of powder flow properties have been developed. Shear cell methodology has been used extensively in the study of pharmaceutical materials. From these methods, a wide variety of parameters can be obtained, including the yield loci representing the shear stress-shear strain relationship, the angle of internal friction, the unconfined yield strength, the tensile strength, and a variety of derived parameters such as the flow factor and other flowability indices. Because of the ability to more precisely control experimental parameters, flow properties can also be determined as a function of consolidation load, time, and other environmental conditions. The methods have been successfully used to determine critical hopper and bin parameters.

##### Basic Methods for Shear Cell

One type of shear cell is the cylindrical shear cell that is split horizontally, forming a shear plane between the lower stationary base and the upper moveable portion of the shear cell ring. After powder bed consolidation in the shear cell (using a well-defined procedure), the force necessary to shear the powder bed by moving the upper ring is determined. Annular shear cell designs offer some advantages over the cylindrical shear cell design, including the need for less material. A disadvantage, however, is that because of its design, the powder bed is not sheared as uniformly; i.e., material on the outside of the annulus is sheared more than material in the inner region. A third type of shear cell (plate-type) consists of a thin sandwich of powder between a lower stationary rough surface and an upper rough surface that is moveable.

All of the shear cell methods have their advantages and disadvantages, but a detailed review is beyond the scope of this chapter. As with the other methods for characterizing powder flow, many variations are described in the literature. A significant advantage of shear cell methodology in general is a greater degree of experimental control. The methodology is rather time-consuming and requires significant amounts of material and a well-trained operator.

##### Recommendations for Shear Cell

The many existing shear cell configurations and test methods provide a wealth of data and can be used very effectively to characterize powder flow. They are also helpful in the design of equipment such as hoppers and bins. Because of the diversity of available equipment and experimental procedures, no specific recommendations regarding methodology are presented in this chapter. It is recommended that the results of powder flow characterization using shear cell methodology include a complete description of equipment and methodology used.

\* Carr, R.L. Evaluating Flow Properties of Solids. *Chem. Eng.* 1965, 72, 163–168.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Robert H. Lafaver, B.A.</a> Scientist 1-301-816-8335	(EGC05) Excipient General Chapters

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#### 1176 PRESCRIPTION BALANCES AND VOLUMETRIC APPARATUS

##### Prescription Balances

note—Balances other than the type described herein may be used if these afford equivalent or better accuracy. This includes micro-, semimicro-, or electronic single-pan balances (see [Weights and Balances](#) (41)). Some balances offer digital or direct-reading features. All balances should be calibrated and tested frequently using appropriate test weights, both singly and in combination.

Description—A prescription balance is a scale or balance adapted to weighing medicinal and other substances required in prescriptions or in other pharmaceutical compounding. It is constructed so as to support its full capacity without developing undue stresses, and its adjustment is not altered by repeated weighings of the capacity load. The removable pans or weighing vessels should be of equal weight. The balance should have leveling feet or screws. The balance may feature dial-in weights and also a precision spring and dial instead of a weighbeam. A balance that has a graduated weighbeam must have a stop that halts the rider or poise at the zero reading. The reading edge of the rider is parallel to the graduations on the weighbeam. The distance from the face of the index plate to the indicator pointer or pointers should be not more than 1.0 mm, the points should be sharp, and when there are two, their ends should be separated by not more than 1.0 mm when the scale is in balance. The indicating elements and the lever system should be protected against drafts, and the balance lid should permit free movement of the loaded weighing pans when the lid is closed. The balance must have a mechanical arresting device.

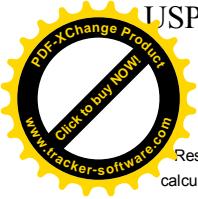
##### Definitions—

Capacity—Maximum weight, including the weight of tares, to be placed on one pan. The N.B.S. Handbook 44, 4th ed., states: "In the absence of information to the contrary, the nominal capacity of a Class A balance shall be assumed to be 15.5 g (1/2 apothecaries' ounce)." Most of the commercially available Class A balances have a capacity of 120 g and bear a statement to that effect.

Weighbeam or Beam—A graduated bar equipped with a movable poise or rider. Metric graduations are in 0.01-g increments up to a maximum of 1.0 g.

Tare Bar—An auxiliary ungraduated weighbeam bar with a movable poise. It can be used to correct for variations in weighing glasses or papers.

Balance Indicator—A combination of elements, one or both of which will oscillate with respect to the other, to indicate the equilibrium state of the balance during weighing.



**Rest Point**—The point on the index plate at which the indicator or pointer stops when the oscillations of the balance cease; or the index plate position of the indicator or pointer calculated from recorded consecutive oscillations in both directions past the zero of the index plate scale. If the balance has a two-pointer indicating mechanism, the position or the oscillations of only one of the pointers need be recorded or used to determine the rest point.

**Sensitivity Requirements (SR)**—The maximum change in load that will cause a specified change, one subdivision on the index plate, in the position of rest of the indicating element or elements of the balance.

**Class A Prescription Balance**—A balance that meets the tests for this type of balance has a sensitivity requirement of 6 mg or less with no load and with a load of 10 g on each pan. The Class A balance should be used for all the weighing operations required in prescription compounding.

In order to avoid errors of 5% or more that might be due to the limit of sensitivity of the Class A prescription balance, do not weigh less than 120 mg of any material. If a smaller weight of dry material is required, mix a larger known weight of the ingredient with a known weight of dry diluent, and weigh an aliquot portion of the mixture for use.

**Testing the Prescription Balance**—A Class A prescription balance meets the following four basic tests. Use a set of test weights, and keep the rider on the weighbeam at zero unless directed to change its position.

1. **Sensitivity Requirement**—Level the balance, determine the rest point, and place a 6-mg weight on one of the empty pans. Repeat the operation with a 10-g weight in the center of each pan. The rest point is shifted not less than one division on the index plate each time the 6-mg weight is added.

2. **Arm Ratio Test**—This test is designed to check the equality of length of both arms of the balance. Determine the rest point of the balance with no weight on the pans. Place in the center of each pan a 30-g test weight, and determine the rest point. If the second rest point is not the same as the first, place a 20-mg weight on the lighter side; the rest point should move back to the original place on the index plate scale or farther.

3. **Shift Tests**—These tests are designed to check the arm and lever components of the balance.

A. Determine the rest point of the indicator without any weights on the pans.

B. Place one of the 10-g weights in the center of the left pan, and place the other 10-g weight successively toward the right, left, front, and back of the right pan, noting the rest point in each case. If in any case the rest point differs from the rest point determined in Step A, add a 10-mg weight to the lighter side; this should cause the rest point to shift back to the rest point determined in Step A or farther.

C. Place a 10-g weight in the center of the right pan, and place a 10-g weight successively toward the right, left, front, and back of the left pan, noting the rest point in each case. If in any case the rest point is different from that obtained with no weights on the pans, this difference should be overcome by addition of the 10-mg weight to the lighter side.

D. Make a series of observations in which both weights are simultaneously shifted to off-center positions on their pans: both toward the outside, both toward the inside, one toward the outside and the other toward the inside, both toward the back, and so on until all combinations have been checked. If in any case the rest point differs from that obtained with no weights on the pan, the addition of the 10-mg weight to the lighter side should overcome this difference.

A balance that does not meet the requirements of these tests must be adjusted.

4. **Rider and Graduated Beam Tests**—Determine the rest point for the balance with no weight on the pans. Place on the left pan the 500-mg test weight, move the rider to the 500-mg point on the beam, and determine the rest point. If it is different from the zero rest point, add a 6-mg weight to the lighter side. This addition should bring the rest point back to its original position or farther. Repeat this test, using the 1-g test weight and moving the rider to the 1-g division on the beam. If the rest point is different, it should be brought back at least to the zero rest point position by the addition of 6 mg to the lighter pan. If the balance does not meet this test, the weighbeam graduations or the rider must be corrected.

Metric or apothecaries' weights for use with a prescription balance should be kept in a special rigid and compartmentalized box and handled with plastic or plastic-tipped forceps to prevent scratching or soiling. For prescription use, analytical weights (Class P or better) are recommended. However, Class Q weights have tolerances well within the limits of accuracy of the prescription balance, and they retain their accuracy for a long time with proper care. Coin-type (or disk-shaped) weights should not be used.

Test weights consisting of two 20-g or two 30-g, two 10-g, one 1-g, one 500-mg, one 20-mg, one 10-mg, and one 6-mg (or suitable combination totaling 6 mg) weights, adjusted to N.B.S. tolerances for analytical weights (Class P or better) should be used for testing the prescription balances. These weights should be kept in a tightly closed box and should be handled only with plastic or plastic-tipped forceps. The set of test weights should be used only for testing the balance or constantly used weights. If properly cared for, the set lasts indefinitely.

#### Volumetric Apparatus

Pharmaceutical devices for measuring volumes of liquids, including burets, pipets, and cylinders graduated either in metric or apothecary units meet the standard specifications for glass volumetric apparatus described in NTIS COM-73-10504 of the National Technical Information Service.<sup>1</sup> Conical graduates meet the standard specifications described in N.B.S.

Handbook 44, 4th Edition, of the National Institute of Standards and Technology.<sup>2</sup> Graduated medicine droppers meet the specifications (see [Medicine Dropper](#) (1101)). An acceptable ungraduated medicine dropper has a delivery end 3 mm in external diameter and delivers 20 drops of water, weighing 1 g at a temperature of 15°. A tolerance of  $\pm 10\%$  of the delivery specification is reasonable.

#### Selection and Use of Graduates—

**Capacity**—The capacity of a graduate is the designated volume, at the maximum graduation, that the graduate will contain, or deliver, as indicated, at the specified temperature.

**Cylindrical and Conical Graduates**—The error in a measured volume caused by a deviation of  $\pm 1$  mm in reading the lower meniscus in a graduated cylinder remains constant along the height of the uniform column. The same deviation of  $\pm 1$  mm causes a progressively larger error in a conical graduate, the extent of the error being further dependent upon the angle of the flared sides to the perpendicular of the upright graduate. A deviation of  $\pm 1$  mm in the meniscus reading causes an error of approximately 0.5 mL in the measured volume at any mark on the uniform 100-mL cylinder graduate. The same deviation of  $\pm 1$  mm can cause an error of 1.8 mL at the 100-mL mark on an acceptable conical graduate marked for 125 mL.

A general rule for selection of a graduate for use is to use the graduate with a capacity equal to or just exceeding the volume to be measured. Measurement of small volumes in large graduates tends to increase errors, because the larger diameter increases the volume error in a deviation of  $\pm 1$  mm from the mark. The relation of the volume error to the internal diameters of graduated cylinders is based on the equation  $V = \pi r^2 h$ . An acceptable 10-mL cylinder having an internal diameter of 1.18 cm holds 109  $\mu$ L in 1 mm of the column. Reading 4.5 mL in this graduate with a deviation of  $\pm 1$  mm from the mark causes an error of about  $\pm 2.5\%$ , and the same deviation in a volume of 2.2 mL in the same graduate causes an error of about  $\pm 5\%$ . Minimum volumes that can be measured within certain limits of error in graduated cylinders of different capacities are incorporated in the design details of graduates in N.B.S. Handbook 44, 4th ed., of the National Institute of Standards and Technology. Conical graduates having a capacity of less than 25 mL should not be used in prescription compounding.

1 NTIS COM-73-10504 is for sale by the National Technical Information Service, Springfield, VA 22151.

2 N.B.S. Handbook 44, 4th ed. (1971), is for sale by the Superintendent of Documents, U. S. Government Printing Office, Washington, DC 20402.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	Rick G. Schnatz Manager, Compounding Pharmacy Expert Committee 1-301-816-8526	(CRX05) Compounding Pharmacy05

## 1177 GOOD PACKAGING PRACTICES

This chapter provides general guidance on packaging considerations for Pharmacopeial preparations that may be stored, transported, and distributed. It describes procedures that should be considered to ensure that proper packaging practices are maintained. It does not affect any applicable requirements under good manufacturing practices, state laws governing pharmacy, the USP General Notices and Requirements or monographs, or provisions under approved labeling.

Definitions for storage conditions and packaging are provided in Preservation, Packaging, Storage, and Labeling under General Notices and Requirements. All equipment used for recording, monitoring, and maintaining these temperature and humidity conditions should be calibrated on a regular basis. This calibration should be traceable to national or international standards (see also the general information chapter [Monitoring Devices—Time, Temperature, and Humidity](#) 1118).

### CONTAINERS

The monograph packaging and storage statement specifies that the container (primary package) should meet the requirements under [Containers—Glass](#) 660, [Containers—Plastic](#) 661, and [Containers—Performance Testing](#) 671, which include the stipulations for determining if a container is "tight" or "well-closed." In most cases, compendial preparations are expected to be packaged in "tight" containers, especially if the article is moisture sensitive. In addition, where necessary, the packaging component should protect the preparation from light, reactive gases, solvent loss, microbial contamination, etc. "Tight" and "well-closed" containers are clearly defined in General Notices and Requirements (see Containers under Preservation, Packaging, Storage, and Labeling), whereas testing protocol and moisture permeation limits to determine if the container meets either of these definitions can be found in [Containers—Glass](#) 660, [Containers—Plastic](#) 661, and [Containers—Performance Testing](#) 671 for single-unit and multiple-unit containers.

A packaging system is composed of a container system with its closure. This system may include several layers of protection for the Pharmacopeial preparation along with any sealing devices, delivery devices, labeling, and package inserts. The General Notices section also provides definitions for types of packaging systems that contain and protect a Pharmacopeial preparation (e.g., single-unit containers, unit-dose containers, etc.). Stability testing is conducted on the dosage forms packaged in the container–closure system proposed for marketing.

One type of permeation test for multiple-unit containers is described in [Containers—Performance Testing](#) 671. This test is intended for drug products being dispensed on prescription in vials with a container–closure system. The results of the test reflect the water vapor permeation through the container and through the closure. Limits have been established to define whether a container for dispensing has tight or well-closed characteristics with regard to water vapor permeation. FDA recommends that manufacturers perform this test on the container–closure system, although it is not specified in USP. In this particular test, the inner seal of the manufacturer's container–closure system is removed prior to testing.

Single-unit containers for capsules and tablets under [Containers—Performance Testing](#) 671 are measured for water vapor permeation according to the criteria for the four classes of containers (classes A–D).

The USP recognizes several official container materials that can be selected on the basis of their properties. Most containers are made of glass or plastic. Glass containers must be evaluated for chemical resistance and light transmission (if indicated) as described in [Containers—Glass](#) 660. In addition, injectable medication containers should be reviewed according to the section [Packaging under Injections](#) 1. Elastomeric closures should be evaluated separately as described in [Elastomeric Closures for Injections](#) 381. Plastic containers should be assessed using different criteria for the three types of plastics as described in the following sections under [Containers—Plastic](#) 661: Polyethylene Containers (PE) for dry oral solid dosage forms, Polyethylene Terephthalate Bottles and Polyethylene Terephthalate C Bottles (PET, PETG) for liquid oral dosage forms, and Polypropylene Containers (PP) for dry solid and liquid oral dosage forms. As articulated in these sections, plastics should undergo testing for light transmission (if appropriate), water vapor permeation (see also [Containers—Performance Testing](#) 671), extraction physicochemical testing, and biological testing (see also [Biological Reactivity Tests, In Vitro](#) 87 and [Biological Reactivity Tests, In Vivo](#) 88). For example, testing water vapor permeation for a PE container is conducted by sealing the container with heat-sealed foil laminate and measuring the water permeation in a humid atmosphere. Given that water vapor does not permeate the foil laminate, this test assesses only the properties of the container. The level of protection provided by a packaging system marketed with a heat-sealed foil laminate inner seal (prior to removal of the foil) is approximated by this test. However, in the case of a PET bottle for liquid preparations, water vapor permeation testing is done by filling containers with water and measuring the water loss rate in a dry atmosphere. Additional testing may be required for certain pharmaceutical dosage forms as well.

The container–closure system for the storage or shipment of a bulk liquid drug substance is typically plastic, stainless steel, a glass-lined metal container, or an epoxy-lined metal container with a rugged, tamper-resistant closure. Qualification of the container–closure system for these types of preparations includes evaluation for solvent and gas permeation, light transmittance, closure integrity, ruggedness in shipment, protection against microbial contamination through the closure, and compatibility and safety of the packaging components as appropriate (see [Containers—Glass](#) 660 and [Containers—Plastics](#) 661).

Other information on container–closure systems may be found in FDA's Guidance for Industry: Container Closure System for Packaging Human Drugs and Biologics, [www.fda.gov](http://www.fda.gov).

### PACKAGING

Packaging for Pharmacopeial articles can be divided into categories according to terminology generally accepted by industry. As mentioned earlier, the General Notices section provides some definitions for different types of containers classified by their characteristics and uses. In addition, the ASTM Committee D10 on packaging publishes terminology, practices, test methods, specifications, guides, and classifications for testing and evaluating packaging (see ASTM D99695, "Standard Terminology of Packaging and Distribution Environments"). Under certain rules and guidelines (e.g., such as 49 CFR, Dangerous Goods, and others), however, alternate terminology is used for the components described below.

For terminology pertaining to repackaging processes, refer to [Packaging Practice—Repackaging a Single Solid Oral Drug Product into a Unit-Dose Container](#) 1146.

**Primary Container**— This container is in direct contact with the Pharmacopeial preparation. The purpose of a primary container, also referred to as an immediate container, is to protect the preparation from environmental hazards during storage and handling. In some cases, the primary container is also a specialized delivery system, such as an aerosol or a metered-dose dispenser (see [Pharmaceutical Dosage Forms](#) 1151). For the majority of oral dosage forms, the primary container consists of a cap and a bottle or a blister or pouch package that can be made from many different materials, including glass, plastic, single or laminated flexible materials, and metal. All components of the primary container must meet the requirements under 21 CFR for direct food contact and, where applicable, the USP requirements under [Containers—Glass](#) 660, [Containers—Plastic](#) 661, and [Containers—Performance Testing](#) 671. A full description of the primary container is included under the "Container/Closure System" section of the New Drug Application (NDA), Abbreviated New Drug Application (ANDA), or other classes of FDA submissions.

**Critical Secondary Container**— This container is not in direct contact with the article, but it provides essential product stability protection. For example, a primary container may be packed inside a critical secondary container such as a pouch to provide moisture, gas, light, or microbial protection not afforded by the primary container. A description of the critical secondary container is included under the "Container/Closure System" section of the NDA, ANDA, or other classes of FDA submissions.

**Secondary Container**— This container encloses one or more primary containers. A secondary container is not always present. If used, it is usually designed for the final market presentation. Secondary containers are often used simply to carry required labeling or to keep individual primary containers together with delivery systems or other add-on features. Secondary containers can also provide protection against damage in the handling and distribution system. The most common secondary container is the standard folding carton. Some products, such as syringes, may be placed in trays prior to packing in a carton. Secondary container materials are not included in the container and closure description and require neither stability studies nor prior approval when making a change in the materials used.

**Additional Packaging**— A wide variety of additional packaging, such as trays and display cartons, may be used to hold primary containers.

**Unit of Sale**— This may be an individual bottle, a carton containing one or more bottles, or a tray with multiple primary containers. A unit of sale may contain more than one item for individual sale. For example, a display tray may have multiples of a single article or a variety of related articles from a single manufacturer, each intended for individual sale. The individual item intended for sale is referred to as a stock-keeping-unit (SKU). SKUs are distinguished by a discrete National Drug Code (NDC). Over-the-counter (OTC) articles contain a Universal Product Code (UPC) for all SKUs. A prescription SKU may be intended for final consumer use and may not be repackaged by a pharmacy. Such packages, often called



"unit of issue" or "unit of use," require child-resistant (CR) packaging as described under 16 CFR 1700, "Poison Prevention Packaging," except for packages exempted by the Consumer Product Safety Commission. The CR feature is typically incorporated by the manufacturer (see [Packaging—Unit-of-Use 1136](#)). OTC articles are regulated under the same rule, but only if they contain certain active ingredients above specified limits. Any regulated product shipped via the United States Postal Service (USPS) must meet the USPS rules under 39 CFR 111.

**Final Exterior Package**— This is typically a corrugated fiberboard box (case) or a wrapper. The shipping case label is affixed to this outermost layer and incorporates all of the bar codes required by the National Wholesale Druggists' Association (NWDA). This final package is normally shipped on pallets to distribution centers, wholesalers, and other large-volume customers. The manufacturer may or may not intend that this package enter the small-package-shipping environment as an individual unit without further protection.

Especially with fiberboard boxes, relative humidity (RH) may have a negative effect on the compression strength of the box, causing loads to shift and potentially damage the article or the outer and inner packaging. Articles stored in refrigerators or freezers, which are environments with high RH, are prone to this type of damage when stacked. The problem may be exacerbated by carton design, stacking pattern, or use of low edge-crush-test corrugated fiberboard. Computer programs are available to determine the acceptable stack height and patterns on the basis of carton weight, style, size, and material. If problems occur, the product manufacturer should be contacted. Source materials and reference information on corrugated fiberboard boxes can be found in the "Fiber Box Handbook" published by the Fiber Box Association.

A wholesaler or other reshipper should not assume that the package received from the manufacturer is suitable for reuse. Many packages are customized for very specific routes and modes of transportation and are not suitable for other applications. Like any other shipping container, insulated cartons and inner protective packaging can be damaged during transit, thus affecting package performance and possibly allowing damage to contents if reused.

#### ENVIRONMENTAL ISSUES

Packaging materials are regulated by a variety of federal, state, and local rules. In general, most pharmaceutical packaging containers can be recycled within local programs. The use of recycled material in primary containers is governed by the FDA, but it is generally not allowed. Pharmaceutical manufacturers commonly follow the most current Coalition of Northeastern Governors' rules (e.g., Model Toxics in Packaging Legislation) regarding heavy metals in packaging and other environmental issues.

Certain classes of Pharmacopeial articles may require special handling. Such articles include products classified as (1) Dangerous Goods under the Department of Transportation (DOT), state, local, or carrier rules; (2) controlled drugs under the Drug Enforcement Administration (DEA); or (3) scheduled substances under state regulations.

#### LABELING

The labeling of shipping containers by manufacturers must be in compliance with the pertinent sections of FDA and DOT rules.

**Dangerous Goods**— The labeling of shipments classified as Dangerous Goods, including all information on the bill of lading or airway bill, must follow the instructions provided by the DOT, the International Air Transport Association (IATA), and the carrier. The exterior package must carry all of the required standard symbols for the class of goods, and the shipping container must comply with the performance standards for the articles enclosed. The shipper of record is responsible for compliance with the Dangerous Goods requirements.

**Controlled Substances**— When Pharmacopeial preparations that contain DEA-scheduled controlled substances are distributed to a patient directly via the USPS, these articles must be marked and labeled in accordance with USPS Domestic Mail Manual, Regulation Article C023, Section 7.2.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Desmond G. Hunt, Ph.D.</a> Scientist 1-301-816-8341	(PS05) Packaging and Storage 05

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1178 GOOD REPACKAGING PRACTICES

#### INTRODUCTION

This chapter is intended to provide guidance to those engaged in repackaging of oral solid drug products; and the chapter provides information to any person who removes drugs from their original container–closure system (new primary package) and repackages them into a different container–closure system for sale and/or for distribution.

This chapter does not apply to pharmacists engaged in dispensing prescription drugs in accordance with state practice of pharmacy. The pharmacist needs to apply

1. the principal information provided in the USP general information chapter [Packaging Practice—Repackaging a Single Solid Oral Drug Product Into a Unit-Dose Container 1146](#) and
2. other beyond-use date references in the subsection Expiration Date and Beyond-Use Date in the Labeling section under [General Notices and Requirements](#).

#### DEFINITIONS

The section Preservation, Packaging, Storage, and Labeling under [General Notices and Requirements](#) provides definitions related to repackaging. For the purposes of this chapter, a repackager, a contract packager, and an equivalent container–closure system are defined as follows:

1. **Repackager**—A repackager is an establishment that repackages drugs and sends them to a second location in anticipation of a need. Repackaging firms repackage preparations for distribution (e.g., for resale to distributors, hospitals, or other pharmacies), a function that is beyond the regular practice of a pharmacy. Distribution is not patient-specific in that there are no prescriptions. Unlike dispensers, repackaging firms are required to register with the FDA and to comply with the Current Good Manufacturing Practice Regulations in 21 CFR 210 and 211.
2. **Contract Packager**—A contract packager is an establishment that is contracted to package or repackaging a drug product into a single- or multi-unit container. These containers should meet all of the applicable requirements in this chapter. A contract packager does not take ownership from the manufacturer and generally receives the assigned expiration date from the contractor.
3. **Equivalent Container–Closure System**—This term refers to a container–closure system that is at least as protective or more protective than the original container–closure system in terms of moisture vapor transmission rate (MVTR), oxygen transmission, light transmission, and compatibility of the container–closure system with the drug product. System equivalency extends to any special protective materials, such as for light transmission, seals, or desiccants associated with the original container–closure system. These values may be determined by the repackager, or they may be obtained from the container–closure vendor for the specific container–closure system under consideration.

#### ESTABLISHING EXPIRATION DATE

In the absence of stability data, the following criteria should be considered by repackagers when assigning an expiration date.

Unit-Dose Packaging



1. The original container–closure system of the drug product to be used for repackaging must be received un-opened and show no outward signs of having been previously opened.
2. The unit-dose container–closure system must meet the testing requirements under [Containers—Performance Testing \(671\)](#) for either Class A or Class B containers.
3. The contents of the original bulk drug product to be repackaged are repackaged at one time unless the repackager has data and/or other scientific information from literature sources demonstrating that the drug product is not sensitive to exposure to moisture, oxygen, or light.
4. The unit-dose container–closure system must meet or exceed the original container's specification for light resistance.
5. The conditions of storage must meet the storage specifications provided in the [USP General Notices](#) and as described in the labeling of the original container–closure system received for repackaging. Where no specific storage conditions are specified, the product must be maintained at controlled room temperature and in a dry place during the repackaging process, including storage.
6. The expiration dating period used for the repackaged product does not exceed (1) 6 months from the date of repackaging; or (2) the manufacturer's expiration date; or (3) 25% of the time between the date of repackaging and the expiration date shown on the manufacturer's bulk article container of the drug being repackaged, whichever is earlier.
7. Nitroglycerin Sublingual Tablets or any other drug product known to have stability problems should not be repackaged. This would include any drug known to be oxygen-sensitive or one that exhibits extreme moisture or light sensitivity. In deciding whether a particular drug product is suitable for repackaging, the repackager should take into consideration any available information from the manufacturer, published literature, the USP, and the FDA.
8. Documentation must be maintained to demonstrate that the preceding criteria are met.
9. Documentation must be maintained that specifies the container–closure packaging material used in repackaging operations.

#### Multiple-Unit Packaging

1. A repackager may use the manufacturer's original expiration date without additional stability testing if the drug product is repackaged into an equivalent container–closure system that is at least as protective as, or more protective than, the original system and complies with criteria established for equivalency.
2. The original container–closure system of the drug product to be used for repackaging must be received un-opened and shows no outward signs of having been previously opened.
3. The contents of the original bulk drug product to be repackaged are repackaged at one time unless the repackager has data and/or other scientific information from literature sources demonstrating that the drug product is not sensitive to exposure to moisture, oxygen, or light.
4. The conditions of storage meet the storage specifications in the [USP General Notices](#) and as described in the labeling of the original container–closure system received for repackaging. When no specific storage conditions are specified, the product should be maintained at controlled room temperature and in a dry place during repackaging operations.
5. The type of container–closure system used for repackaging must be at least as protective or more protective than the original container–closure system in terms of moisture vapor transmission rate (MVTR), oxygen transmission, light transmission, and compatibility of the container–closure system with the drug product. System equivalency extends to any special protective materials, such as for light transmission, seals, or desiccants associated with the original container–closure system.
6. The container–closure system must meet or exceed the original container–closure system's results for light transmission.
7. Nitroglycerin Sublingual Tablets or any other drug product known to have stability problems should not be repackaged. This would include any drug known to be oxygen-sensitive or one that exhibits extreme moisture or light sensitivity. In deciding whether a particular drug product is suitable for repackaging, the repackager should take into consideration any available information from the manufacturer, published literature, the USP, and the FDA.
8. Documentation must be maintained to demonstrate that the preceding criteria are met.
9. Documentation must be maintained that specifies the container–closure packaging material used in repackaging operations.

#### REFERENCES FOR REPACKAGING REGULATIONS AND GUIDANCES

The references listed below are not meant to be all inclusive: specific repackaging operations may have additional requirements.

- Food, Drug, and Cosmetic Act
- Food and Drug Administration Regulations and Guidances

##### Enforcement Policy: 21 CFR, Part 7

General Labeling Provisions: 21 CFR, Part 201, Subpart A

Drug Establishment Registration and Listing: 21 CFR, Part 207.20

Current Good Manufacturing Regulations: 21 CFR, Parts 210–211

Special Requirements for Specific Human Drugs: 21 CFR, Part 250

Controlled Substances: 21 CFR, Part 1300

Potable Water: 40 CFR, Part 141

FDA Compliance Policy Guides, including the following:

Sub Chapter 430 Labeling and Repackaging

Sub Chapter 460 Pharmacy Issues

Sub Chapter 480 Stability/Expiration Dating

- Applicable USP Chapters

↳ [660 Containers—Glass](#)

↳ [661 Containers—Plastics](#)

↳ [671 Containers—Performance Testing](#)

↳ [681 Repackaging into Single-Unit Container and Unit-Dose Container for Nonsterile Solid and Liquid Dosage Forms](#)

↳ [1079 Good Storage and Shipping Practices](#)

↳ [1146 Packaging Practice—Repackaging a Single Solid Oral Drug Product into a Unit-Dose Container](#)

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Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Desmond G. Hunt, Ph.D.</a> Scientist 1-301-816-8341	(PS05) Packaging and Storage 05

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↳ [1181 SCANNING ELECTRON MICROSCOPY](#)

Scanning electron microscopy (SEM) is an electron optical imaging technique that yields both topographic images and elemental information when used in conjunction with energy-dispersive X-ray analysis (EDX) or wavelength-dispersive X-ray spectrometry (WDS). SEM is useful for characterizing the size and morphology of microscopic specimens. Together, image and X-ray analyses are important for the identification of small particles. Elemental analyses using SEM/EDX or SEM/WDS are useful for qualitative and semiquantitative determination of elemental content. Accurate quantitation is possible only for bulk samples with smooth surfaces and thus is not practical for particle specimens.

Typically, SEM analysis requires a small amount (10–10 to 10–12 g) of a solid specimen that is coated with a conductive substance to inhibit sample charging. The sample is placed in an evacuated chamber and scanned in a controlled raster pattern by an electron beam. Interaction of the electron beam with the specimen produces a variety of physical phenomena that, when detected, are used to form images and provide elemental information about the specimen. These phenomena include (1) emission of secondary electrons (SES), (2) reflection of backscattered electrons (BSES), (3) characteristic X-ray emission, (4) emission of Auger electrons, (5) cathodoluminescence (CL), (6) conduction of current, (7) charging from induced voltages (IVS) or adsorbed electrons (AES), (8) electron transmission, (9) heat generation, and (10) electromotive forces (see [Figure 1](#)).

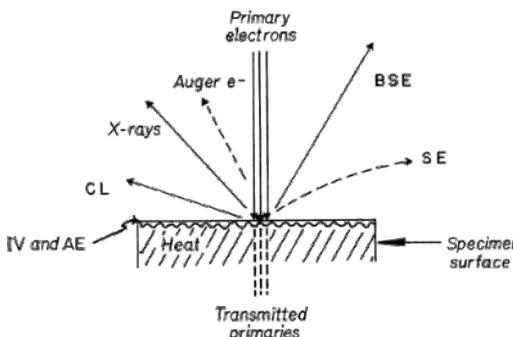


Figure 1. Interaction Diagram.

Of these, SES and BSES are the most important for constructing SEM images, and X-ray emission analyses are the most common methods for detecting the presence of particular elements. Use of ancillary instrumentation to detect the variety of phenomena other than (1), (2), or (3) above greatly increases the cost and complexity of the SEM system and will not be addressed here.

#### ELECTRON BEAM–SAMPLE INTERACTION

**Imaging**— Images are formed in a SEM system by detection and manipulation of electrons. SE are emitted from a specimen surface as the result of inelastic collisions between primary (incident) electrons (PE) and electrons within a specimen. When the energy imparted to a specimen electron exceeds the work function of a sample, that electron is emitted as an SE. Most SES have energies of 5 to 20 eV; electrons in this low-energy range can be efficiently collected, yielding high signal-to-noise images. Because such low-energy electrons can penetrate only short distances through the specimen, SES originate from within 2 to 30 nm of the surface and generate highly resolved images. The actual PES penetration depth is dependent on PES accelerating voltage, specimen elemental composition, specimen density, and specimen mounting angle. Excitation volumes of 0.5 to 5  $\mu\text{m}$  in diameter are common.

Backscattered electrons are PES that have been reflected from the sample. The PES can undergo multiple collisions prior to exiting from the specimen; therefore, BSES have energies over a broad range and emerge from relatively deep penetration ( $\approx$ 0.1 to 5  $\mu\text{m}$ ) (see [Figure 2](#)).

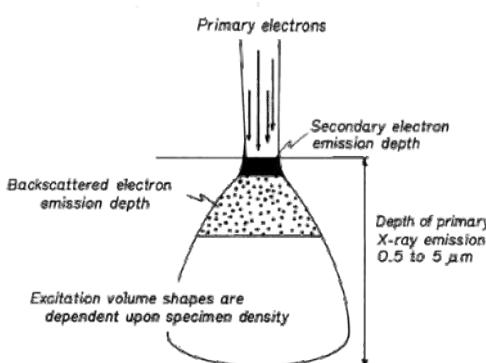


Figure 2. Bulk Penetration.

These high-energy (15 to 25 keV) BSES are collected less efficiently than SES, and they yield images with poorer resolution. The efficiency of BSES reflection is a function of the atomic number (Z) of the specimen atoms; thus, the contrast of BSES images depends on elemental composition. The penetration depth of all electrons is affected by elemental composition, specimen density, specimen tilt, and incident beam energy (accelerating voltage). For example, the SE images of sodium phosphate and zinc phosphate crystals are quite similar. However, the heavier nuclei of the zinc species produce more efficient BSE reflection and BSES images with higher contrast. BSE images of heavy- versus light-element phases, or mixtures of species, show dramatic contrast differences that are representative of elemental heterogeneity.

Although single-angstrom resolution is possible, practical SEM image resolution is limited to  $\geq 100 \text{ \AA}$  ( $\geq 0.1 \mu\text{m}$  for X-ray images). These limits depend not only on instrument performance but also on operator acuity. Resolution is optimized under the following conditions: minimum working distance, high accelerating voltage, excellent grounding, excellent mechanical alignment, excellent electronic alignment, minimum incident spot diameter, minimum final aperture diameter, and cleanest column conditions. Sample preparations can be viewed in a variety of orientations and detector modes. Often the examination of a specimen at an oblique angle reveals features unobserved by an electron beam normal to the surface. This is especially true of specimens that have flat, featureless surfaces or that are poor conductors, e.g., glass surfaces. The PE accelerating voltage can be varied to change the PE penetration depth. This procedure is useful for characterizing specimens that are laminated or otherwise heterogeneous between surface and bulk content.

Coating a sample allows observation of a specimen's topography, undisturbed by flare and distortion caused by thermal effects and insufficient grounding. Coatings such as gold, gold-palladium, and carbon are often used because they are highly conductive, easy to apply, and relatively inert. Either evaporation or sputter-coating systems can be used to apply metal films; carbon films must be evaporated. Metal coatings give superior resolution, although their fluorescence can interfere with elemental analysis. Specimen charging affects not only image quality but also X-ray fluorescence yield.

**X-ray Emission Analysis**— When a PE encounters an orbital electron in an atom, the resultant collision can either promote that orbital electron to a higher energy level or ionize the atom. Stabilization of an atom by relaxation of a higher energy electron to fill a vacancy results in the emission of an X-ray photon. These X-ray energies are discrete and element-specific; they equal the differences between the shell electron energies for the various shells of a given element. For instance, an ejected K-shell electron can be stabilized by a higher energy L-shell electron, yielding a net energy ( $\text{EL} - \text{EK}$ ), which is specific for the X-ray photon energy of the elemental K line. X-ray emission lines are classified according to the electron shell in which the vacancy existed, e.g., K, L, M. The lines are further categorized according to the shell from which the relaxing electron originates. Thus, a K $\alpha$  X-ray line

arises from a vacancy in a K-shell that is filled from an L-shell; a  $K\beta$  X-ray line arises from a K-shell vacancy filled from an M-shell, and so on (see [Figure 3](#)).

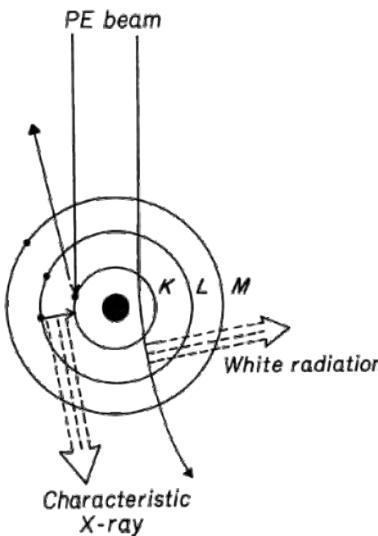
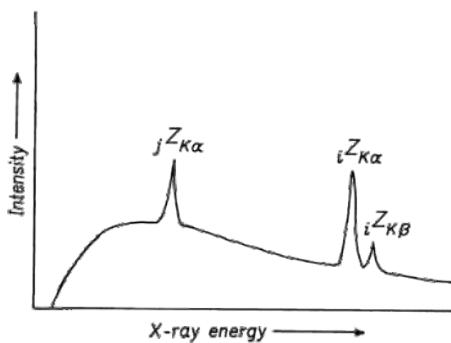


Figure 3. Atom Model.

Since each shell above K possesses a number of energy levels, electron transitions yield a number of lines, such as  $K\alpha_1$ ,  $K\alpha_2$ ,  $K\beta_1$ , and  $K\beta_2$ . The existence of several X-ray emission lines for each element (a few for  $Z \leq 11$  and many for  $Z \geq 11$ ) is useful in overcoming detection problems due to (1) interelement spectral interferences, e.g., titanium  $K\alpha$  and barium  $L\alpha$ , (2) sample matrix effects on energy or intensity, and (3) insufficient PE energy to excite some elemental lines, e.g., lead, K lines.

The energies normally encountered in a SEM/EDX (or WDS) analysis range from 0.28 keV ( $\approx 447$  nm) for carbon  $K\alpha$  to the upper end of the instrument accelerating voltage, typically  $\leq 40$  keV ( $\leq 1$  nm). The natural line width, which is inversely proportional to the lifetime of the upper electronic state, is governed primarily by the transition probabilities for X-ray emission and Auger electron emission. Interaction of X-ray photons with electrons within the specimen can result in Compton scattering to produce a broadened line shifted to lower energy. X-ray photons are also emitted as a result of inelastic acceleration of electrons by atomic nuclei within a specimen. These X-ray photons, termed bremsstrahlung or white radiation, have a broad, continuous energy distribution; and their characteristic lines are superimposed on this background signal.

For lighter elements,  $Z \leq 11$ , the low-energy X-ray photons originating from K-shell transitions can be detected only with wavelength-dispersive spectrometers or specially configured energy-dispersive detectors. All other elements emit easily detectable X-ray photons. Heavier elements,  $Z \geq 16$ , emit two or more detectable lines corresponding to K- and L-shell transitions; and  $Z > 57$  emit three or more detectable lines corresponding to K-, L-, and M-shell transitions. For a given element, X-ray intensities generally vary as follows:  $K\alpha > K\beta > L\alpha > L\beta$ , etc. (see [Figure 4](#)).



The elemental content of a sample has a bearing on the selection of conditions for analysis. The most useful range of accelerating voltage is  $\approx 3$  to 20 kV; most elements of interest can be ionized by electrons with energies in this range. The energy required in order to excite X-ray emission from a given line is termed its critical excitation potential. The critical excitation potential for a K line can be approximated by the sum of the primary line energies ( $K\alpha_1 + L\alpha_1 + M\alpha_1$ ). Selection of an accelerating voltage equal to 1.5 times this sum is usually sufficient for semiquantitative analyses. For example, copper has  $K\alpha$  at 8.05 keV +  $L\alpha$  at 0.93 keV = 8.98 keV:  $1.5 \times 8.98$  keV = 13.47 keV. Selection of 15-kV accelerating voltage yields sufficient energy to ionize the K-shell of copper atoms and generate a useful analytical signal.

Interelement interferences originate from many effects. High-energy X-rays emitted from heavy atoms can ionize lighter elements to produce secondary X-ray emission from the lighter species. Lower high-Z element fluorescence and higher low-Z element fluorescence can be observed, in contrast to that expected from the PE-induced signal of a pure element. Conversely, X-ray emission from a light element may be absorbed by a heavier matrix to yield a negative bias in the light-element signal. These effects always exist in heterogeneous specimens and must be corrected for during any quantitative analysis. A common algorithm, ZAF, may be used to correct for Z-dependent interferences due to absorption and secondary X-ray emission.

#### APPARATUS

The SEM system consists of three electronic groups: (1) illumination, (2) optics, and (3) scanning control-display (see [Figure 5](#)).

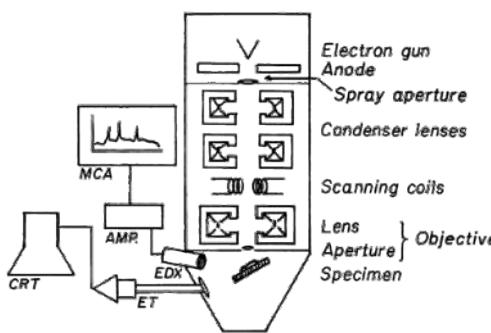


Figure 5. Optics Diagram.

The image is produced by mapping a specimen with an incident electron beam, rastered in a two-dimensional array. The electron beam is generated by emission from one of three types of sources, listed in order of increasing current density, vacuum requirements, and cost: (1) a tungsten filament cathode, (2) a LaB<sub>6</sub> cathode, or (3) a field emission gun. By far the most common SEM source is the tungsten filament, although the high current density of the LaB<sub>6</sub> source is especially useful where high resolution or detection of low-Z elements is required.

The optics consist of condenser and objective lenses, in conjunction with selected apertures. The size of the final aperture controls the beam diameter and, accordingly, the image resolution and total current at a specimen. Selection of an objective aperture is an important choice. Small apertures are required for high resolution and large apertures provide high current for optimal X-ray emission intensity. In many systems, the objective aperture can be adjusted during use with a sliding or rotating holder. Flexibility in trading resolution for specimen current is also important because sample characteristics affect these two criteria differently. This feature is beneficial to users requiring high magnification and elemental detection, especially of  $Z \leq 11$  elements.

Image magnification is controlled by altering the area of the electron beam raster; smaller areas yield higher magnification, because the cathode-ray tube (CRT) area remains constant. An Everhart-Thornley (ET) detector is used for electron detection; the resultant images are most similar to those of reflected light microscopy. An ET detector consists of a Faraday cage and a scintillator disk connected by a light pipe to a photomultiplier tube. The Faraday cage serves three functions: (1) at positive bias it attracts SE; (2) at negative bias it repels

SE to enable the ET detector to collect BSE signals alone; and (3) it shields the PE beam from the scintillator potential. Various scintillator coatings are used. For example, phosphorus-based coatings yield intense, high-contrast images. Aluminum-based coatings, although less sensitive, can withstand the high SE flux generated during elemental analyses. Solid-state detectors provide up to 10 times greater sensitivity for BSE collection. They can be placed at a variety of positions and distances with respect to a specimen.

#### Procedure

**Preparation**— Samples for analysis are easily prepared, especially with an optical aid or a stereomicroscope.

**Particles**— Place isolated or selected particles onto the SEM sample substrate (e.g., a pyrolytic carbon or metal pedestal). In all cases, the specimen should be attached to an exposed area of the substrate with a suitable liquid cement\* (see Figure 6).

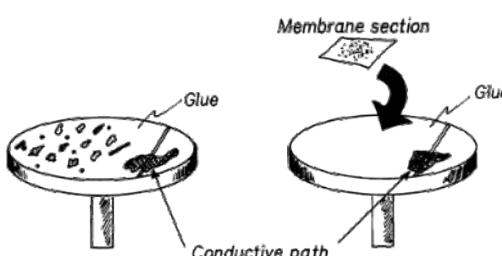


Figure 6. SEM Pedestal Mount.

Use silver or carbon paint to provide a conductive path between the cement surface and the substrate. A single preparation can accommodate from one to several hundred particles, placed in an identifiable pattern.

**Particles** isolated from liquid samples by membrane filtration can be examined by placing a filter membrane on a sample substrate, as described above (see Figure 6). This preparation is especially useful for examining a random sample of particles from a liquid. Membrane or film filters rather than depth filters are recommended, since small particles are easily lost in the open pores of a depth filter. Portions of a random isolate can be used for the SEM examination, and the remainder can be saved for other tests. Alternatively, particles can be dispersed in a solvent and concentrated onto a substrate. If a membrane filter is precoated and used to collect particles, a sample can be examined directly without further coating. This procedure alleviates excessive handling of a specimen and lessens exposure of the specimen to coater (sputter or evaporation) environments and to background conductive film signals.

**Bulk Materials**— Scatter a loose powder over double-sided sticky tape on a substrate; excess material can be blown off with a clean air jet. Liquid adhesives can be used instead of tape. However, rapid filming of an adhesive surface can occur and can hinder particle adhesion. Also, particles may sink into an adhesive before it dries. Pack loose powder into small holes cut into the surface of a metal pedestal. This technique is favored for semiquantitative analyses because it produces a relatively flat surface, is for practical purposes infinitely thick to the electron beam, provides effective grounding, and requires a minimal amount of sample.

**Cement** or clamp large materials directly onto the mounting substrate. Nearly any sample that will fit into a vacuum chamber, withstand evacuated conditions, and be effectively grounded is amenable to analysis. For all the above methods, specimen coating is required. In the absence of coating, a preparation must be viewed at sufficiently low energies, usually  $\leq 5$  kV, to yield suitable micrograph quality.

**Analysis**— The method of probing each particle and obtaining a composition is dependent on the level of information required. Several factors must be considered if semiquantitative analysis is desired. The presence of multiple elements, the type of elements contained in the specimen, and the size and surface characteristics of each particle are a few of the considerations.

Any specimen  $\geq 10$   $\mu$ m in diameter can be probed quickly and without subsequent data reduction. Particles between 10  $\mu$ m and 0.2  $\mu$ m must be considered more carefully, since their size approaches that of the excitation volume of the probe. Elemental characterization of a particle should be conducted on a specimen sufficiently thick that the particle volume is equal to or greater than the volume of X-ray production. Depth of signal excitation (dX) determines the volume of X-ray production. Conditions such as accelerating voltage, source current, and spot size that are appropriate for analyzing a given specimen volume can be determined empirically, if particles are mounted on a metal substrate from which fluorescence can be detected. Substrate fluorescence indicates that the excitation volume has exceeded the volume of the particle. Quantitatively, dX depends on particle characteristics and SEM conditions as follows:

$$d_X = 0.033 \frac{A_{ave}}{dZ_{ave}} (E_O^{1.69} - E_C^{1.69})$$

where  $d_X$  is the depth of signal excitation,  $Z_{ave}$  is the average atomic number,  $A_{ave}$  is the average atomic mass,  $E_O$  is the energy of incident electrons,  $E_C$  is the critical excitation energy of the measured X-ray line, and  $d$  is the density of the particle.

Elemental content is estimated directly from elemental line intensities. Use the following procedures.

- (1) Mount the specimen(s) by any of the above procedures and ensure good electronic and physical alignment of the SEM.
- (2) Tilt or align the specimen toward the detector window at an angle that optimizes collection of the X-rays, i.e., 45° for horizontal detectors.
- (3) Select the symmetrical center of the particle and use the raster mode, rather than the point mode, for the analysis. This eliminates topographical variations and generates an average signal for the sample. Choose a magnification at which the raster is  $\leq 50\%$  of the particle area to eliminate background signal from the substrate.
- (4) Adjust the accelerating voltage to produce adequate signal-to-noise for detection of the element of choice.
- (5) Integrate the signal as long as necessary to achieve statistically significant results. This period can be determined through analysis of reference materials using the SEM conditions of choice (40 seconds per particle is a good working rule).
- (6) Ensure sufficient specimen current. A good working rule is 20% to 40% dead time, as measured by the detector multichannel analyzer (MCA).
- (7) Subtract a background spectrum that is representative of the coating, chamber, and handling.
- (8) Perform an analysis, using exactly the same conditions as above on a reference standard that contains the element of choice.

(9) Perform an analysis, using exactly the same conditions as above on an internal reference (such as the substrate or other metal), for use in normalizing the analytical conditions.

The weight percent of a given element (E) in the specimen can be calculated as

$$\text{Weight \%} = \frac{(I_E/I_{ref}) \text{ Sample}}{(I_E/I_{ref}) \text{ Standard}} \times C^{Estd} \times 100\%$$

where  $I_E$  is the intensity of the element of interest,  $I_{ref}$  is the intensity of the internal reference element, and  $C^{Estd}$  is the concentration of the element of interest in the standard specimen. More accurate quantitative analysis must take into account the effects of matrix type, of interelements, of counting times, and of takeoff angle. The ZAF algorithm used to correct for these effects involves multiplying the measured weight percent by a series of correction factors.

\* Any "superglue," copper diallylphthalate, double-sided sticky tape, or other types of mounting resins can be used. The cement with the least residual organic phase will have the longest stability.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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## 1184 SENSITIZATION TESTING

### INTRODUCTION

This chapter considers sensitization and hypersensitization in the context of medical devices and implants, and describes methodologies for testing such articles for their potential to cause sensitization.

There are four types of hypersensitization reactions according to the Gell and Coombs classification system. Type I reactions involve the fixation of IgE to mast cells that subsequently release pharmacologically active substances, such as histamine. Type II reactions are the result of IgG and/or IgM binding to target cells, followed by complement fixation and cell lysis. Type III reactions are caused by the presence of antigen-antibody complexes that cause physical damage such as kidney damage due to glomerular blockage. Type IV reactions are cell-mediated (involve the action of T cells and their interaction with the human lymphocyte antigens). Type IV reactions are also called delayed-type hypersensitivity reactions.

[Table 1](#) below summarizes the types of reactions, the mediators of the reactions, and examples of representative diseases.

Table 1. The Four Types of Hypersensitization Reactions\*; Mediators, and Disease Examples

Reaction Class	Mediators	Disease Examples
Type I	IgE molecules bound to mast cells interact with antigen to release pharmacologically active substances	Hay fever, bronchial asthma, other atopic reactions
Type II	IgM and/or IgG molecules interact with target cells, fix complement, cell lysis	Various drug allergies, erythroblastosis fetalis, hemolytic anemia, thrombocytopenia
Type III	Antigen-antibody complexes, complement	Arthus reaction, serum sickness, allergic glomerulonephritis
Type IV	T lymphocytes, antigen, monocytes, macrophages	Contact dermatitis

\* According to Gell and Coombs classification scheme

A multi-step process, delineated in chapter [The Biocompatibility of Materials Used in Drug Containers, Medical Devices, and Implants](#) (1031) is followed in determining which, if any, toxicological tests need to be performed on a given article. In some cases, sufficient evidence to satisfy toxicology requirements may be available from previously marketed articles (See Figure 1 in chapter [1031](#)). Important factors addressed in Figure 1 (chapter [1031](#)) include the type and extent of contact with the body, the chemical composition, the manufacturing process, the sterilization process, and, as mentioned above, similarity to previously marketed articles.

If further toxicological testing is necessary, the classification of medical devices provided in Table 2 from general information chapter [1031](#) is important, because the degree and extent of toxicological testing that is required is strongly influenced by the nature and duration of the bodily contact with the article. The classification derived from Table 2 in chapter [1031](#), coupled with the length of exposure to the article, is used in Tables 3–5 of chapter [1031](#) to determine which toxicological tests need to be performed. [Table 2](#) below presents information extracted from Tables 3–5 of chapter [1031](#) and indicates those circumstances for which sensitization testing should be considered.

Table 2. Articles For Which Sensitization Testing Should Be Considered Based on Article Category and Length of Exposure

Device Category	Body Contact	Contact Duration



Surface devices	Skin	A <sup>a</sup> , B <sup>b</sup> , C <sup>c</sup>
	Mucosal membrane	A, B, C
	Breached or compromised surfaces	A, B, C
External communicating devices	Blood path, indirect	A, B, C
	Tissue, bone, or dentin communicating	A, B, C
	Circulating blood	A, B, C
Implant devices	Tissue or bone	A, B, C
	Blood	A, B, C

a A: limited (less than 24 hours)  
b B: prolonged (24 hours to 30 days)  
c C: permanent (more than 30 days)

There are nine test methodologies reviewed in this chapter. [Table 3](#) lists the methods and the species with which they are performed.

Table 3. Test Methodologies That May Be Used in Sensitization Testing, and Species Required for Test

Test	Species Used in Test
Magnusson & Kligman Maximization	Guinea pig
Standard Buehler	Guinea pig
Open Epicutaneous	Guinea pig
Freund's Complete Adjuvant	Guinea pig
Optimization	Guinea pig
Split Adjuvant	Guinea pig
Local Lymph Node Assay	Mouse
Mouse Ear Swelling	Mouse
Vitamin A Enhancement	Mouse

Given the preponderance of testing performed with either the Magnusson & Kligman Guinea Pig Maximization Test (GPMT) or Buehler Tests (BT), those tests will be reviewed in detail in this chapter. A brief summary of the remaining tests is provided as alternatives to the more frequently used procedures.

Each test should be periodically validated in the performing laboratory using positive controls such as hexyl cinnamic aldehyde, mercaptobenzothiazole, or benzocaine (positive controls recommended by the Organization for Economic Cooperation and Development [OECD]).

#### MAGNUSSON & KLIGMAN GUINEA PIG MAXIMIZATION TEST (GPMT)

##### Animals

Either male and female albino guinea pigs or both may be used. All animals should be in good health and weigh between 300 g and 500 g at the start of the experiment. The females should not be pregnant, nor should they have borne young previously. Prior to use, it is essential to acclimatize the animals to the laboratory conditions for at least 5 days. All animals should be handled in accordance with the guidelines in the appropriate regulatory requirements established for the humane treatment of animals. At least 10 test animals and 5 control animals should be used. To obtain sufficient analytical power (i.e., to detect weak sensitizers) it may be necessary to use 20 test animals and 10 control animals. Additional animals may be required to establish the proper doses to administer (see Determination of Test Article Concentration).

##### Housing and Feeding

The animal room should be held at  $20 \pm 3^\circ$ , at 30% to 70% relative humidity, with 12 hours of light and dark. Animals may be housed individually or in group housing. Standard laboratory diets may be used (those satisfactory for guinea pigs ensure an adequate amount of ascorbic acid). Drinking water should be available ad libitum.

##### Animal Pretest Preparation

Animals should be randomized via a validated randomization method. For example, such methods may utilize random number tables or computer-generated random numbers. Sites on the animals intended for test article application (intrascapular region) should have the hair removed in a manner that does not abrade the skin. This may be accomplished via clipping, shaving, or with chemical depilatories. The chemical depilatory must not elicit irritation of its own. General observations of the animals prior to use in the test should be recorded, including any indication of ill health (do not use such animals in tests), and body weights.

##### Test Article Preparation<sup>4</sup>

The use of this test requires that the test article can be injected intradermally. When the test article is not suitable for direct administration, extracts should be prepared according to the procedure provided in general chapter [Biological Reactivity Tests, In Vivo](#) (88).

##### Determination of Test Article Concentration

The purpose of this preliminary study is to determine the concentrations of Test Article Preparation to be used during the initial induction phase and the second challenge phase of a GPMT study. Two or three animals may be used for the concentration determination.

A range of concentrations of the test article, or extracts of the article, should be injected intradermally (0.1 mL per site), using the solvent that will be employed in the Test Procedure. The concentration that causes only mild to moderate irritation (no extensive skin destruction, with no evidence of overt systemic toxicity to the animals) should be used in the Intradermal Injection Induction Phase of the Test Procedure.

Using two or more animals, apply via occlusive dressings and patches, a range of concentrations of test article or extracts of the article. Remove the dressings/patches after 24 hours, and examine the sites for erythema. Choose the concentration that causes only slight erythema for the Topical Application Induction Phase of the Test Procedure. Use the highest concentration of test article or extract that does not cause erythema for the Challenge Phase of the Test Procedure. If the irritation threshold is not reached, then select the highest possible concentration for the Topical Application Induction Phase and Challenge Phase of the Test Procedure.

##### Test Procedure

###### intradermal injection induction phase

This phase requires three pairs of injections administered intradermally, with the test and control injection of each pair on opposite sides intrascapularly. Each injection should contain 0.1 mL, with injection pairs 1 and 2 administered nearer to the head, and injection pair 3 administered slightly farther towards the tail. The pairs are nominally within an area of 8 cm<sup>2</sup>.

The pairs of injections consist of the following:

Injection pair 1: A 1:1 (v/v) mixture of Freund's Complete Adjuvant (FCA), an oil–water emulsion containing mycobacteria, and the appropriate solvent/vehicle (see [Biological Reactivity](#)).

pair 1:



Tests, In Vivo (88). Control animals receive a mixture of FCA and physiological saline (1:1).

The Test Article Preparation in the concentration as specified in Determination of Test Article Concentration, using the appropriate solvent/vehicle. Control animals receive only the solvent/vehicle.

Injection pair 3: The Test Article Preparation in the concentration as specified in Determination of Test Article Concentration in a 1:1 (v/v) mixture with FCA. Control animals receive an injection of a 1:1 (v/v) mixture of FCA and solvent/vehicle.

#### topical application induction phase

Seven days ( $\pm 1$  day) after completion of the Intradermal Injection Induction Phase, administer the test sample by topical application to the intrascapular region of each animal. For both test and control animals, if the Test Article Preparation does not cause skin irritation, apply 10% sodium lauryl sulfate in petrolatum approximately 24 hours before the start of the Topical Application Induction Phase to induce a local irritation.

Test animals should have 2-  $\times$  4-cm pieces of filter paper or absorbent gauze fully loaded with the Test Article Preparation (prepared within 24 hours of use) using the concentration selected in Determination of Test Article Concentration applied to each injection site. The filter paper or absorbent gauze should be secured to the animals using occlusive dressings.

Control animals receive the same treatment, except that the appropriate solvent/vehicle is used instead of the test article.

Remove the dressings and patches approximately 48 hours after application.

#### challenge phase

This phase should occur 14  $\pm$  1 days after the Topical Application Induction Phase. Hair should be removed from the test application sites. Filter paper patches or chambers are soaked with a freshly prepared Test Article Preparation in the concentration specified in Determination of Test Article Concentration. This is done for all test and control animals. The patches or chambers are secured with an occlusive dressing and removed after 24  $\pm$  2 hours.

#### Observations

At approximately 24, 48, and 72 hours after removal of the challenge patches, the application sites should be examined for signs of reactions. Of particular importance are instances where the reaction of the test animals exceeds that of the control animals. All signs of reactivity should be recorded, with particular attention paid to signs of erythema and edema. A true edematous reaction will blanch under gentle pressure. The longer the period of blanching, the greater the severity of edema.

#### Interpretation

There is more than one way of evaluating and grading the results from GPMT. [Tables 4, 5, and 6](#) list details for three such grading systems. Grades of 1 or higher in the test animals, with grades of less than 1 in control animals, are indicative of sensitization. If control animals display grade 1 reactivity, and if the test animals display reactivity above the greatest reactivity seen in the control animals, sensitization due to the test article is again suspected. The percentages in [Table 4](#) need to be revised if there are only 10 test animals (i.e., the categories would be 0, <10%, 10%–30%, 31%–60%, 61%–80%, and 81%–100%). If there are 20 test animals, then multiples of 5% are appropriate.

Table 4. Classification Based on Percent of Responsive Test Animals

% of Positives in Test Group	Assigned Grade Class
0	Nonsensitizer
<8	1 Weak
8–28	2 Mild
29–64	3 Moderate
65–80	4 Strong
81–100	5 Extreme

Table 5. Classification Based on Erythema and Edema Formation

Erythema and Eschar	Grade
No erythema	0
Slight or equivocal erythema	<1
Well-defined erythema	2
Moderate erythema	3
Severe erythema to slight eschar formation	4
Edema	
No edema	0
Slight or equivocal edema	<1
Well-defined edema	2
Moderate edema	3
Severe edema	4

Table 6. Classification Based on Erythema Formation Alone

Erythema formation	Grade
No erythema	0
Discrete or patchy erythema	1
Moderate and confluent erythema	2
Intense erythema and swelling	3

The results should be submitted for statistical analysis (e.g., chi-square contingency table) to determine if the differences in scores between treated and control animals are significant.

The response of the test group versus the control group should be compared statistically. (The Mann-Whitney U test can be used for the comparison.)

#### Rechallenge

The extent of any response in the negative control group, under experimental conditions, shows the irritation potential of the Test Article Preparation. In this case, test and control animals should be rechallenged 1 week later on the untreated side of the animal, with a reduced concentration of the Test Article Preparation. A sensitized guinea pig will react to some degree to both challenges. A weak reaction occurring at a single time point in only one challenge should cast strong doubt as to whether that guinea pig is truly sensitized.<sup>2</sup>

#### STANDARD BUEHLER TESTS (SBT)



## Animals

See Animals in the Magnusson & Kligman Guinea Pig Maximization Test (GPMT).

### Housing and Feeding

See Housing and Feeding in the Magnusson & Kligman Guinea Pig Maximization Test (GPMT).

### Animal Pretest Preparation

See Animal Pretest Preparation under Magnusson & Kligman Guinea Pig Maximization Test (GPMT). The fur of the guinea pig may be removed from one flank by clipping.

### Test Article Preparation

See Test Article Preparation in the Magnusson & Kligman Guinea Pig Maximization Test (GPMT).

### Determination of Test Article Concentration

The purpose of this preliminary study is to determine the concentrations of Test Article Preparation to be used during the initial induction phase and the second challenge phase of an SBT study. Two or three animals may be used for the concentration determination.

A range of concentrations of the test article, or extracts of the article, should be applied using patches (for example, four 4 cm<sup>2</sup> absorbent pads) or chambers. The patches should be held in place using tape (if necessary) and occlusive dressings. The patches should be removed after approximately 6 hours, and any residues of the test chemical are removed from the test site. Observations are made at that time, and at 24 and 48 hours.

The concentration that causes only mild to moderate irritation (slight erythema, with no evidence of overt toxicity to the animals) and can be applied repeatedly to the same site should be used in the Induction Phase of the Test Procedure. Use the highest concentration of test article or extract that does not cause erythema for the Challenge Phase of the Test Procedure.

### Test Procedure

#### induction phase

Apply 0.4 mL of the Test Article Preparation in an appropriate solvent/vehicle at the dose identified in Determination of Test Article Concentration. Use patches similar to those used in Determination of Test Article Concentration. The patches should be applied to one flank (hair clipped off) and held in place occlusively for 6 hours. The animals may need to be restrained to ensure occlusion. Patches and any visible residues should be removed after 6 hours. Control animals also receive patches, but these contain only the appropriate solvent/vehicle. This process should be repeated three times a week for both test and control animals on the same site for three consecutive weeks (weekly intervals are used in the modified Buehler Test).

#### challenge phase

This phase should be carried out 14 days after the last application of the Induction Phase. Clip the hair off the previously untested flank of each animal 24 hours before the challenge application. As in the Induction Phase, apply patches containing the test article (concentration specified in Determination of Test Article Concentration) or solvent/vehicle alone to the untested areas of the test and control animals. To obtain well-defined edges at the application sites, commercial chambers with a lipped edge are preferred. Secure the patches with occlusive dressings, and keep them in place for 6 hours. Remove all patches after 6 hours.

### Observations

At 22 ± 2 hours after removal of the patches, the application sites should have the animal's fur removed via clipping or depilation. After approximately 2 more hours, grade the sites (Tables 4, 5, or 6 may be employed). All signs of reactivity should be recorded, with particular attention paid to signs of erythema and edema. Repeat the grading once again after 24 to 48 hours more have elapsed. The response of the test group versus the control group can be compared statistically. (The Mann-Whitney U test can be used for the comparison.)

### Interpretation

The results should be submitted for a statistical analysis (e.g., chi-square contingency table) to determine if the differences in scores between treated and control animals are significant.

See Interpretation in the Magnusson & Kligman Guinea Pig Maximization Test (GPMT).

### Rechallenge

See Rechallenge in the Magnusson & Kligman Guinea Pig Maximization Test (GPMT).

## OTHER SENSITIZATION TEST PROCEDURES

The Magnusson & Kligman Guinea Pig Maximization Test and the Standard Buehler Tests are the most frequently performed sensitization tests. However, there are a number of other methods that may be useful in the assessment of the potential for sensitization. Some may be applicable to both solid test articles and extracts, some only to extracts.

Where the use of guinea pigs is called for in the following tests, the animals and their housing should meet the requirements as specified for Animals in the Magnusson & Kligman Guinea Pig Maximization Test. The fur of the guinea pig should be removed from test sites as indicated for Animal Pretest Preparation in the Magnusson & Kligman Guinea Pig Maximization Test.

### Draize Test

This was the first predictive test accepted by the regulatory agencies, and is still in use. The test uses guinea pigs and the test article is administered via intradermal injections.

#### test article preparation

This test requires that the test article be in the form of a solution that may be directly applied to the animal's skin. Therefore, extracts of the material would need to be made. See [Biological Reactivity Tests, In Vivo](#) (88) for information on the preparation procedure.

#### induction phase

One flank of each of 20 guinea pigs is shaved, then 0.05 mL of a 0.1% solution of test article is injected into the anterior flank. The next day, and then every other day thereafter up to day 20, 0.1 mL of the test article is injected into a new site on the same flank.

#### challenge phase

This phase begins 2 weeks after the final injection of the Induction Phase. The untreated flank is shaved, then 0.05 mL of test article is injected into each of the 20 guinea pigs. Twenty previously untreated animals serve as the controls, and receive injections of the test article as well.

#### observations

The test sites of all control and test animals are evaluated for erythema at 24 and 48 hours after the challenge injections. The degree of reaction in test animals is compared to the reaction in control animals. A larger and/or more intense response by the test animals versus the control animals is indicative of sensitization.

### Open Epicutaneous Test

This test uses guinea pigs. The goal is to determine the dose required to induce sensitization by simulating human usage via topical application of the test article.



#### test material preparation

This test requires that the test article be in the form of a solution that may be directly applied to the animal's skin. Therefore, extracts of the material need to be made. See [Biological Reactivity Tests, In Vivo](#) (88) for information on the preparation procedure.

#### preliminary testing

A series of concentrations of test article is applied to 2 cm<sup>2</sup> areas of skin on the anterior flank of 6 to 8 guinea pigs (0.025 mL per application). The test sites should be examined for erythema 24 hours after test article administration. The highest concentration that does not cause irritation (maximum nonirritant concentration) and the lowest concentration causing erythema in approximately 25% of the animals (minimum irritant concentration) are determined.

#### induction phase

The test article (or control vehicle) is applied to 8 cm<sup>2</sup> areas of the flank skin of 6 to 8 guinea pigs daily for 3 weeks, or five times a week for 4 weeks. The amount per application is 0.01 mL. A set of increasing concentrations is again employed, ranging from the minimum irritant concentration using a stepwise progression. The test article should be applied to the same sites each time, unless irritation develops, in which case a new site on the same flank should be used. Control animals receive the same series of treatments using the vehicle instead of the test article.

#### challenge phase

Each animal is challenged on the untreated flank 24 to 72 hours after the last Induction Phase treatment using 0.025 mL applied to 2 cm<sup>2</sup> areas. A set of increasing concentrations is used, from minimum irritant concentration to the maximum nonirritant concentration, and five lower concentrations are also used.

#### observations

The test sites are evaluated at 24, 48, and 72 hours post-treatment. The maximum concentration that does not cause irritation in the control group is determined. Animals from the test groups that develop inflammatory responses at concentrations lower than the maximum nonirritating concentration in the controls should be considered to be sensitized.

#### Freund's Complete Adjuvant Test

This test is based upon the use of intradermal injections using the test article in a mixture of Freund's complete adjuvant and distilled water (50:50).

#### test material preparation

Because this test uses intradermal injections, extracts of the test material need to be made in order to use this procedure. See [Biological Reactivity Tests, In Vivo](#) (88) for information on the extraction procedure.

#### preliminary testing

The minimum irritating and the maximum nonirritating concentrations are determined in the same manner as for Preliminary Testing in the Open Epicutaneous Test.

#### induction phase

The test area consists of six 2 cm<sup>2</sup> areas across the shoulders of the guinea pigs. Two groups of 10 to 20 guinea pigs each should be used. The test group animals are injected intradermally with 0.1 mL of a 5% solution of the test article extract in FCA/water. Control animals receive injections with FCA/water without the test article. These injections are repeated every 4 days until a total of three injections have been given.

#### challenge phase

This phase should begin 2 weeks after the last injection of the Induction Phase. Topical applications of 0.025 mL of test article at the minimum irritating and the maximum nonirritating concentrations, plus two lower concentrations, are administered to 2 cm<sup>2</sup> areas of the shaved flank. The test sites should remain uncovered.

#### observations

The test sites are examined for the presence of erythema 24, 48 and 72 hours after the topical applications. The minimum nonirritating concentration in the control animals should be determined. Those test animals that display erythema at concentrations lower than the minimum nonirritating concentration in the control animals should be considered to be sensitized.

#### Optimization Test

This test has some similarities to the older Draize Test. Unlike the Draize Test, however, this test uses both intradermal and topical treatments, and includes adjuvant for some induction injections.

#### test material preparation

As with other test procedures that incorporate intradermal injections, the test article needs to be in a form suitable for injection. See [Biological Reactivity Tests, In Vivo](#) (88) for information on the extraction procedure.

#### induction phase

Twenty test and 20 control guinea pigs are used. A total of 10 intradermal injections should be given to each animal. Test animals receive 0.1 mL of a mixture of 0.1% test article and 0.9% saline (50:50) on day 1, with one injection into a shaved flank, and another into a portion of shaved dorsal skin. Two and 4 days later, one intradermal injection of the test article in saline is given to eight new dorsal sites. Every other day during weeks 2 and 3, the test article is injected intradermally into 10 sites over the shoulders in a 50:50 mixture of saline and FCA. The same sequence of injections is given to the 20 control animals, except that no test article is included with the saline or saline/FCA injections.

#### challenge phase

Thirty-five days after the first injection, the animals are challenged topically with 0.1 mL of the 0.1% solution of test article in saline (for test animals). The control animals receive saline injections only. At 45 days after the first injection, a second topical challenge is given. A nonirritating concentration of test article (0.05 mL) is applied topically to a 1 cm<sup>2</sup> area of untreated skin. This site should then be covered with a 2 cm<sup>2</sup> piece of filter paper, after which an occlusive dressing should be applied. The patch should be removed after 24 hours.

#### observations

Twenty-four hours after each injection during week 1, the thickness of a fold of skin over the injection sites for each animal should be measured using a caliper (mm), and the two largest cross-diameters of each erythematous reaction should be recorded (mm). The reaction volumes are calculated by multiplying the fold thickness by the products of the two cross-diameters (expressed as  $\mu$ L). The mean reaction (+1 SD) volume during week 1 should be calculated for each animal.

Challenge reaction volumes are calculated for each animal following the injections at day 35. If an animal develops a challenge reaction volume greater than its mean reaction volume + 1 SD, it should be considered sensitized.

Following the patch testing challenge, the test sites are evaluated for erythema and edema. Evaluations should be made using [Table 5](#).

The number of positive animals should be compared statistically with the pseudopositive control animals. This should be done for both intradermal injection results and patch testing results. The Fisher exact test may be used.

The results from the intradermal injections and the patch testing, following separate statistical analysis, may be combined and evaluated using [Table 7](#) in order to classify a test article as a strong, moderate, or weak sensitizer; or not a sensitizer.

Table 7. Classification Scheme for Test Articles Based on the Optimization Test



Intradermal % of Positive Animals	Patch Test % of Positive animals	Classification
S*, > 75	and/or S, > 50	Strong sensitizer
S, 50–75	and/or S, 30–50	Moderate sensitizer
S, 30–50	N.S. <sup>‡</sup> , 0–30	Weak sensitizer
N.S., 0–30	N.S., 0	Not a sensitizer

\* S = significant; N.S. = not significant

#### Split Adjuvant Test

This test makes use of both FCA and skin damage. The test article is applied topically.

##### test material preparation

Because this test employs topical test article applications, the article can be either in solid or liquid form. If extracts are to be made, see chapter [Biological Reactivity Tests, In Vivo](#) (88) for extraction procedures.

##### induction phase

Ten to 20 guinea pigs are used for both test and control groups. An area of back skin immediately behind the scapulas should be shaved to the extent that the skin becomes glistening. The shaved areas should then be treated with dry ice for 5 to 10 seconds. A dressing made of loose mesh gauze with stretch adhesive and a 2- x 2-cm opening should be placed over the treated area, then secured with adhesive tape. The test article (0.2 mL of viscous materials, 0.1 mL of liquids, or solid material) is placed within the opening in the dressing on top of the treated skin. Two layers of #2 filter paper should be placed over the test article, then backed by occlusive tape. Then the filter paper/occlusive backed material should be secured to the surrounding dressing with adhesive tape. After 2 days have passed, the filter paper should be lifted from the test sites, and the test article reapplied on the same site. The filter paper and backing should be secured once again. After 2 more days, the filter paper should be lifted and two injections of 0.075 mL of FCA should be administered into the edges of the test site. Then the test material is once again applied, and the filter paper/backing resecured. The test article should be reapplied once more on day 7 and the filter paper/backing resealed. On day 9, the filter paper and all associated dressing material should be removed.

##### challenge phase

On day 22 following the induction treatment, 0.5 mL of test material (or the solid article) should be applied to a 2- x 2-cm area of shaved midback. The test sites should be covered by filter paper and backed by adhesive tape. This should be held in place with an elastic bandage secured with adhesive tape. Control animals receive the same challenge phase treatment. The preparation should be removed after 24 hours.

##### observations

Twenty-four, 48, and 72 hours after the removal of the challenge phase preparation, the test sites should be evaluated for erythema and edema. The grading scheme of [Table 5](#) could be employed.

#### Mouse Ear Swelling Test

There are a number of potential advantages in using mice versus guinea pigs for sensitization methods. The classic guinea pig tests tend to be costly and require a long time to complete. Moreover, with the dependence upon relatively subjective scoring based on edema and erythema, methodological robustness, and ruggedness may be questionable. This test uses mice and employs both topical exposures and injections.

##### animals

Female, 6- to 8-week old CF-1, Balb/c, or Swiss mice should be used. They may be group housed in direct bedding cages. Acclimatization should be for at least 5 to 7 days. Food (appropriate mouse feed) and water should be available ad libitum. No animals with damaged pinnae should be used in the study. The thickness of both ears of each animal should be measured and recorded at this time.

##### test material preparation

As with other test procedures that incorporate intradermal injections, the test article needs to be in a form suitable for injection. See [Biological Reactivity Tests, In Vivo](#) (88) for information on the extraction procedure.

##### preliminary testing

The minimally irritating and maximally nonirritating concentrations of test article for this procedure should be determined. This is done by using four groups of two mice and examining the effects of at least four concentrations of test article.

##### induction phase

The abdomens of the animals should be shaved, then tape-striped using a surgical adhesive tape until the test area is glistening. A single injection of 0.05 mL of FCA is subdivided into two injection sites administered intradermally within the shaved/stripped area, but along the borders. After the adjuvant injections, 100  $\mu$ L of test article (using the minimally irritating concentration) or vehicle (controls) is applied to the center of the shaved test areas. After the test areas dry, the mice should be returned to their cages. The tape stripping and application of test article (but not FCA) is repeated each day for the next 3 days.

##### challenge phase

This phase should occur 7 days after the final topical induction application. The test article (highest nonirritating concentration) should be applied topically (20  $\mu$ L) to one ear, while the opposite ear receives 10  $\mu$ L of vehicle alone. This should be done for both test and control animals.

##### observations

The thickness of both ears of each animal should be recorded after 24 and 48 hours postchallenge. The measurements should be made with a caliper (a spring-loaded caliper is preferable). A sensitized animal is one in which the test article-treated ear is at least 20% thicker than its opposite ear. For the test to be valid, the test article-treated ears of control animals should not be more than 10% thicker than the opposite ears. If the control animal ears do not meet the requirements, the test should be repeated using lower concentrations.

#### Local Lymph Node Test

This test is based on the observation that exposure of the mice to sensitizers can cause hyperplasia of T cells within the auricular lymph nodes of mice. The method combines both in vivo and in vitro phases, and requires the use of radioisotopes. An unusual aspect of this test is that no challenge phase is required.

##### animals

Four groups of four mice at least, male or female CBA/ca mice (only one sex in a given test) between the ages of 8 to 12 weeks should be used.

##### test material preparation

Although in theory one could apply a solid test article to the dorsal surface of the ear of a mouse, in practice an extract of such an article should be used. See [Biological Reactivity Tests, In Vivo](#) (88) for information on the extraction procedure.



#### preliminary testing

A nontoxic concentration of test article should be used. If not already established, a preliminary test for overt toxicity may be required to establish a suitable dose.

#### induction phase

Twenty-five  $\mu$ L of the appropriate test article concentration, or vehicle (controls), should be applied to the dorsal surface of each pinna for 3 consecutive days. Five days after the first treatment, the animals should be injected, via the tail vein, with 2.5 mL of phosphate buffered saline containing 20  $\mu$ Ci of 3H-methyl thymidine. Five hours after the isotopic injection, the animals should be euthanized. The draining auricular lymph nodes should be removed from each animal of each test and control group. The nodes from all animals within a given group should be combined, such that a single cell suspension can be made from each group of animals. The cell suspension can be made by passing the nodes through a 200-mesh stainless steel gauze using a syringe plunger. The cells should then be centrifuged at 190  $\times$  g for 10 minutes, resuspended in 3 mL of 5% trichloroacetic acid (TCA), and held overnight at 4°.

The resulting precipitate should be recovered by centrifugation, and the pelleted precipitate should be resuspended in 1 mL of 5% TCA. The suspension should then be placed in scintillation vials with 10 mL of scintillation fluid, and the disintegrations/minute (dpm) counted with a  $\beta$ -counter.

#### observations

The ratio of dpm for each test group should be compared to the dpm for the control group. If the ratio equals or exceeds 3 for any test group, the concentration of test article used with that group may be considered to be sensitizing.

#### Vitamin A Enhancement Test

This test is similar to the Mouse Ear Swelling Test in that test articles are applied topically to the abdomen, with a challenge application to the ears, followed by measurements of ear thickness. A principal difference is the use of mouse feed supplemented with vitamin A acetate. The purpose of the supplementation is to increase the reactivity of the immune system, thereby increasing the potential sensitization reaction.

#### animals

Male, 3- to 4-week old Balb/c mice should be maintained on a diet supplemented with vitamin A acetate. The diet may be prepared by mixing each kg of feed with 0.477 g of gelatinized vitamin A acetate. The feed mixture should be used within 3 weeks of preparation. Mice intended for use in sensitization studies should have been on the supplemented diet for at least 4 weeks. The mice at the time of the sensitization study should therefore be between 7 and 10 weeks old. The thickness of both ears of each animal should be measured and recorded at this time.

#### test material preparation

Although, in theory, one could apply a solid test article to the dorsal surface of the ear of a mouse, in practice an extract of such an article should be used. See [Biological Reactivity Tests, In Vivo](#) 88 for information on the extraction.

#### preliminary testing

The maximally nonirritating dose and minimally irritating concentrations should be determined using separate groups of animals. This could be done as described for Preliminary Testing in the Mouse Ear Swelling Test.

#### induction phase

The fur of the abdomen and thorax of 10 mice per group should be shaved. Then 100  $\mu$ L of test article (at the minimally irritating concentration) should be applied to the test areas on days 0, 2, 4, 7, and 11. Control animals receive 100  $\mu$ L of vehicle alone on the same schedule.

#### challenge phase

This phase should occur 4 days after the final application of the Induction Phase. Twenty-five  $\mu$ L of test article (at the maximally nonirritating concentration) should be applied to each ear of each animal in the test and control groups.

#### observations

Ear thickness for both ears of each animal should be recorded after 24 and 48 hours postchallenge. The measurements should be made with a caliper (a spring-loaded caliper is preferable). The percent increase in ear thickness should be calculated for each ear by subtracting the pretreatment measurement from the post-treatment measurement, dividing the result by the pretreatment measurement, then multiplying by 100. The response of the test group versus the control group should be compared statistically. (The Mann-Whitney U test could be used for the comparison.)

The results of individual animals should also be calculated. If an increase in ear thickness for an animal from the test group is at least 50% greater than the largest increase of a control animal, that is indicative of sensitization. As an overall evaluation, should the results of the study provide a significant result of the statistical test at  $p < 0.01$  for the control versus test group comparisons, or if at least two test animals have ear thickness increases in excess of 50% of the maximum control thickness changes and the group comparison showed a  $p < 0.05$ , sensitization is indicated for the test article.

1 For further information on sample preparation, see ANSI/AAMI/ISO/CEN Standard 10993-12—1996: Biological Evaluation of Medical Devices—Part 12: Sample Preparation and Reference Materials

2 Baskett D.A. Guinea pig predictive tests for contact hypersensitivity. In *Immunotoxicology and Immunopharmacology*, 2nd ed.; Dean, J.H., Luster, M.I., Munson, A.E., Kimber, I., Eds; Raven Press, Ltd: New York, 1994; pp 693-702.

#### Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

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#### 1191 STABILITY CONSIDERATIONS IN DISPENSING PRACTICE

note—Inasmuch as this chapter is for purposes of general information only, no statement in the chapter is intended to modify or supplant any of the specific requirements pertinent to Pharmacopeial articles, which are given elsewhere in this Pharmacopoeia.

Aspects of drug product stability that are of primary concern to the pharmacist in the dispensing of medications are discussed herein.

Pharmacists should avoid ingredients and conditions that could result in excessive physical deterioration or chemical decomposition of drug preparations, especially when compounding (see [Pharmaceutical Compounding—Nonsterile Preparations](#) 795). The stability and clinical effect of manufactured dosage forms can be greatly compromised by seemingly negligible alterations or inappropriate prescription compounding. Pharmacists should establish and maintain compounding conditions that include the ensuring of drug stability to help prevent therapeutic failure and adverse responses.

Stability—Stability is defined as the extent to which a product retains, within specified limits, and throughout its period of storage and use (i.e., its shelf-life), the same properties and



characteristics that it possessed at the time of its manufacture. Five types of stability generally recognized are shown in the accompanying table.

Criteria for Acceptable Levels of Stability	
Type of Stability	Conditions Maintained Throughout the Shelf Life of the Drug Product
Chemical	Each active ingredient retains its chemical integrity and labeled potency, within the specified limits.
Physical	The original physical properties, including appearance, palatability, uniformity, dissolution, and suspending ability, are retained.
Microbiological	Sterility or resistance to microbial growth is retained according to the specified requirements. Antimicrobial agents that are present retain effectiveness within the specified limits.
Therapeutic	The therapeutic effect remains unchanged.
Toxicological	No significant increase in toxicity occurs.

#### FACTORS AFFECTING PRODUCT STABILITY

Each ingredient, whether therapeutically active or pharmaceutically necessary, can affect the stability of drug substances and dosage forms. The primary environmental factors that can reduce stability include exposure to adverse temperatures, light, humidity, oxygen, and carbon dioxide. The major dosage form factors that influence drug stability include particle size (especially in emulsions and suspensions), pH, solvent system composition (i.e., percentage of "free" water and overall polarity), compatibility of anions and cations, solution ionic strength, primary container, specific chemical additives, and molecular binding and diffusion of drugs and excipients. In dosage forms, the following reactions usually cause loss of active drug content, and they usually do not provide obvious visual or olfactory evidence of their occurrence.

**Hydrolysis**— Esters and  $\beta$ -lactams are the chemical bonds that are most likely to hydrolyze in the presence of water. For example, the acetyl ester in aspirin is hydrolyzed to acetic acid and salicylic acid in the presence of moisture, but in a dry environment the hydrolysis of aspirin is negligible. The aspirin hydrolysis rate increases in direct proportion to the water vapor pressure in an environment.

The amide bond also hydrolyzes, though generally at a slower rate than comparable esters. For example, procaine (an ester) will hydrolyze upon autoclaving, but procainamide will not. The amide or peptide bond in peptides and proteins varies in the lability to hydrolysis.

The lactam and azomethine (or imine) bonds in benzodiazepines are also labile to hydrolysis. The major chemical accelerators or catalysts of hydrolysis are adverse pH and specific chemicals (e.g., dextrose and copper in the case of ampicillin hydrolysis).

**Epimerization**— Members of the tetracycline family are most likely to incur epimerization. This reaction occurs rapidly when the dissolved drug is exposed to a pH of an intermediate range (higher than 3), and it results in the steric rearrangement of the dimethylamino group. The epimer of tetracycline, epitetracycline, has little or no antibacterial activity.

**Decarboxylation**— Some dissolved carboxylic acids, such as p-aminosalicylic acid, lose carbon dioxide from the carboxyl group when heated. The resulting product has reduced pharmacological potency.

$\beta$ -Keto decarboxylation can occur in some solid antibiotics that have a carbonyl group on the  $\beta$ -carbon of a carboxylic acid or a carboxylate anion. Such decarboxylations will occur in the following antibiotics: carbenicillin sodium, carbenicillin free acid, ticarcillin sodium, and ticarcillin free acid.

**Dehydration**— Acid-catalyzed dehydration of tetracycline forms epianhydrotetracycline, a product that both lacks antibacterial activity and causes toxicity.

**Oxidation**— The molecular structures most likely to oxidize are those with a hydroxyl group directly bonded to an aromatic ring (e.g., phenol derivatives such as catecholamines and morphine), conjugated dienes (e.g., vitamin A and unsaturated free fatty acids), heterocyclic aromatic rings, nitroso and nitrite derivatives, and aldehydes (e.g., flavorings). Products of oxidation usually lack therapeutic activity. Visual identification of oxidation, for example, the change from colorless epinephrine to its amber colored products, may not be visible in some dilutions or to some eyes.

Oxidation is catalyzed by pH values that are higher than optimum, polyvalent heavy metal ions (e.g., copper and iron), and exposure to oxygen and UV illumination. The latter two causes of oxidation justify the use of antioxidant chemicals, nitrogen atmospheres during ampul and vial filling, opaque external packaging, and transparent amber glass or plastic containers.

**Photochemical Decomposition**— Exposure to, primarily, UV illumination may cause oxidation (photo-oxidation) and scission (photolysis) of covalent bonds. Nifedipine, nitroprusside, riboflavin, and phenothiazines are very labile to photo-oxidation. In susceptible compounds, photochemical energy creates free radical intermediates, which can perpetuate chain reactions.

**Ionic Strength**— The effect of the total concentration of dissolved electrolytes on the rate of hydrolysis reactions results from the influence of ionic strength on interionic attraction. In general, the hydrolysis rate constant is inversely proportional to the ionic strength with oppositely charged ions (e.g., drug cation and excipient anions) and directly proportional to the ionic strength with ions of like charge. A reaction that produces an ion of opposite charge to the original drug ion because of the increasing ionic strength, can increase the drug hydrolysis rate as the reaction proceeds. High ionic strength of inorganic salts can also reduce the solubility of some other drugs.

**pH Effect**— The degradation of many drugs in solution accelerates or decelerates exponentially as the pH is decreased or increased over a specific range of pH values. Improper pH ranks with exposure to elevated temperature as a factor most likely to cause a clinically significant loss of drug, resulting from hydrolysis and oxidation reactions. A drug solution or suspension, for example, may be stable for days, weeks, or even years in its original formulation, but when mixed with another liquid that changes the pH, it degrades in minutes or days. It is possible that a pH change of only 1 unit (e.g., from 4 to 3 or 8 to 9) could decrease drug stability by a factor of 10 or greater.

A pH buffer system, which is usually a weak acid or base and its salt, is a common excipient used in liquid preparations to maintain the pH in a range that minimizes the drug degradation rate. The pH of drug solutions may also be either buffered or adjusted to achieve drug solubility. For example, pH in relation to pKa controls the fractions of the usually more soluble ionized and less soluble nonionized species of weak organic electrolytes.

The influence of pH on the physical stability of two phase systems, especially emulsions, is also important. For example, intravenous fat emulsion is destabilized by acidic pH.

**Interionic (IonN<sup>+</sup>-IonN<sup>-</sup>) Compatibility**— The compatibility or solubility of oppositely charged ions depends mainly on the number of charges per ion and the molecular size of the ions. In general, polyvalent ions of opposite charge are more likely to be incompatible. Thus, an incompatibility is likely to occur upon the addition of a large ion with a charge opposite to that of the drug.

**Solid State Stability**— Solid state reactions are relatively slow; thus, stability of drugs in the solid state is rarely a dispensing concern. The degradation rate of dry solids is usually characterized by first-order kinetics or a sigmoid curve. Therefore, solid drugs with lower melting point temperatures should not be combined with other chemicals that would form a eutectic mixture.

When moisture is present, the solid drug decomposition may change to zero-order chemical kinetics because the rate is controlled by the relatively small fraction of the drug that exists in a saturated solution, which is located (usually imperceptibly) at the surface or in the bulk of the solid drug product.

**Temperature**— In general, the rate of a chemical reaction increases exponentially for each  $10^{\circ}$  increase in temperature. This relationship has been observed for nearly all drug hydrolysis and some drug oxidation reactions. The actual factor of rate increase depends on the activation energy of the particular reaction. The activation energy is a function of the specific reactive bond and the drug formulation (e.g., solvent, pH, additives). As an example, consider a hydrolyzable drug that is exposed to a  $20^{\circ}$  increase in temperature, such as that from cold to controlled room temperature (see General Notices and Requirements). The shelf life of the drug at controlled room temperature should be expected to decrease to one-fourth to one-twenty-fifth of its shelf life under refrigeration.

The pharmacist should also be aware that inappropriately cold temperatures may cause harm. For example, refrigeration may cause extreme viscosity in some liquid drugs and cause supersaturation in others. Freezing may either break or cause a large increase in the droplet size of emulsions; it can denature proteins; and in rare cases, it can cause less soluble



polymorphic states of some drugs to form.

#### STABILITY STUDIES IN MANUFACTURING

The scope and design of a stability study vary according to the product and the manufacturer concerned. Ordinarily the formulator of a product first determines the effects of temperature, light, air, pH, moisture, trace metals, and commonly used excipients or solvents on the active ingredient(s). From this information, one or more formulations of each dosage form are prepared, packaged in suitable containers, and stored under a variety of environmental conditions, both exaggerated and normal. See [Pharmaceutical Stability](#) 1150. At appropriate time intervals, samples of the product are assayed for potency by use of a stability-indicating method, observed for physical changes, and, where applicable, tested for sterility and or for resistance to microbial growth and for toxicity and bioavailability. Such a study, in combination with clinical and toxicological results, enables the manufacturer to select the optimum formulation and container and to assign recommended storage conditions and an expiration date for each dosage form in its package.

#### Responsibility of Pharmacists

Pharmacists help to ensure that the products under their supervision meet acceptable criteria of stability by (1) dispensing oldest stock first and observing expiration dates, (2) storing products under the environmental conditions stated in the individual monographs, labeling, or both, (3) observing products for evidence of instability, (4) properly treating and labeling products that are repackaged, diluted, or mixed with other products, (5) dispensing in the proper container with the proper closure, and (6) informing and educating patients concerning the proper storage and use of the products, including the disposition of outdated or excessively aged prescriptions.

**Rotation of Stock and Observance of Expiration Dates**— Proper rotation of stock is necessary to ensure the dispensing of suitable products. A product that is dispensed infrequently should be closely monitored so that old stocks are given special attention, particularly with regard to expiration dates. The manufacturer can guarantee the quality of a product up to the time designated as its expiration date only if the product has been stored in the original container under recommended storage conditions.

**Storage under Recommended Environmental Conditions**— In most instances, the recommended storage conditions are stated on the label, in which case it is imperative to adhere to those conditions. They may include a specified temperature range or a designated storage place or condition (e.g., "refrigerator," or "controlled room temperature") as defined in the General Notices. Supplemental instructions, such as a direction to protect the product from light, also should be followed carefully. Where a product is required to be protected from light and is in a clear or translucent container enclosed in an opaque outer covering, such outer covering is not to be removed and discarded until the contents have been used. In the absence of specific instructions, the product should be stored at controlled room temperature (see **Storage Temperature** in the General Notices). The product should be stored away from locations where excessive or variable heat, cold, or light prevails, such as those near heating pipes or fluorescent lighting.

**Observing Products for Evidence of Instability**— Loss of potency usually results from a chemical change, the most common reactions being hydrolysis, oxidation-reduction, and photolysis. Chemical changes may also occur through interaction between ingredients within a product, or rarely between product and container. An apparent loss of potency in the active ingredient(s) may result from diffusion of the drug into, or its combination with, the surface of the container-closure system. An apparent gain in potency usually is caused by solvent evaporation or by leaching of materials from the container-closure system.

The chemical potency of the active ingredient(s) is required to remain within the limits specified in the monograph definition. Potency is determined by means of an assay procedure that differentiates between the intact molecule and its degradation products. Chemical stability data should be available from the manufacturer. Although chemical degradation ordinarily cannot be detected by the pharmacist, excessive chemical degradation sometimes is accompanied by observable physical changes. In addition, some physical changes not necessarily related to chemical potency, such as change in color and odor, formation of a precipitate, or clouding of solution, may serve to alert the pharmacist to the possibility of a stability problem. It should be assumed that a product that has undergone a physical change not explained in the labeling may also have undergone a chemical change, and such a product is never to be dispensed. Excessive microbial growth, contamination, or both, may also appear as a physical change. A gross change in a physical characteristic such as color or odor is a sign of instability in any product. Other common physical signs of deterioration of dosage forms include the following.

**Solid Dosage Forms**— Many solid dosage forms are designed for storage under low-moisture conditions. They require protection from environmental water and therefore should be stored in tight containers (see **Containers** in the General Notices) or in the container supplied by the manufacturer. The appearance of fog or liquid droplets, or clumping of the product, inside the container signifies improper conditions. The presence of a desiccant inside the manufacturer's container indicates that special care should be taken in dispensing. Some degradation products, for example, salicylic acid from aspirin, may sublime and be deposited as crystals on the outside of the dosage form or on the walls of the container.

**hard and soft gelatin capsules**— Since the capsule formulation is encased in a gelatin shell, a change in gross physical appearance or consistency, including hardening or softening of the shell, is the primary evidence of instability. Evidence of release of gas, such as a distended paper seal, is another sign of instability.

**uncoated tablets**— Evidence of physical instability in uncoated tablets may be shown by excessive powder and/or pieces (i.e., crumbling as distinct from breakage) of tablet at the bottom of the container (from abraded, crushed, or broken tablets); cracks or chips in tablet surfaces; swelling; mottling; discoloration; fusion between tablets; or the appearance of crystals that obviously are not part of the tablet itself on the container walls or on the tablets.

**coated tablets**— Evidence of physical instability in coated tablets is shown by cracks, mottling, or tackiness in the coating and the clumping of tablets.

**dry powders and granules**— Dry powders and granules that are not intended for constitution into a liquid form in the original container may cake into hard masses or change color, which may render them unacceptable.

**powders and granules intended for constitution as suspensions**— Dry powders and granules intended for constitution into solutions or suspensions require special attention. Usually such forms are antibiotics or vitamins that are particularly sensitive to moisture. Since they are always dispensed in the original container, they generally are not subject to contamination by moisture. However, an unusual caked appearance necessitates careful evaluation, and the presence of a fog or liquid droplets inside the container generally renders the preparation unfit for use. Presence of an objectionable odor also may be evidence of instability.

**effervescent tablets, granules, and powders**— Effervescent products are particularly sensitive to moisture. Swelling of the mass or development of gas pressure is a specific sign of instability, indicating that some of the effervescent action has occurred prematurely.

**Liquid Dosage Forms**— Of primary concern with respect to liquid dosage forms are homogeneity and freedom from excessive microbial contamination and growth. Instability may be indicated by cloudiness or precipitation in a solution, breaking of an emulsion, nonresuspendable caking of a suspension, or organoleptic changes. Microbial growth may be accompanied by discoloration, turbidity, or gas formation.

**solutions, elixirs, and syrups**— Precipitation and evidence of microbial or chemical gas formation are the two major signs of instability.

**emulsions**— The breaking of an emulsion (i.e., separation of an oil phase that is not easily dispersed) is a characteristic sign of instability; this is not to be confused with creaming, an easily redispersible separation of the oil phase that is a common occurrence with stable emulsions.

**suspensions**— A caked solid phase that cannot be resuspended by a reasonable amount of shaking is a primary indication of instability in a suspension. The presence of relatively large particles may mean that excessive crystal growth has occurred.

**tinctures and fluidextracts**— Tinctures, fluidextracts, and similar preparations usually are dark because they are concentrated, and thus they should be scrutinized carefully for evidence of precipitation.

**sterile liquids**— Maintenance of sterility is of course critical for sterile liquids. The presence of microbial contamination in sterile liquids usually cannot be detected visually, but any haze, color change, cloudiness, surface film, particulate or flocculent matter, or gas formation is sufficient reason to suspect possible contamination. Clarity of sterile solutions intended for ophthalmic or parenteral use is of utmost importance. Evidence that the integrity of the seal has been violated on such products should make them suspect.

**Semisolids (Creams, Ointments, and Suppositories)**— For creams, ointments, and suppositories, the primary indication of instability is often either discoloration or a noticeable change in consistency or odor.

**creams**— Unlike ointments, creams usually are emulsions containing water and oil. Indications of instability in creams are emulsion breakage, crystal growth, shrinking due to evaporation of water, and gross microbial contamination.



ointments— Common signs of instability in ointments are a change in consistency and excessive “bleeding” (i.e., separation of excessive amounts of liquid) and formation of granules or grittiness.

suppositories— Excessive softening is the major indication of instability in suppositories, although some suppositories may dry out and harden or shrivel. Evidence of oil stains on packaging material should warn the pharmacist to examine individual suppositories more closely by removing any foil covering. As a general rule (although there are exceptions), suppositories should be stored in a refrigerator (see Storage Temperature in the General Notices).

Proper Treatment of Products Subjected to Additional Manipulations— In repackaging, diluting a product or mixing it with another product, the pharmacist may become responsible for its stability.

Repackaging— In general, repackaging is inadvisable. However, if repackaging is necessary, the manufacturer should be consulted concerning potential problems. In the filling of prescriptions, it is essential that suitable containers be used. Appropriate storage conditions and, when appropriate, an expiration date and beyond use date should be indicated on the label of the prescription container. Single-unit packaging calls for care and judgment and for strict observance of the following guidelines: (1) use appropriate packaging materials, (2) if stability data on the new package are not available, repackage at any one time only sufficient stock for a limited time, (3) include on the unit-dose label a lot number and an appropriate beyond-use date, (4) if a sterile product is repackaged from a multiple-dose vial into unit-dose (disposable) syringes, discard the latter if not used within 24 hours, unless data are available to support longer storage, (5) if quantities are repackaged in advance of immediate need, maintain suitable repackaging records showing name of manufacturer, lot number, date, and designation of persons responsible for repackaging and for checking (see General Notices), (6) if safety closures are required, use container closure systems that ensure compliance with compendial and regulatory standards for storage.

Dilution or Mixing— If a product is diluted, or if two products are mixed, the pharmacist should observe good professional and scientific procedures to guard against incompatibility and instability. For example, tinctures such as those of belladonna and digitalis contain high concentrations of alcohol to dissolve the active ingredient(s), and they may develop a precipitate if they are diluted or mixed with aqueous systems. Pertinent technical literature and labeling should be consulted routinely; it should be current literature, because at times formulas are changed by the manufacturer. If a particular combination is commonly used, consultation with the manufacturer(s) is advisable. Since the chemical stability of extemporaneously prepared mixtures is unknown, the use of such combinations should be discouraged; if such a mixture involves an incompatibility, the pharmacist might be responsible. Oral antibiotic preparations constituted from powder into liquid form should never be mixed with other products.

Combining parenteral products necessitates special care, particularly in the case of intravenous solutions, primarily because of the route of administration. This area of practice demands the utmost in care, aseptic technique, judgment, and diligence. Because of potential unobservable problems with respect to sterility and chemical stability, all extemporaneous parenteral preparations should be used within 24 hours unless data are available to support longer storage.

Informing and Educating the Patient— As a final step in meeting responsibility for the stability of drugs dispensed, the pharmacist is obligated to inform the patient about the proper storage conditions (for example, in a cool, dry place—not in the bathroom) for both prescription and nonprescription products, and to suggest a reasonable estimate of the time after which the medication should be discarded. When beyond-use dates are applied, the pharmacist should emphasize to the patient that the dates are applicable only when proper storage conditions are observed. Patients should be encouraged to clean out their drug storage cabinets periodically.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Desmond G. Hunt, Ph.D.</a> Scientist 1-301-816-8341	(PS05) Packaging and Storage 05

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#### 1196 PHARMACOPEIAL HARMONIZATION

This general information chapter provides information about the concept of harmonization by the Pharmacopeial Discussion Group (PDG). The chapter provides: (1) the PDG Policy Statement; (2) the PDG Working Procedures; (3) a discussion; (4) a status report; and (5) a glossary.

#### HARMONIZATION POLICY

The following policy statement was approved by the PDG at its September 2002 meeting.

##### General Information

In 1989, the PDG was formed with representatives from the European Directorate for the Quality of Medicines in the Council of Europe, the United States Pharmacopeial Convention, Inc., and the Japanese Pharmacopoeia in the Ministry of Health and Welfare—now the Ministry of Health, Labor, and Welfare (MHLW). Since that time, the PDG generally meets twice a year to work on pharmacopeial harmonization topics. In May 2001, the PDG welcomed the World Health Organization as an observer. While not part of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), the PDG usually meets in conjunction with the ICH and provides the ICH Steering Committee with reports of its progress. To facilitate harmonization of some ICH Quality guidelines and the Quality section of the Common Technical Document, the PDG representatives sometimes attend ICH expert working group discussions as observers.

##### Purpose

A pharmacopeial monograph for an active ingredient or excipient, preparation, or other substance used in the manufacture or compounding of a medicinal product generally provides a name, definition, description, and sometimes packaging, labeling, and storage statements. Thereafter, the monograph provides tests, procedures, and acceptance criteria that constitute the specification. For frequently cited procedures, a monograph may refer to a general chapter for editorial convenience. The PDG works to harmonize excipient monographs and general chapters. This will reduce manufacturers' burden of performing analytical procedures in different ways, using different acceptance criteria. At all times, the PDG works to maintain an optimal level of science consistent with protection of the public health.

##### Definition of Harmonization

The PDG has defined harmonization of a pharmacopeial monograph or general chapter as follows:

“A pharmacopeial general chapter or other pharmacopeial document is harmonized when a pharmaceutical substance or product tested by the document's harmonized procedure yields the same results and the same accept/reject decision is reached.”

When using a fully harmonized pharmacopeial monograph or general chapter, an analyst will perform the same procedures and reach the same accept/reject decisions irrespective of which PDG pharmacopeia is referenced. This approach is called interchangeability, and each pharmacopeia will identify, in an appropriate manner, such a monograph or general chapter.

When full harmonization of a pharmacopeial monograph or general chapter is not possible, the PDG works to harmonize it using an approach termed harmonization by attribute. In this approach, some elements of a monograph or general chapter may be harmonized, but others may not. When a monograph is harmonized by attribute, a combination of approaches is needed. For nonharmonized elements, reliance on the individual PDG pharmacopeia is necessary.

##### Process

Harmonization of pharmacopeial documents in the PDG is based upon decisions of the expert bodies of each pharmacopeia. The PDG works transparently in many ways, principally through the public notice and comment procedures of each pharmacopeia. The details are described below under PDG Working Procedures.

##### Implementation



The implementation of a harmonized document varies in the three PDG regions, depending upon their legal requirements, need for translation, and publication schedules. Each pharmacopeia generally allows some period of time after publication to implement official harmonized texts to allow manufacturers and other users to achieve conformity.

Harmonization is not achieved until the text becomes official in all three pharmacopeias.

#### Revision of Harmonized Monographs

The pharmacopeias participating in the PDG have agreed not to revise unilaterally any harmonized document after publication. Should revisions be necessary for any appropriate reasons, the initiating pharmacopeia notifies the PDG, and revision proceeds according to the PDG Working Procedures.

#### PDG WORKING PROCEDURES

Working Procedures of the PDG were updated at the October 2006 PDG meeting.

##### General

Harmonization may be carried out retrospectively for existing monographs or chapters or prospectively for new monographs or chapters.

The three pharmacopeias have a commitment to respect the agreed working procedures and the associated time deadlines as an essential part of the harmonization procedure.

Harmonization of pharmacopeial documents in the PDG is performed on the basis of decisions of the expert bodies of each pharmacopeia. The PDG works transparently in many ways, but principally through the public notice and comment procedures of each pharmacopeia.

Where necessary, meetings of experts are held to identify potential solutions to difficult problems.

The specific stages of the PDG procedure (Process) involved in harmonization are described below.

##### Stage 1: Identification

On the basis of an inquiry among its users, the PDG identifies subjects to be harmonized among PDG pharmacopeias and nominates a coordinating pharmacopeia for each subject.

The PDG distributes the work by consensus among the three pharmacopeias and strives for a balance in the distribution of assignments to coordinating pharmacopeias.

##### Stage 2: Investigation

For a subject to be harmonized retrospectively, the coordinating pharmacopeia collects the information on the existing specifications in the three pharmacopeias, on the grades of products marketed, and on the potential analytical procedures.

The coordinating pharmacopeia prepares a draft monograph or chapter, accompanied by a report giving the rationale for the proposal with validation data.

Stage 2 ends with the proposal draft, which is mentioned in this procedure as a Stage 3 draft. The Stage 3 draft, accompanied by supporting comments or data that explain the reasons for each test procedure or limit proposed, is sent by the coordinating pharmacopeia to the secretariats of the other two PDG pharmacopeias.

##### Stage 3: Proposal for Expert Committee Review

The three pharmacopeias forward the Stage 3 draft to their expert committee (through meetings or consultation by correspondence).

Comments by the experts resulting from this preliminary survey are sent to their respective pharmacopeial secretariat, preferably within 2 months. However, the comment period should not exceed 4 months. Within 2 months of receipt of the comments, the pharmacopeial secretariat should consolidate the comments and forward them to the coordinating pharmacopeia.

The coordinating pharmacopeia reviews the comments received and prepares a harmonized document (Stage 4 draft) accompanied by a commentary discussing comments received about the previous text and providing reasons for action taken in response to those comments.

The Stage 4 draft, as far as possible written in global style—a style easily understood by a variety of readers—together with the commentary, is sent to the secretariats of the other pharmacopeias (end of Stage 3).

##### Stage 4: Official Inquiry

The Stage 4 draft and the commentary are published in the revision document or forum of each pharmacopeia in a section entitled International Harmonization. The draft is published in its entirety.

The corresponding secretariats may have to add information essential to the understanding of the implementation of the texts (e.g., the description of an analytical procedure or of reagents that do not exist in the pharmacopeia), and a translation is added by the European and Japanese Pharmacopeias. The style may be adapted to that of the pharmacopeia concerned, or global style may be used. The three pharmacopeias endeavor to publish the drafts simultaneously or as closely as possible.

Comments regarding this draft are sent by readers of the revision document to their respective pharmacopeial secretariat, preferably within 4 months and at most within 6 months of its publication.

Each pharmacopeia analyzes the comments received and submits its consolidated comments to the coordinating pharmacopeia within 2 months of the end of the review or comment period.

The coordinating pharmacopeia reviews the comments received and prepares a draft harmonized document (Stage 5A draft), accompanied by a commentary discussing comments received regarding the previous text and providing reasons for action taken in response to those comments.

The Stage 5A draft, together with the commentary, is sent to the secretariats of the other two PDG pharmacopeias.

##### Stage 5. Consensus

###### 5a. provisional

The Stage 5A draft is reviewed and commented on by the other two PDG pharmacopeias within 4 months of receipt. The three pharmacopeias shall do their utmost to reach full agreement at this stage to obtain a final consensus document.

If a consensus has not been reached, the coordinating pharmacopeia prepares a revised version (Stage 5A/2), taking into consideration relevant, substantiated comments on the Stage 5A document from the two other pharmacopeias. The revised document (Stage 5A/2), together with the commentary, is sent to the secretariats of the other two PDG pharmacopeias. The revised document is reviewed and commented on by the other two PDG pharmacopeias, preferably within 2 months of receipt. This review or comment and revision process of the 5A document is repeated (Stage 5A/n) until the three PDG pharmacopeias reach a consensus or until the coordinating pharmacopeia considers that harmonization by attribute should be applied.

If the coordinating pharmacopeia considers certain attributes in the monograph or provisions in a general chapter (especially for retroactive harmonization) are such that it will not be possible to harmonize within a reasonable time period, harmonization by attribute will be applied. If harmonization by attribute is applied, a special cover page (see the table in the Appendix) indicating harmonization is included with the draft. The text contains harmonized attributes and provisions, and nonharmonized and local attributes are not included. The nonharmonized attributes are clearly indicated in the text as such. The table is prepared as follows: if three pharmacopeias agree on the attribute, there will be a (+) in all columns; if two pharmacopeias agree that the attribute should be included and have agreed on the method and limit, there will be a (+) in the column for those two pharmacopeias, and a (-) in the column for the pharmacopeia that will not stipulate the test.

For nonharmonized or local requirements, if three pharmacopeias agree that the attribute should be included, but have not come to agreement on the method or limit: state attribute



under "nonharmonized attributes." If only one pharmacopeia will include an attribute: state under "local requirement."

If the Stage 5A draft is substantially different from the Stage 4 draft, the PDG may decide that it should be published again in the revision documents; the draft then reverts technically to Stage 4, revised.

#### 5b. draft sign-off

When agreement is reached, the 5B draft is sent by the coordinating pharmacopeia to the other pharmacopeias no later than 4 weeks before a PDG meeting for final confirmation. The document is then presented for sign-off at the PDG meeting. This document includes nonharmonized attributes clearly marked as such.

#### Stage 6: Regional Adoption and Implementation

note—The last two stages of the implementation of the "harmonized" chapters and monographs take place independently according to the procedures established by each pharmacopeial organization.

##### 6a. regional adoption and publication

The document is submitted for adoption to the organization responsible for each pharmacopeia. Each pharmacopeia incorporates the harmonized draft according to its own procedures.

Adopted texts are published by the three pharmacopeias in their supplements, or where applicable, in a new edition.

If necessary, the Stage 5B draft may be adopted with some amendments (local requirements) corresponding to a general policy in the national or regional (European) area. If a pharmacopeia includes a local attribute after the sign-off of a text, it will inform the PDG. It is, however, preferred to include the nonharmonized text in Stage 5B as an alert to the other pharmacopeias that there will be some differences in text in the final document.

Users of the pharmacopeias are appropriately informed of the harmonization status of monographs and general chapters. In the European Pharmacopoeia (EP) and USP–NF, for general chapters, this is done via a preliminary paragraph. For the Japanese Pharmacopoeia (JP), a notification is made by the MHLW, and information is given in a general chapter.

##### 6b. implementation

The pharmacopeias will inform each other of the date of implementation in their particular region.

The date of implementation of a harmonized document varies in the three PDG regions depending on their legal requirements, need of translation, and publication schedules. Each pharmacopeia generally allows some period of time after publication for implementation to allow manufacturers and other users to achieve conformity. Harmonization is not achieved until the text becomes official in all three pharmacopeias.

##### 6c. indication of harmonization

Each pharmacopeia will introduce a statement indicating the harmonization status. EP and USP reference the corresponding text of the other PDG pharmacopeias. JP references the harmonized text. In case of residual differences, these are indicated by a specific symbol (black diamond ♦).

Harmonization is achieved when all pharmacopeias have highlighted harmonization and any residual differences, based on a general policy in the national or regional area.

Concurrent to Stages 6A, B, and C, a dialogue is opened between PDG and ICH Q4B Expert Working Group for the purpose of obtaining regulatory acceptance of the harmonized text.

The coordinating pharmacopeia provides documents to ICH Q4B EWG as defined in the ICH Q4B Guideline.

#### Stage 7: Inter-Regional Acceptance

Following the Q4B evaluation process, a formal notification of regulatory acceptance is posted by ICH.

A topic-specific annex to the Q4B guideline for each monograph or chapter concerned is processed for publishing and implementation by each regional authority.

#### Revision

The procedure for the revision of harmonized monographs and chapters is as follows.

The pharmacopeias participating in the PDG have agreed not to revise unilaterally any harmonized document (monograph or chapter) after sign-off or after publication.

A pharmacopeia requesting the revision of a monograph or chapter shall apply the following criteria for justification of the revision:

- Public health and safety reasons.
- Insufficient supply of pharmacopeial-quality product on the market.
- Specified analytical reagents or equipment unavailable.
- New methods of preparation of products or reagents not covered by the current monograph.
- Analytical procedures capable of being replaced by more appropriate, accurate, or precise procedures.

The PDG as a whole has to agree to initiate the revision. A coordinating pharmacopeia will be nominated. The coordinating pharmacopeia will prepare a Stage 3 draft, based on the validation of data provided by the pharmacopeia requesting the revision.

The PDG Working Procedures will then be followed. The revisions of a sign-off document prepared for this or other reasons are indicated as revision 1, 2, 3, etc.

In case of health and safety issues, and whenever agreed to by the PDG, an accelerated procedure shall be applied (shortening or eliminating stages).

#### Discussion

Harmonization of general chapters and monographs benefits manufacturers of pharmaceutical products intended for human use, regulatory agencies, and ultimately, practitioners and patients. Benefits are derived from (1) reduced development effort; (2) simplification of regulatory filings; and (3) reduced release testing.

Pharmacopeial harmonization amplifies the work of the ICH, particularly for Quality topics. While the PDG is not part of the ICH, the PDG periodically provides updates to the ICH Steering Committee, and in the past participated in a joint task force. This task force focused on harmonization of general chapters considered important to the ICH harmonized document Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances (Q6A). USP also participates in the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Products (VICH). As with the ICH, some of the quality guidelines developed in VICH depend upon harmonization of pharmacopeial general chapters. A major difference between the PDG and ICH/VICHs is that the ICH/VICH guidelines generally are applicable only to ingredients and drug products not previously registered in an ICH/VICH region or nation, whereas the PDG harmonization applies to all marketed products in the applicable region or nation.

In the case of harmonization by attribute, nonharmonization or divergence will be indicated in USP–NF and EP by the symbol ♦. For these nonharmonized attributes, reliance upon the individual pharmacopeia is required. A monograph or general chapter in one PDG pharmacopeia may unilaterally include additional local or national attributes that are not included in the corresponding text of the other pharmacopeias. Such text is not considered by the PDG to be a divergence from the PDG harmonized text.

As with other USP–NF revisions, draft harmonization texts are published for comment in Pharmacopeial Forum. Final harmonized official text in USP–NF is presented in the latest edition, Supplement, or Interim Revision Announcement. The current status of all harmonization projects appears in [Table 1](#) and [Table 2](#). These status tables will be updated in subsequent editions of USP–NF and its Supplements.

In the U.S., cases of noncompliance or dispute are resolved through performance of the official procedure in USP or NF. If the procedure and its acceptance criteria are harmonized in the PDG, then a manufacturer may follow the relevant compendial instructions in USP–NF, EP, or JP.

Table 1. Status of Harmonization—Excipient



## Monographs

Excipient Name	Coordinating Pharmacopeia	Harmonization Stage
Alcohol (Rev 2)	EP	4
Benzyl Alcohol (Rev 1)	EP	6
Dehydrated Alcohol (Rev 2)	EP	4
Butylparaben	EP	6
Calcium Carbonate	USP	4
Calcium Disodium Eddetate	JP	6
Calcium Phosphate Dibasic (and anhydrous)	JP	6
Carmellose Calcium (Rev 1)	USP	6
Carmellose Sodium	USP	4
Carmellose	JP	4
Cellulose Acetate (Rev 1)	USP	6
Cellulose Acetate Phthalate	USP	6
Microcrystalline Cellulose (Rev 1)	USP	6
Cellulose, Powdered (Rev 1)	USP	6
Citric Acid, Anhydrous (Rev 1)	EP	6
Citric Acid, Monohydrate (Rev 1)	EP	6
Copovidone	JP	4
Croscarmellulose Sodium	USP	6
Crospovidone	EP	4
Ethylcellulose	EP	6
Ethylparaben	EP	6
Gelatin	EP	3
Glucose Monohydrate/Anhydrous	EP	3
Glycerin	USP	3
Glyceryl Monostearate	USP	2
Hydroxyethyl Cellulose	EP	4/2
Hydroxypropyl Cellulose	USP	4
Hydroxypropyl Cellulose, Low Substituted	USP	4
Hydroxypropylmethyl Cellulose	JP	6
Hypromellose Phthalate	USP	6
Lactose, Anhydrous (Rev 2)	USP	6
Lactose, Monohydrate	USP	6
Magnesium Stearate	USP	5A
Mannitol	EP	3
Methylcellulose	JP	6
Methylparaben	EP	6
Petrolatum	USP	4
Petrolatum, White	USP	4
Polyethylene Glycol	USP	4
Polysorbate 80	EP	4 rev
Povidone	JP	6
Propylene Glycol	EP	4
Propylparaben	EP	6
Saccharin	USP	6
Saccharin, Calcium	USP	6
Saccharin, Sodium (Rev 1)	USP	6
Silicon Dioxide	JP	4 rev



Silicon Dioxide, Colloidal	JP	4 rev
Sodium Chloride (Rev 2)	EP	6
Sodium Lauryl Sulfate	USP	3
Sodium Starch Glycolate (Rev 1)	USP	6
Starch, Corn (Rev 2)	USP	6
Starch, Potato	EP	6
Starch, Pregelatinized	JP	3
Starch, Rice	EP	6
Starch, Wheat	EP	6
Stearic Acid	EP	5B
Sucrose	EP	4
Sterile Water for Injection in Containers	USP	3
Talc	EP	6
Titanium Dioxide	JP	5A2

Table 2. Status of Harmonization—General Chapters

Chapter Title	Coordinating Pharmacopeia	Harmonization Stage
Amino Acid Determination	USP	6
Bacterial Endotoxins (Rev 1)	JP	4 rev
Bulk Density and Tapped Density	EP	5A2
Conductivity	EP	2
Color (Instrumental Method)	EP	3
Density of Solids	EP	5B
Disintegration	USP	6
Dissolution (Rev 1)	USP	6
Capillary Electrophoresis	EP	6
Polyacrylamide Gel Electrophoresis	EP	6
Extractable Volume (Rev 1)	EP	6
Heavy Metals	USP	3
Inhalation	EP	4
Isoelectric Focusing	EP	6
Laser Diffraction Measure of Particle Size	EP	4
Limits for Nonsterile Products	EP	6
Microbial Contamination	EP	6
Tests for Specified Microorganisms	EP	6
Microbial Enumeration	EP	6
Optical Microscopy	USP	6
Particle Size Distribution Estimation by Analytical Sieving (Rev 1)	USP	5B
Particulate Contamination (Rev 1)	EP	6
Peptide Mapping	USP	6
Porosimetry by Mercury Intrusion	EP	4
Powder Fineness	USP	5A
Powder Flow	USP	6
Protein Determination	USP	6
Residue on Ignition (Rev 2)	JP	6
Specific Surface Area	EP	6
Sterility Tests	EP	6
Tablet Friability	USP	6
Thermal Behavior of Powders	EP	3
Uniformity of Content/Mass	USP	6
Uniformity of Delivered Dose of Inhalations	EP	2
Water-Solid Interaction	EP	3



## Glossary

Harmonized monograph or general chapter— Text that has reached Stage 5B and that has been countersigned by the three PDG pharmacopeias.

Total harmonization— A monograph or a general chapter that is identical in the three PDG pharmacopeias in terms of identical tests, test procedures, and acceptance criteria.

Harmonization by attributes— A monograph or a general chapter that contains a combination of harmonized and nonharmonized tests or sections.

Interchangeability— A test or a section of a monograph or a general chapter that is not the same among the PDG pharmacopeias, but the accept/reject decision is the same regardless of which one of the tests or sections of the PDG pharmacopeia is used.

Local or national divergence— A monograph or a general chapter in one of the PDG pharmacopeias contains specific attributes of local or national origin in addition to the PDG harmonized/nonharmonized attributes.

Nonharmonized attributes— Attributes that could not be agreed upon by the three PDG pharmacopeias because of regional differences, regulatory differences, nonavailability of reagents, etc.

## Appendix

## Example

## Pharmacopeial Discussion Group

## Sign-Off Document

Name: \_\_\_\_\_

Attributes	EP	JP	USP
1	+	+	+
2	+	+	-
3	+	+	+
4	+	+	+
5	+	+	+
6	+	+	+
7	+	-	+
8	+	+	+
9	+	+	+
10	+	-	+
11	+	+	+

## Legend

+ will adopt and implement; - will not stipulate

## Nonharmonized attributes

## Reagents and reference materials

Each pharmacopeia will adapt the text to take account of local reference materials and reagent specifications.

Date:

Signatures:

European Pharmacopoeia Japanese Pharmacopoeia United States Pharmacopeia

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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Pharmacopeial Forum: Volume No. 33(4) Page 751

**1207 STERILE PRODUCT PACKAGING—INTEGRITY EVALUATION**

This information chapter addresses the maintenance of the microbiological integrity of sterile product packaging until the time of use of its contents. The scope of application of this chapter includes the container and closure systems of drug products and the sterile barrier packaging of medical devices, including in vitro diagnostic products. Specially designed or novel container–closure and barrier packaging systems, which are generally more complex than classical packaging and delivery systems, are also discussed in this chapter. The establishment of sterile product integrity is necessary to ensure the maintenance of two extremely important product conditions: total product attributes within label claim specifications and product sterility prior to use.

Product package integrity testing continues throughout the life cycle of the product. Generally, this integrity testing should occur during three phases: (1) the initial development of the product packaging system, (2) routine manufacturing, and (3) shelf life stability assessments. Generally, during initial packaging development both physical and microbial studies are conducted to assess integrity. It is at this time that comparative information about physical and microbial challenge methods may be obtained.

During routine manufacturing, physical measurements may be conducted in accordance with an established sampling plan to determine whether the packaging or capping system or both are operating consistently within predetermined performance acceptance ranges. Packaging integrity tests conducted during shelf life stability assessments are physical tests that confirm the integrity of the packaging system supported by acceptance values established during packaging development. Microbial ingress testing may be unnecessary for shelf life stability assessment if comparative physical and microbial testing conducted during the packaging development stage has established physical acceptance values that would preclude microbial ingress. Reconfirmation of product packaging integrity should occur when there are major changes in the package design and materials or whenever there are changes in the manufacturing processing conditions, including sterilization conditions.



## PRODUCT PACKAGE DEVELOPMENT PHASE

Testing during the product development phase is frequently intensive, because it establishes product design limitations before full-scale manufacturing occurs. At this phase, an appropriate product package design relative to the end use of the product is selected, and manufacturing process variables are defined. The effect of the design and process variables on the maintenance of package integrity is assessed. Approximate design tolerances are assessed to ensure sterility of the fluid pathway or contents during processing and prior to usage. Product packaging integrity evaluations should take into consideration the maximum stress conditions encountered during the manufacturing and sterilization processes. Testing should also validate the integrity of the design when exposed to anticipated extreme conditions of storage, shipment, and distribution. During this phase, physical methods are developed to assess the integrity of the proposed package, and they are used later in routine manufacturing testing or marketed product stability testing.

## ROUTINE MANUFACTURING PHASE

During this phase, maintenance of specified production, engineering, and microbiological conditions is monitored through standard operating procedures. Physical testing methods, on-line or not, may be intermittently used to supplement process control measurements to ensure that product packaging values are maintained within acceptable limits established during the product development phase. During routine manufacturing, monitoring for continuous unit product integrity or finished product release package integrity may be unnecessary when critical production processes are well controlled. Microbiological testing of package integrity is usually not expected during routine manufacturing that operates within specification limits previously determined to produce acceptable packaging.

## MARKETED PRODUCT STABILITY PHASE

Physical testing methods may be used to evaluate product packaging systems that are included in a marketed product shelf life stability program. Such systems should be evaluated at stability conditions, at the beginning of the product shelf life as well as at the expiration date of the product, and at other times and intervals defined by regulatory requirements or guidelines. Sole testing of product sterility will not ensure maintenance of product package integrity over the shelf life of the product when performed as part of the packaging stability program.

## PHYSICAL AND MICROBIOLOGICAL TESTING

During the initial evaluation of product packaging, both physical and microbiological testing are frequently used.<sup>1</sup> Physical testing to assess product packaging integrity has a variety of advantages over microbial testing, depending on the test method and the packaging being evaluated. These advantages may include greater sensitivity, ease of use, rapid speed of testing, or lower cost. Physical testing can be used to evaluate product packaging throughout its life cycle to ensure that total product attributes are maintained within optimum predefined limits. Microbial ingress testing in concert with physical testing should also be considered at the initial stages of product packaging development. However, physical testing methods with sensitivity comparable to or greater than that of microbial methods are preferred for evaluating product packaging in a marketed product shelf life stability program.

Physical testing methods provide rapid assessment of packaging integrity during routine testing of large numbers of product stability samples.

### Comparison of Microbial and Physical Methods

A comparative evaluation should determine whether microbial intrusion or ingress into the product packaging could occur at those physical integrity test value ranges that have been deemed acceptable for the finished product. This determination should be based on a comparison between microbiological data and the values obtained from physical integrity test method. The comparison of physical integrity testing to microbial ingress testing for assessment of product packaging integrity can be obtained either by direct comparison or by studies that demonstrate that the physical test measures defects that are too small for microbial passage.

Due to its design or material composition, a product packaging system may not permit or yield graded physical test responses when a range of defects is created in the packaging. In some situations, even upon creating artificial defects in a product packaging system, the resulting range of physical testing values may remain approximately the same. In other words, the test method may yield a qualitative rather than a quantitative measure of package leakage. Also, microbial ingress may not occur until exaggerated physical defects are created in the packaging. In such cases, a direct correlation of microbial ingress to a series of physical value ranges is not possible. When this occurs, the testing results become merely pass or fail results conducted on packaging with known defects.

### Selection of Evaluation Methods

Physical and microbiological methods for product package integrity testing should take into consideration the design of the closure system; the manufacturing method, including the sterilization process; and the intended use of the product. Any particular physical or microbial method may not be applied to all product packaging systems.

During initial integrity evaluations of a packaging system, a number of physical tests may be used. These physical tests may include, but are not limited to, pressure and vacuum decay tests, dye immersion tests, liquid chemical tracer tests, gas ionization of evacuated containers, high-voltage leak detection of plastic or glass containers, visual examination for glass cracks, and gas leakage or package headspace analysis. Other tests that may be valuable in evaluating package seal quality include screw-cap removal torque, elastomeric stopper residual seal force, or heat seal strength.

Microbiological tests may include closure immersion testing for rigid container systems, inoculated shipment testing for certain packaging, and an aerobiological challenge for tortuous path barrier packaging.

### Dual Function Container–Closure Systems<sup>2</sup>

Dual function container–closure systems are characterized by the addition of one or more intended functions to that of a container and require special consideration for integrity evaluation. For instance, in the case of small, flexible or rigid containers with an appended device component that allows direct patient injection and drug delivery, a delivery function is added to the container function. Thus, frequently, one compartment of the dual container–closure system is designed to contain the drug or solution prior to use or activation of the system—product containment compartment. Another compartment, different in function and design, either directly delivers the product from the container portion to a fluid pathway for direct injection of the patient or communicates with a sterile pathway of another access device. For example, a prefilled syringe contains a solution (container compartment) and a device component (delivery compartment) physically separated from the container compartment and used to directly administer the drug to the patient.

Therefore, dual container–closure systems typically have at least two compartments that require microbial barrier properties, and packaging integrity after sterilization and/or aseptic filling should be demonstrated for both compartments. In many cases, different portions of the dual system require different integrity testing methods. The selection of the integrity testing method is determined primarily on the basis of the intended objectives or performance requirements of the particular compartment. For example, the solution or drug-containing compartment of the dual container–closure system must be enclosed or sealed in a manner that precludes leakage of product or microbial ingress during and following the manufacturing process (see Selection of Evaluation Methods). On the other hand, the delivery portion of the dual container–closure system frequently contains a fluid pathway that is empty during the sterilization or aseptic filling process and is intended to remain dry until the product container portion is activated prior to use. A covering, a sheath, or perhaps a cap designed to vent during sterilization and storage protects the delivery compartment from airborne microbial ingress throughout the life of the article. However, this portion of the device is frequently not designed to prevent liquid ingress. Liquid ingress can be precluded by secondary packaging or by the physical design of the system itself. Microbial integrity testing of the delivery portion of such a dual container–closure system may include a nonimmersion microbial method or physical integrity test. Microbial testing would include, for example, an aerosolized microbial challenge under defined pressure changes.

1 A review of physical and microbiological methods related to the evaluation of product packaging integrity appears in *Pharmaceutical Package Integrity*, Parenteral Drug Association's Technical Report No. 27, 1998.

2 For more information, see ANSI/AAMI/ISO Standard 11607-2000, 2nd ed., *Packaging for Terminally Sterilized Medical Devices*.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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### 1208 STERILITY TESTING—VALIDATION OF ISOLATOR SYSTEMS

This chapter provides guidelines for the validation of isolator systems for use in sterility testing of compendial articles. [note—In the context of this chapter, “decontaminated” refers to an item or surface that has been subjected to a process that eliminates viable bioburden.]

Isolators—devices that create controlled environments in which to conduct Pharmacopeial sterility tests—have been used since the mid-1980s. An isolator is supplied with air through a HEPA or better air filter and is able to be reproducibly decontaminated. Closed isolators, which are systems with no direct opening to the external environment, are normally used for sterility testing, although open isolators which allow the egress of materials through a defined opening that precludes the entry of contamination by means of air overpressure may be used. Closed isolators use only decontaminated interfaces or a rapid-transfer port for the transfer of materials. Isolators are constructed of flexible plastics (such as polyvinyl chloride), rigid plastics, glass, or stainless steel.

Isolator systems protect the test article and supplies from contamination during handling by essentially eliminating direct contact between the analyst and the test articles. All transfers of material into and out of the isolator are accomplished in an aseptic fashion while maintaining complete environmental separation. Aseptic manipulations within the isolator are made with half-suits, which are flexible components of the isolator wall that allow the operator a full range of motion within the isolator, or by gloves and sleeves. Operators are not required to wear special clean-room clothing for conducting sterility tests within isolators; standard laboratory clothing is adequate, although a pair of sterile gloves is frequently worn under the isolator gloves as an added precaution against contamination entering the isolator enclosure and for hygiene purposes. The interior of the isolator is treated with sporicidal chemicals that result in the elimination of all viable bioburden on exposed surfaces.

#### ISOLATOR DESIGN AND CONSTRUCTION

##### Air Handling Systems

An isolator used for sterility testing is equipped with microbial retentive filters (HEPA filters or better are required). At rest, the isolator meets the particulate air-quality requirements for an ISO Class 5 area as defined in ISO 14644-1 through -3\* (see [Microbiological Evaluation of Clean Rooms and Other Controlled Environments](#) 1116). However, the isolator need not meet Class 5 conditions during an operation that may generate particulates, and no requirements for air velocity or air exchange rate exist. The isolator should be sealed well enough during decontamination that the dissemination of sporicidal vapors or gases into the surrounding environment is kept to appropriately low levels. When direct openings to the outside environment exist, constant air overpressure conditions maintain sterile conditions within the isolator. In general, both open and closed isolators are maintained at positive pressure relative to the surrounding environment, and overpressures of 20 Pa or more are typical. The user should never exceed the maximum pressure recommended by the isolator manufacturer. Airflow within isolators used for sterility testing is either unidirectional or turbulent.

##### Transfer Ports and Doors

Isolators may be attached to a “pass-through” decontaminator or transfer isolator to enable the direct transfer of sterile media, sterile dilution fluids, and sterile supplies from the decontaminator into the isolator system. Rapid transfer ports (RTPs) enable two isolators, i.e., the work station and transfer isolator, to be connected to one another, so that supplies can be moved aseptically from one isolator to another. Aseptic connections between two isolators or an isolator and an RTP-equipped container can be made in unclassified environments using RTPs. The nonsterile surfaces of the RTP are connected using locking rings or flanges. A compressed gasket assembly provides an airtight seal, thereby preventing the ingress of microorganisms.

When the two RTP flanges are linked to form an airtight passage, a narrow band of gasket remains that could harbor microbial contamination. This exposed gasket should be routinely disinfected immediately after the connection is made, and before materials are transferred through the RTP. Good aseptic technique is used when transferring materials and care is taken not to touch the gasket with the materials being transferred or with the gloved hands.

Preventive maintenance and lubrication of the gasket assemblies on the flanges is performed according to the RTP manufacturer’s recommendations. The RTP gaskets are changed at the recommended frequency and periodically checked for damage, because cut or torn gaskets cannot make a truly airtight seal.

##### Selection of a Location for the Isolator

Isolators for sterility testing need not be installed in a classified clean room, but it is important to place the isolator in an area that provides limited access to nonessential staff. The appropriate location provides adequate space around the isolator for moving transfer isolators, staging of materials, and general maintenance. No environmental monitoring of the surrounding room is required.

Temperature and humidity control in the room is important to operator safety and comfort and is critical for the effective utilization of certain decontamination technologies. Uniform temperature conditions in the room are desirable when temperature-sensitive decontamination methods are employed. Care should be taken in locating the isolator so that cold spots are avoided that might result in excessive condensation when condensing vapors are used for decontamination.

#### VALIDATION OF THE ISOLATOR SYSTEM

The isolator system must be validated before its use in sterility testing as part of a batch release procedure. To verify that the isolator system and all associated equipment are suitable for sterility tests, validation studies are performed in three phases: installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ). The following sections contain points to consider in the validation of isolator systems for sterility testing. The assignment of test functions to a particular phase of the validation program (i.e., IQ, OQ, and PQ) is not critical, as long as proper function of the isolator is demonstrated and documented before its use in compendial Assays.

##### Installation Qualification (IQ)

The IQ phase includes a detailed description of the physical aspects of the system, such as the dimensions, internal configuration, and materials of construction. The unit layout is diagrammed with interfaces and transfer systems clearly and dimensionally indicated. Compliance with design specifications for utility services, such as air supply, vacuum, external exhaust, and temperature and humidity control, is verified. Other equipment used with the isolator system is also described in detail; if any revisions to design specifications are made, these are included. Equipment manuals and copies are catalogued and stored where they can be retrieved and reviewed. Compliance of drawings to design specifications is verified.

All drawings and process and instrumentation diagrams are catalogued, stored, and are retrievable.

All documentation is reviewed to verify that it precisely reflects the key attributes of the installed system. This establishes a general benchmark for the isolator system’s compliance with design specifications and installation requirements.

Potential process-control or equipment problems that could cause system failure during operation are identified and documented during failure-mode analysis and hazard analysis. The system is modified, if necessary, to minimize the risk of failure, and critical control point methods are established.

The results of the IQ are summarized in an Installation Qualification Report. The following documentation is suggested.

Equipment—The equipment is listed with its relevant design specifications. The IQ verifies that equipment meeting the appropriate design specifications was received and that it was installed according to the manufacturer’s requirements.

Construction Materials—The construction materials of critical system components are checked for compliance with design specifications. The compatibility of the intended decontamination method with the construction materials is verified.



#### Instruments— System instruments are listed with their calibration status.

Utility Specifications— All utilities required for operation—as defined in the operating manuals and process and instrumentation diagrams—are checked for availability and compliance with design specifications. Any connection between utility systems and the isolator system is inspected and conformance of these connections to specifications is verified.

Filter Certification— HEPA filters and other microbial retentive filters are tested and certified; copies of test results and certificates are included in the IQ summary. Purchase orders are reviewed and conformance of the air filtration system to specifications is verified.

Computer Software— All computer software associated with the isolator system is listed with its name, size, and file revision number. The master computer disks are checked for proper labeling and stored securely.

#### Operational Qualification (OQ)

The OQ phase verifies that the isolator system operates in conformance to functional specifications.

Operational Performance Check— This test verifies that all alert and alarm functions comply with their functional specifications. The system's ability to comply with all set points and adjustable parameters is verified.

Isolator Integrity Check— The integrity of the isolator is maintained during all normal operating conditions. A leak test is performed to verify the compliance with the manufacturer's functional specifications and to ensure safety prior to charging the isolator with a decontaminating sporicidal chemical. To safeguard against adventitious contamination, isolators are operated at a suitable positive pressure during normal operation. Validation studies must show that the air pressure set point can be maintained and controlled during operation.

Decontamination Cycle Verification— A decontamination cycle that is the function of the decontamination equipment in concert with the isolator(s) is verified.

Different decontamination methods can be used to eliminate bioburden from isolator systems and supplies. Among the chemicals that have been used to treat isolators are peracetic acid, chlorine dioxide, ozone, and hydrogen peroxide; each has different requirements for exposure conditions and process control. It is critical to comply with the manufacturer's operational requirements for the selected decontamination method and to describe them in the functional specifications. The temperature inside the isolator is also important, particularly for hydrogen peroxide vapor decontamination, where it is critical to maintain the concentration relative to the condensation point. Some sterilization chemicals, such as chlorine dioxide and ozone, require the addition of moisture to the isolator prior to decontamination. When elevated relative humidity is required, the ability to control it must be verified during OQ.

It is also important to verify the concentration and distribution of the decontaminating chemical. When applied in gaseous or vapor form, the distribution may be evaluated using chemical indicators, spectroscopic methods, or electronic sensors.

Gas and vapor decontamination methods may require fans in the isolator to distribute the chemical evenly. The location and orientation of these fans are adjusted to ensure optimum air distribution. If the isolator utilizes a recirculating unidirectional airflow system, distribution fans may not be required, but this should be evaluated on a case-by-case basis. Because shelving units, equipment, glove-and-sleeve assemblies, and half-suits have an impact on distribution patterns, distribution checks are done with the isolator fully loaded with equipment and supplies, and the setup of these units is defined and documented.

Many installations use smaller transfer isolators as portable surface decontamination units. In these transfer isolators, test articles and supplies are treated chemically to eliminate bioburden before transfer through an RTP into the testing isolator. Its loading configuration is defined, and configuration drawings are reviewed and verified during the OQ. [note—The decontaminating chemicals used in isolators work on the surfaces of materials; therefore, any surface that is occluded will not be treated and could contain viable bioburden. Special precautions should be in place for treating surfaces known to be occluded with a sporicide if such surfaces may be revealed during the conduct of sterility tests.]

Decontamination agents need to be removed from the isolator after the exposure period, which is accomplished by a current of fresh air provided either by the decontamination equipment or by utilizing the isolator air handling system. Aeration is accomplished either in an open loop, in which the gas is exhausted through a vent to the atmosphere, or in a closed loop, in which the chemical is removed and destroyed by the decontamination equipment. The aeration system is checked; if an open-loop configuration is used, the external exhaust system's flow and safety are checked.

Decontamination Cycle Development— When the OQ is completed, decontamination cycle development is performed to establish the parameters necessary for process control during routine decontamination cycles. Any of the methods generally used in the industry for the validation of decontamination processes—including bioburden-based, fractional cycle, and overkill methods—are adequate. The decontamination process is challenged with biological indicators (BIs). The spore population and resistance of the BIs to the decontamination conditions being applied are known. Wherever possible, a D value estimate is done for each B1 system or, alternatively, a survivor curve for the BI system is obtained (see [Biological Indicators—Resistance Performance Tests](#) (55)); it is acceptable to obtain the D value from the BI vendor.

#### Performance Qualifications (PQ)

The PQ phase verifies that the system is functioning in compliance with its operator requirement specifications. At the completion of the PQ phase, the efficacy of the decontamination cycle and, if appropriate, the adequacy of decontaminating chemical venting are verified. All PQ data are adequately summarized, reviewed, and archived.

Cleaning Verification— In general, cleaning is not critical for sterility testing applications. However, residual products are a concern in multiproduct testing, particularly for aggressive antimicrobial agents, because these materials could interfere with the ability of subsequent tests to detect low levels of contamination in the product. Concerns about contamination with the product are heightened when it is an inherently antimicrobial powder, because powders are more readily disseminated. Cleaning to a level at which no visible contamination is present is adequate for sterility test isolator systems and is a suitable operator requirement specification. The cleaning method, frequency, equipment, and materials used to clean the isolator are documented.

Decontamination Validation— The interior surfaces of the isolator, the equipment within the isolator, and the materials brought into the isolator are treated to eliminate all bioburden. The decontamination methods used to treat isolators, test articles, and sterility testing supplies are capable of reproducibly yielding greater than a three-log reduction against highly resistant biological indicators (see [Biological Indicators for Sterilization](#) (1035)), as verified by the fraction negative or total kill analysis methods. Total kill analysis studies are suitable for BIs with a population of 103 spores per unit, while fraction negative studies are suitable for BIs with a population of 105 or greater. A sufficient number of BIs are used to prove statistical reproducibility and adequate distribution of the decontaminating agent. Particular attention is given to areas that pose problems relative to the concentration of the agent. A larger number of BIs may be required in isolators that are heavily loaded with equipment and materials. The ability of the process to reproducibly deliver a greater than three-log kill is confirmed in three consecutive validation studies.

The operator establishes a frequency for re-decontamination of the isolator. The frequency may be as short as a few days or as long as several weeks, depending on the sterility maintenance effort (see Maintenance of Asepsis within the Isolator Environment).

#### PACKAGE INTEGRITY VERIFICATION

Some materials are adversely affected by decontaminating agents, which can result in inhibition of microbial growth. Of concern are the penetration of decontaminating agents into product containers; accessory supplies such as filter sets and tubing; or any material that could come in contact with product, media, or dilution fluids used in the sterility test. It is the responsibility of the operator to verify that containers, media, and supplies are unaffected by the decontamination process. Screw-capped tubes, bottles, or vials sealed with rubber stoppers and crimp overseals have proven very resistant to the penetration of commonly used decontaminating agents. Wrapping materials in metal foil or placing them in a sealed container will prevent contact with the decontaminating agent; however, these procedures may also result in some surfaces not being decontaminated. In some cases, the use of shorter duration decontamination cycles and reduced concentrations may be necessary to minimize penetration of decontaminating agents into the package or container. Cycles that provide a less than three-log kill of resistant BIs may be acceptable provided microbiological analysis of the environment proves that the isolator(s) are free of recoverable bioburden.

In many cases, the operator will choose to treat the surfaces of product containers under test with the decontaminating agent in order to minimize the likelihood of bioburden entering the isolator. It is the responsibility of the operator to demonstrate, via validation studies, that exposure of product containers to the decontaminating agent does not adversely affect the ability of the sterility test to detect low levels of contamination within these test articles. It is suggested that the ability of the package to resist contamination be examined using both chemical and microbiological test procedures. Bacteriostasis and fungistasis validation tests must be performed using actual test articles that have been exposed to all phases of the



decontamination process (see [Sterility Tests](#) 71). This applies to medicinal device packages as well as pharmaceutical container and closure systems.

Validation studies determine whether both sterility test media and environmental control media meet the requirements for Growth Promotion Test of Aerobes, Anaerobes, and Fungi under [Sterility Tests](#) 71.

#### MAINTENANCE OF ASEPSIS WITHIN THE ISOLATOR ENVIRONMENT

The ability of the isolator system to maintain an aseptic environment throughout the defined operational period must be validated. In addition, a microbiological monitoring program must be implemented to detect malfunctions of the isolator system or the presence of adventitious contamination within the isolator. Microbiological monitoring usually involves a routine sampling program, which may include, for instance, sampling following decontamination on the first day of operation and sampling on the last day of the projected maintenance of asepsis period. Periodic sampling throughout the use period can be performed to demonstrate maintenance of asepsis within the isolator.

The surfaces within the isolator can be monitored using either contact plates for flat surfaces or swabs for irregular surfaces. However, because media residues could impose a risk on isolator asepsis, these tests are generally best done at the end of the test period. If performed concurrently with testing, care is used to ensure that any residual medium is removed from isolator surfaces, and that those surfaces are carefully cleaned and disinfected. Active air samples and settling plates may be used, but they may not be sufficiently sensitive to detect the very low levels of contamination present within the isolator enclosure.

A potential route for contamination to enter the isolator is during the introduction of supplies and samples into the enclosure. Validating that all materials taken into the isolator enclosure are free of microbial contamination is critical, as is periodic inspection of gaskets to detect imperfections that could allow ingress of microorganisms. Gloves and half-suit assemblies are another potential source of microbial contamination. Gloves are of particular concern because they are used to handle both sterility testing materials and test articles.

Resistance to puncture and abrasion should be considered in the selection of gloves and sleeves. Hypalon materials are resistant to both chemical sporicides used in the decontamination of isolators and to punctures and are available in several thicknesses to provide adequate tactile feel through the gloves while maintaining their integrity.

Very small leaks in gloves are difficult to detect until the glove is stretched during use. There are several commercially available glove leak detectors; the operator ensures that the detectors test the glove under conditions as close as possible to actual use conditions. Microbiological tests are used to supplement or substitute physical tests. [note—Standard "finger dab plates" may not be sensitive enough to detect low levels of contamination. Submersion of the gloves in 0.1% peptone water followed by filtration of the diluent and plating on growth media can detect loss of integrity in the gloves that would otherwise go unnoticed.]

Continuous nonviable particulate monitoring within the isolator's enclosure is ideal, because it can quickly detect filter failure. A second choice is periodic monitoring using a portable particle counter. Sampling for particles must be done in a manner that poses no risk to the maintenance of asepsis within the isolator.

#### INTERPRETATION OF STERILITY TEST RESULTS

A sterility test resulting in a false positive in a properly functioning and validated isolator is very unlikely if bioburden is eliminated from the isolator interior with a high degree of assurance; if gloves, sleeves, and half-suits are free of leaks; and if the RTPs are functioning properly. Nevertheless, isolators are mechanical devices and good aseptic techniques are still required. A decision to invalidate a false positive is made only after fully complying with the requirements of Observation and Interpretation of Results under [Sterility Tests](#) 71.

#### TRAINING AND SAFETY

As with sterility testing conducted in conventional clean rooms, operators are trained in procedures that are specific to their isolator. Use of proper aseptic techniques is vital to the conduct of sterility tests in isolators, just as it is in clean rooms. Therefore, training in proper aseptic techniques is required for all sterility testing technicians. All training sessions and the evaluation of the operator's performance are documented in the individual's training record. Training of all personnel in the appropriate safety procedures necessary for the operation and maintenance of the isolation system is imperative.

Personnel safety in the use of a decontaminating agent must be assessed. Material Safety Data Sheets, or equivalent documents, are available in the immediate area where the decontaminating agent is being used. All storage and safety precautions are followed. An operational readiness inspection of the safety of the isolator and all associated equipment is performed and documented prior to placing the unit in service.

\* International Organization for Standardization (ISO) International Standards 14644-1, -2, -3, and -7

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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Pharmacopeial Forum: Volume No. 30(6) Page 2162

1209 STERILIZATION—CHEMICAL AND PHYSICOCHEMICAL INDICATORS AND INTEGRATORS

#### INTRODUCTION

The Federal Code of Regulations, Part 211 on Good Manufacturing Practices for Finished Pharmaceuticals in section 211.165 states: "There shall be appropriate laboratory testing, as necessary, of each batch of drug product required to be free of objectionable microorganisms." This statement has been interpreted to mean that an alternate laboratory control test for sterility is required for any batch load of a product that is parametrically released. An appropriate laboratory test for each batch may be a biological indicator, which is included in each batch of product that is terminally sterilized (see [Biological Indicators for Sterilization](#) 1035), or a physicochemical indicator or integrator. This requirement may also be met by a primary product release system that includes the documented recording of thermometric measurements systems that are calibrated with a NIST traceability system and that demonstrate a  $\pm 0.5^{\circ}$  performance capability.

The presence of this chapter in the USP does not mean that chemical indicators and integrators are primary release requirements for parametric released product. The recorded and documented measurements from established thermometric measurement systems and associated process controllers (that have been calibrated and used during initial and periodic validation studies, as well as in routine production) can be considered to be primary, product-release systems for parametric release.

#### PERFORMANCE

Performance standards within lots and between lots of physicochemical indicators or integrators from a given manufacturer should be consistent. They should not interact physically or chemically with any container or product when placed adjacent to the product for sterilization in the sterilizer load, and should not alter the strength, quality, or purity of the sterilized article. The safety of personnel handling the physicochemical indicators or integrators should also be assessed, and, if need be, appropriate precautions should be taken.

Similar to biological indicators, chemical indicators are considered Class II devices and require the indicator manufacturer to obtain a device 510K approval prior to commercial use.

#### PHYSICOCHEMICAL INDICATORS



Recorded process engineering data can be supplemented by the presence in each sterilized batch of a physicochemical indicator. A physicochemical indicator is defined as a device that responds in a measurable fashion to one or more critical sterilization parameters.

A number of different kinds of indicators dependent on chemical or physicochemical means have been developed for monitoring sterilization cycles. Some products are used in a sterilization apparatus for monitoring whether the contents have been exposed to a selected factor (i.e., temperature) of the particular sterilization cycle, but may not show the duration or intensity of such exposure. Chemical and physicochemical indicators are used to monitor a physical parameter of a sterilization apparatus and can be placed on the outside of the packages of articles to be sterilized, or they may be distributed within the sterilizer load. In the latter case, one can evaluate to some extent the effect of the packaging material and configuration of the load on the selected parameter.

#### PHYSICOCHEMICAL INTEGRATORS

A physicochemical integrator is defined as a device that responds to a sterilization process critical parameter, which results in a measurable or quantifiable value that can be correlated to some standard of microbial lethality. Physicochemical integrators have been designed to broadly match the predictable inactivation of those spore preparations in biological indicators that have high and defined resistance to the sterilizing agent.

The manufacturers of physicochemical integrators should provide data to demonstrate that the labeled performance characteristics tests of the integrators are met. Users of physicochemical integrators should verify that specific measured values directly correlate to successful microbial lethality in a validated sterilization cycle.

A physicochemical integrator indicates whether or not the critical combination of physical parameters of a validated sterilization cycle has been met or exceeded. The integrator is not generally used as a substitute for a biological indicator in the development and validation of sterilization cycles. An indication by a physicochemical integrator that the critical combination of physical parameters of a stated sterilization cycle has been achieved should not be considered equivalent to the inactivation of spores of a variety of biological indicators. However, the physicochemical integrator can detect whether the sterilization process has been continued for too long, at too high a temperature or gas concentration, or has been overexposed to radiation.

The interval between the lower range and the upper range of time, or any other designated set of parameters, resembles the survival time and kill time window characteristics of a biological indicator. This interval should not be wider than that desirable for the designated parameter, but may be narrower if the manufacturer is able to achieve consistent performance over a narrower range. Even where a sterilization apparatus with consistent performance is used, cases may occur where the determined performance characteristics of the integrator differ from the label claims. This could represent a difference between the user's apparatus performance and the manufacturer's apparatus used for verifying the label claims. Closer conformity to the label claims may also be shown with any highly developed apparatus, such as a BIER vessel.<sup>1,2</sup> Hence, the integrator requires its own precautions in use and has appropriate interpretive criteria within its performance characteristics. Tests for performance characteristics of physicochemical integrators include determination under applicable defined conditions of (a) the maximum time of exposure at which none of the specimens indicates that adequate exposure to the cycle has occurred, and (b) a minimum time of exposure at which all specimens show that adequate exposure to the cycle has occurred. An intermediate time of exposure, where about half the number of specimens show adequate exposure, could indicate an approaching exposure endpoint for the physicochemical integrator.

Because an indicator reflects only the interaction of the physical parameters of sterilization, it will not be affected by some of the factors that may influence the resistance of the microbial load on the products to be sterilized (e.g., progeny resistance, spore population, inoculum substrate, oil, salts, proteins, or residues or configurations), all of which may protect a contaminated area from penetration by the sterilizing agent. (Hence, the inappropriateness of these devices for cycle development.) There are other factors, however, that may affect a biological indicator that could also affect a physicochemical integrator (e.g., interfering configuration of a pack in which the integrator was placed, variations in the applied timing or temperature control, or failure of the apparatus to reach the set temperature or meet other requirements).

Defective performance of the sterilization apparatus generally can be ascertained from gauges and from records of temperature, pressure, time of exposure, and gas concentration, whichever are applicable. The integrator can only indicate inadequate, adequate, or excessive exposure to a combination of critical sterilization parameters. Where an integrator shows inadequate exposure to the sterilization parameters, it is necessary to ascertain whether the gauges and recordings reflect accurately the sterilization conditions within the sterilization chamber. Variations between sterilization vessels, which might affect the efficiency of a selected sterilization cycle, might be detectable by parallel exposure of several integrators in a number of locations in each sterilizer load.

Physicochemical integrators for steam sterilization are designed to react predictably to a particular combination of physical sterilization parameters: temperature, steam pressure, and time of exposure. Deviation to some extent of one or more of these critical parameters, not compensated by modification of other parameters, causes the integrator to indicate failure to reach the preset integrated limits.

Physicochemical integrators for ethylene oxide sterilization are designed with similar general principles as the integrators for steam sterilization, but to react predictably to the particular combination of the physical sterilization parameters: humidity, temperature, sterilizing gas concentration, and time of exposure. Deviation to some extent of one or more of these critical parameters, not compensated by modification of other parameters, causes the integrator to indicate failure to reach the preset integrated limits.

Physicochemical integrators have been designed to match broadly the predictable inactivation of spore preparations that have a high and defined resistance to the sterilizing agent. For steam sterilization, a strain of *Bacillus stearothermophilus* is used (see [Biological Indicator for Steam Sterilization, Paper Carrier](#)), and for ethylene oxide sterilization, a strain of *Bacillus subtilis*, subspecies *niger*, is used (see [Biological Indicator for Ethylene Oxide Sterilization, Paper Carrier](#)). Since there are no standard preparations of these strains, the performance characteristics of these physicochemical integrators must be interpreted in relation to a specific validated sterilization cycle.

Performance standards within lots and between lots of physicochemical integrators from a given manufacturer should be consistent. The integrators should not interact physically or chemically with container or product when placed adjacent to the product for sterilization in the sterilizer load, and shall not alter the strength, quality, or purity of the sterilized article beyond official requirements. Users should obtain information from integrator manufacturers on whether or not sterilization in the presence of physicochemical integrators may affect particular articles to be sterilized. In addition, safety in using these integrators should be verified.

#### Moist Heat Sterilization

The use of steam sterilization physicochemical integrators to supplement the information obtained through physical assessment of the critical operating parameters should be part of parametric release of moist heat sterilized products. These should be designed to enable the assurance that the lethality delivery specified for the process has been met or exceeded. The consistency of the performance of physicochemical integrators for moist heat sterilization should be ensured through the testing of performance characteristics that include testing of the performance of the indicator or integrator system at various pre-set selected moist heat process conditions.

Critical elements of this type of physicochemical integrator would include an organic compound sensitive to the combination of temperature and steam, a polymeric material penetrable by saturated steam, and a wicking device under the polymeric material that is in contact with the organic compound. As steam passes through the polymeric material, the organic compound melts in a predictable fashion in steps dependent on the steam temperature during the cycle. The liquefied material travels along the wick for a distance that can be measured on a scale. This organic compound has a stated melting temperature range. Some integrators may, for example, have melting ranges of 132.2° to 134.5° or of 137.0° to 142.0°. Other melting ranges could also be specified so as to indicate the sterilization parameters to which it has actually been exposed. The combination of the sterilization parameters, applied for the exposure time required for a stated moist heat sterilization cycle, is indicated on the front of the article by the linear travel of the melt.

These types of physicochemical integrators can also be used for so-called "flash" moist heat sterilization cycles in which the successive steps of the sterilization process are rapidly carried out in such a manner as to achieve the required lethality for the validated process. Other types of physicochemical integrators for moist heat sterilization can be used if they are also calibrated against a specified validated moist heat sterilization cycle.

#### Ethylene Oxide Sterilization

Physicochemical integrators for ethylene oxide sterilization should be designed to match broadly the predictable inactivation of spore preparations that have a high and defined resistance to ethylene oxide sterilization. The inactivation of spores of a strain of *Bacillus subtilis*, subspecies *niger*, can be used as a model, although other spores of relevant microorganisms can also be used. The critical elements of physicochemical integrators for ethylene oxide sterilization are a base with an organic compound along a linear indicator strip sensitive to a combination of temperature, humidity, and sterilizing gas concentration. Where the organic compound is exposed to a sterilizing gas mixture at a specific



temperature and humidity, a chemical reaction triggers the appearance of a color along the linear indicator bar. This is dependent on the time of exposure under the conditions of ethylene oxide sterilization in a predictable fashion. The absence of fading or of decolorization of the indicator bar for a stated period after the sterilization cycle has been completed would confirm adequate humidification in the cycle. The integrator should be capable of detecting deviations from the prescribed parameters of temperature, sterilizing gas concentration, humidity, and time of exposure that may affect sterilization. It does not show the required reactions if exposed to reduced amounts of gas concentration, temperature, and humidity, even if exposed for prolonged periods.

Other types of physicochemical integrators for ethylene oxide sterilization based on different principles or mechanisms of integration of critical parameters of sterilization could be used if they are also calibrated against a specified validated ethylene oxide sterilization cycle.

The consistency of performance of physicochemical integrators for ethylene oxide sterilization has to be ensured through the testing of performance characteristics at various pre-set selected times for a given ethylene oxide sterilization cycle.

1 Standard for BIER/Steam Vessels, 27 March 1981, Association for the Advancement of Medical Instrumentation (AAMI), 3330 Washington Boulevard, Suite 400, Arlington, VA 22201-4598.

2 Standard for BIER/EO Vessels, 27 March 1992, Association for the Advancement of Medical Instrumentation (AAMI), 3330 Washington Boulevard, Suite 400, Arlington, VA 22201-4598.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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#### 1211 STERILIZATION AND STERILITY ASSURANCE OF COMPIENDIAL ARTICLES

This informational chapter provides a general description of the concepts and principles involved in the quality control of articles that must be sterile. Any modifications of or variations in sterility test procedures from those described under [Sterility Tests 71](#) should be validated in the context of the entire sterility assurance program and are not intended to be methods alternative to those described in that chapter.

Within the strictest definition of sterility, a specimen would be deemed sterile only when there is complete absence of viable microorganisms from it. However, this absolute definition cannot currently be applied to an entire lot of finished compendial articles because of limitations in testing. Absolute sterility cannot be practically demonstrated without complete destruction of every finished article. The sterility of a lot purported to be sterile is therefore defined in probabilistic terms, where the likelihood of a contaminated unit or article is acceptably remote. Such a state of sterility assurance can be established only through the use of adequate sterilization cycles and subsequent aseptic processing, if any, under appropriate current good manufacturing practice, and not by reliance solely on sterility testing. The basic principles for validation and certification of a sterilizing process are enumerated as follows:

1. Establish that the process equipment has capability of operating within the required parameters.
2. Demonstrate that the critical control equipment and instrumentation are capable of operating within the prescribed parameters for the process equipment.
3. Perform replicate cycles representing the required operational range of the equipment and employing actual or simulated product. Demonstrate that the processes have been carried out within the prescribed protocol limits and finally that the probability of microbial survival in the replicate processes completed is not greater than the prescribed limits.
4. Monitor the validated process during routine operation. Periodically as needed, requalify and recertify the equipment.
5. Complete the protocols, and document steps (1) through (4) above.

The principles and implementation of a program to validate an aseptic processing procedure are similar to the validation of a sterilization process. In aseptic processing, the components of the final dosage form are sterilized separately and the finished article is assembled in an aseptic manner.

Proper validation of the sterilization process or the aseptic process requires a high level of knowledge of the field of sterilization and clean room technology. In order to comply with currently acceptable and achievable limits in sterilization parameters, it is necessary to employ appropriate instrumentation and equipment to control the critical parameters such as temperature and time, humidity, and sterilizing gas concentration, or absorbed radiation. An important aspect of the validation program in many sterilization procedures involves the employment of biological indicators (see [Biological Indicators for Sterilization 1035](#)). The validated and certified process should be revalidated periodically; however, the revalidation program need not necessarily be as extensive as the original program.

A typical validation program, as outlined below, is one designed for the steam autoclave, but the principles are applicable to the other sterilization procedures discussed in this informational chapter. The program comprises several stages.

The installation qualification stage is intended to establish that controls and other instrumentation are properly designed and calibrated. Documentation should be on file demonstrating the quality of the required utilities such as steam, water, and air. The operational qualification stage is intended to confirm that the empty chamber functions within the parameters of temperature at all of the key chamber locations prescribed in the protocol. It is usually appropriate to develop heat profile records, i.e., simultaneous temperatures in the chamber employing multiple temperature-sensing devices. A typical acceptable range of temperature in the empty chamber is  $\pm 1^{\circ}$  when the chamber temperature is not less than  $121^{\circ}$ . The confirmatory stage of the validation program is the actual sterilization of materials or articles. This determination requires the employment of temperature-sensing devices inserted into samples of the articles, as well as either samples of the articles to which appropriate concentrations of suitable test microorganisms have been added, or separate BIs in operationally fully loaded autoclave configurations. The effectiveness of heat delivery or penetration into the actual articles and the time of the exposure are the two main factors that determine the lethality of the sterilization process. The final stage of the validation program requires the documentation of the supporting data developed in executing the program.

It is generally accepted that terminally sterilized injectable articles or critical devices purporting to be sterile, when processed in the autoclave, attain a 10-6 microbial survivor probability, i.e., assurance of less than 1 chance in 1 million that viable microorganisms are present in the sterilized article or dosage form. With heat-stable articles, the approach often is to considerably exceed the critical time necessary to achieve the 10-6 microbial survivor probability (overkill). However, with an article where extensive heat exposure may have a damaging effect, it may not be feasible to employ this overkill approach. In this latter instance, the development of the sterilization cycle depends heavily on knowledge of the microbial burden of the product, based on examination, over a suitable time period, of a substantial number of lots of the presterilized product.

The D value is the time (in minutes) required to reduce the microbial population by 90% or 1 log cycle (i.e., to a surviving fraction of 1/10), at a specific temperature. Therefore, where the D value of a BI preparation of, for example, *Bacillus stearothermophilus* spores is 1.5 minutes under the total process parameters, e.g., at  $121^{\circ}$ , if it is treated for 12 minutes under the same conditions, it can be stated that the lethality input is 8D. The effect of applying this input to the product would depend on the initial microbial burden. Assuming that its resistance to sterilization is equivalent to that of the BI, if the microbial burden of the product in question is 102 microorganisms, a lethality input of 2D yields a microbial burden of 1 (10

$^{\circ}$  theoretical), and a further 6D yields a calculated microbial survivor probability of 10-6. (Under the same conditions, a lethality input of 12D may be used in a typical "overkill" approach.) Generally, the survivor probability achieved for the article under the validated sterilization cycle is not completely correlated with what may occur with the BI. For valid use, therefore, it is essential that the resistance of the BI be greater than that of the natural microbial burden of the article sterilized. It is then appropriate to make a worst-case assumption and treat the microbial burden as though its heat resistance were equivalent to that of the BI, although it is not likely that the most resistant of a typical microbial burden isolates will demonstrate a heat resistance of the magnitude shown by this species, frequently employed as a BI for steam sterilization. In the above example, a 12-minute cycle is considered adequate for sterilization if the product had a microbial burden of 102 microorganisms. However, if the indicator originally had 106 microorganisms content, actually a 10-2 probability of survival could be expected; i.e., 1 in 100 BIs may yield positive results. This type of situation may be avoided by selection of the appropriate BI. Alternatively, high content indicators may be used on the basis of a predetermined acceptable count reduction.



The D value for the *Bacillus stearothermophilus* preparation determined or verified for these conditions should be reestablished when a specific program of validation is changed.

Determination of survival curves (see [Biological Indicators](#) (1035)), or what has been called the fractional cycle approach, may be employed to determine the D value of the biological indicator preferred for the specific sterilization procedure. The fractional cycle approach, may also be used to evaluate the resistance of the microbial burden. Fractional cycles are studied either for microbial count-reduction or for fraction negative achievement. These numbers may be used to determine the lethality of the process under production conditions. The data can be used in qualified production equipment to establish appropriate sterilization cycles. A suitable biological indicator such as the *Bacillus stearothermophilus* preparation may be employed also during routine sterilization. Any microbial burden method for sterility assurance requires adequate surveillance of the microbial resistance of the article to detect any changes, in addition to periodic surveillance of other attributes.

#### METHODS OF STERILIZATION

In this informational chapter, five methods of terminal sterilization, including removal of microorganisms by filtration and guidelines for aseptic processing, are described. Modern technological developments, however, have led to the use of additional procedures. These include blow-molding (at high temperatures), forms of moist heat other than saturated steam and UV irradiation, as well as on-line continuous filling in aseptic processing. The choice of the appropriate process for a given dosage form or component requires a high level of knowledge of sterilization techniques and information concerning any effects of the process on the material being sterilized.<sup>1</sup>

##### Steam Sterilization

The process of thermal sterilization employing saturated steam under pressure is carried out in a chamber called an autoclave. It is probably the most widely employed sterilization process.<sup>2</sup> The basic principle of operation is that the air in the sterilizing chamber is displaced by the saturated steam, achieved by employing vents or traps. In order to displace air more effectively from the chamber and from within articles, the sterilization cycle may include air and steam evacuation stages. The design or choice of a cycle for given products or components depends on a number of factors, including the heat lability of the material, knowledge of heat penetration into the articles, and other factors described under the validation program (see above). Apart from that description of sterilization cycle parameters, using a temperature of 121°, the F0 concept may be appropriate. The F0, at a particular temperature other than 121°, is the time (in minutes) required to provide the lethality equivalent to that provided at 121° for a stated time. Modern autoclaves generally operate with a control system that is significantly more responsive than the steam reduction valve of older units that have been in service for many years. In order for these older units to achieve the precision and level of control of the cycle discussed in this chapter, it may be necessary to upgrade or modify the control equipment and instrumentation on these units. This modification is warranted only if the chamber and steam jacket are intact for continued safe use and if deposits that interfere with heat distribution can be removed.

##### Dry-Heat Sterilization

The process of thermal sterilization of Pharmacopeial articles by dry heat is usually carried out by a batch process in an oven designed expressly for that purpose. A modern oven is supplied with heated, filtered air, distributed uniformly throughout the chamber by convection or radiation and employing a blower system with devices for sensing, monitoring, and controlling the critical parameters. The validation of a dry-heat sterilization facility is carried out in a manner similar to that for a steam sterilizer described earlier. Where the unit is employed for sterilizing components such as containers intended for intravenous solutions, care should be taken to avoid accumulation of particulate matter in the chamber. A typical acceptable range in temperature in the empty chamber is ±15° when the unit is operating at not less than 250°.

In addition to the batch process described above, a continuous process is frequently employed to sterilize and depyrogenate glassware as part of an integrated continuous aseptic filling and sealing system. Heat distribution may be by convection or by direct transfer of heat from an open flame. The continuous system usually requires a much higher temperature than cited above for the batch process because of a much shorter dwell time. However, the total temperature input during the passage of the product should be equivalent to that achieved during the chamber process. The continuous process also usually necessitates a rapid cooling stage prior to the aseptic filling operation. In the qualification and validation program, in view of the short dwell time, parameters for uniformity of the temperature, and particularly the dwell time, should be established.

A microbial survival probability of 10–12 is considered achievable for heat-stable articles or components. An example of a biological indicator for validating and monitoring dry-heat sterilization is a preparation of *Bacillus subtilis* spores. Since dry heat is frequently employed to render glassware or containers free from pyrogens as well as viable microbes, a pyrogen challenge, where necessary, should be an integral part of the validation program, e.g., by inoculating one or more of the articles to be treated with 1000 or more USP Units of bacterial endotoxin. The test with *Limulus lysate* could be used to demonstrate that the endotoxic substance has been inactivated to not more than 1/1000 of the original amount (3 log cycle reduction). For the test to be valid, both the original amount and, after acceptable inactivation, the remaining amount of endotoxin should be measured. For additional information on the endotoxin assay, see [Bacterial Endotoxins Test](#) (85).

##### Gas Sterilization

The choice of gas sterilization as an alternative to heat is frequently made when the material to be sterilized cannot withstand the high temperatures obtained in the steam sterilization or dry-heat sterilization processes. The active agent generally employed in gaseous sterilization is ethylene oxide of acceptable sterilizing quality. Among the disadvantages of this sterilizing agent are its highly flammable nature unless mixed with suitable inert gases, its mutagenic properties, and the possibility of toxic residues in treated materials, particularly those containing chloride ions. The sterilization process is generally carried out in a pressurized chamber designed similarly to a steam autoclave but with the additional features (see below) unique to sterilizers employing this gas. Facilities employing this sterilizing agent should be designed to provide adequate post sterilization degassing, to enable microbial survivor monitoring, and to minimize exposure of operators to the potentially harmful gas.<sup>3</sup>

Qualification of a sterilizing process employing ethylene oxide gas is accomplished along the lines discussed earlier. However, the program is more comprehensive than for the other sterilization procedures, since in addition to temperature, the humidity, vacuum/positive pressure, and ethylene oxide concentration also require rigid control. An important determination is to demonstrate that all critical process parameters in the chamber are adequate during the entire cycle. Since the sterilization parameters applied to the articles to be sterilized are critical variables, it is frequently advisable to precondition the load to achieve the required moisture content in order to minimize the time of holding at the required temperature before placement of the load in the ethylene oxide chamber. The validation process is generally made employing product inoculated with appropriate (BIs) such as spore preparations of *Bacillus subtilis*. For validation they may be used in full chamber loads of product, or simulated product. The monitoring of moisture and gas concentration requires the utilization of sophisticated instrumentation that only knowledgeable and experienced individuals can calibrate, operate, and maintain. The BI may be employed also in monitoring routine runs.

As is indicated elsewhere in this chapter, the BI may be employed in a fraction negative mode to establish the ultimate microbiological survivor probability in designing an ethylene oxide sterilization cycle using inoculated product or inoculated simulated product.

One of the principal limitations of the ethylene oxide sterilization process is the limited ability of the gas to diffuse to the innermost product areas that require sterilization. Package design and chamber loading patterns therefore must be determined so that there is minimal resistance to gas diffusion.

##### Sterilization by Ionizing Radiation

The rapid proliferation of medical devices unable to withstand heat sterilization and the concerns about the safety of ethylene oxide have resulted in increasing applications of radiation sterilization. It is applicable also to drug substances and final dosage forms. The advantages of sterilization by irradiation include low chemical reactivity, low measurable residues, and the fact that there are fewer variables to control. In fact, radiation sterilization is unique in that the basis of control is essentially that of the absorbed radiation dose, which can be precisely measured. Because of this characteristic, new procedures have been developed to determine the sterilizing dose. These, however, are still under review and appraisal, particularly with regard to the need, or otherwise, for additional controls and safety measures. Irradiation causes only a minimal temperature rise but can affect certain grades and types of plastics and glass.

The two types of ionizing radiation in use are radioisotope decay (gamma radiation) and electron-beam radiation. In either case the radiation dose established to yield the required degree of sterility assurance should be such that, within the range of minimum and maximum doses set, the properties of the article being sterilized are acceptable.

For gamma irradiation, the validation of a procedure includes the establishment of article materials compatibility, establishment of product loading pattern and completion of dose mapping in the sterilization container (including identification of the minimum and maximum dose zones), establishment of timer setting, and demonstration of the delivery of the required sterilization dose. For electron-beam irradiation, in addition, the on-line control of voltage, current, conveyor speed, and electron beam scan dimension must be validated.



For gamma radiation sterilization, an effective sterilizing dose that is tolerated without damaging effect should be selected. Although 2.5 megarads (Mrad) of absorbed radiation was historically selected, it is desirable and acceptable in some cases to employ lower doses for devices, drug substances, and finished dosage forms. In other cases, however, higher doses are essential. In order to validate the efficacy particularly of the lower exposure levels, it is necessary to determine the magnitude (number, degree, or both) of the natural radiation resistance of the microbial population of the product. Specific product loading patterns must be established, and absorbed minimum and maximum dosage distribution must be determined by use of chemical dosimeters. (These dosimeters are usually dyed plastic cylinders, slides, or squares that show color intensification based directly on the amount of absorbed radiation energy; they require careful calibration.)

The setting of the preferred absorbed dose has been carried out on the basis of pure cultures of resistant microorganisms and employing inoculated product, e.g., with spores of *Bacillus pumilus* as biological indicators. A fractional experimental cycle approach provides the data to be utilized to determine the D10 value of the biological indicator. This information is then applied in extrapolating the amount of absorbed radiation to establish an appropriate microbial survivor probability. The most recent procedures for gamma radiation sterilization base the dose upon the radiation resistance of the natural heterogeneous microbial burden contained on the product to be sterilized. Such procedures are currently being refined but may provide a more representative assessment of radiation resistance, especially where significant numbers of radiation-resistant organisms are present.<sup>4</sup> These range from inoculation with standard resistant organisms such as *Bacillus pumilus* to subprocess (sublethal) dose exposure of finished product samples taken from production lines. Certain hypotheses are common to all these methods. Although the total microbial population present on an article generally consists of a mixture of microorganisms of differing sensitivity to radiation, the step of subjecting the article to a less than totally lethal sterilization dose eliminates the less resistant microbial fraction. This results in a residual relatively homogeneous population with respect to radiation resistance and yields consistent and reproducible results of determinations with the residual population. The amount of laboratory manipulation required is dependent upon the particular procedure used.

One such procedure requires the enumeration of the microbial population on representative samples of independently manufactured lots of the article. The resistance of the microbial population is not determined, and dose setting is based on a standard arbitrary radiation resistance assigned to the microbial population, derived from data obtained from manufacturers and from the literature. The assumption is made that the distribution of resistances chosen represents a more severe challenge than the natural microbial population on the product to be sterilized. This assumption, however, is verified by experiment. After verification, the appropriate radiation sterilization dose is read from a table.

Another and, more elaborate method does not require the enumeration of the microbial population but uses a series of incremental dose exposures to allow a dose established to be such that approximately one out of 100 samples irradiated at that dose will be nonsterile. This is not the ultimate sterilization dose, but it provides the basis on which to determine the sterilization dose by extrapolation from the dose yielding one out of 100 nonsterile samples, using an appropriate resistance factor that characterizes the remaining microorganism-resistant population. A periodic audit is conducted to check that the findings continue to be operative.

More elaborate procedures, requiring more experimentation and including the isolation of microbial cultures, include one in which, after determining the substerilization dose (yielding one out of 100 nonsterile samples), the resistance of the surviving microorganisms is used to determine the sterilizing dose. Another is based on different determinations, starting with a substerilization incremental dose that results in not more than 50% of the samples being nonsterile. After irradiation of sufficient samples at this dose, a number of microbial isolates are obtained. The radiation resistance of each of these is determined. The sterilization dose is then calculated using the resistance determinations and the 50% sterilizing dose initially determined. Audit procedures are required for these methods, as for the others described.

Where the required minimum radiation dose has been determined and delivery of that dose has been confirmed (by chemical or physical dosimeters), release of the article being sterilized could be effected within the overall validation of sterility assurance (which may include such confirmation of applied dosage, the use of biological indicators, and other means).

#### Sterilization by Filtration

Filtration through microbial retentive materials is frequently employed for the sterilization of heat-labile solutions by physical removal of the contained microorganisms. A filter assembly generally consists of a porous matrix sealed or clamped into an impermeable housing. The effectiveness of a filter medium or substrate depends upon the pore size of the porous material and may depend upon adsorption of bacteria on or in the filter matrix or upon a sieving mechanism. There is some evidence to indicate that sieving is the more important component of the mechanism. Fiber-shedding filters, particularly those containing asbestos, are to be avoided unless no alternative filtration procedures are possible. Where a fiber-shedding filter is required, it is obligatory that the process include a nonfiber-shedding filter introduced downstream or subsequent to the initial filtration step.

**Filter Rating**— The pore sizes of filter membranes are rated by a nominal rating that reflects the capability of the filter membrane to retain microorganisms of size represented by specified strains, not by determination of an average pore size and statement of distribution of sizes. Sterilizing filter membranes (those used for removing a majority of contaminating microorganisms) are membranes capable of retaining 100% of a culture of 107 microorganisms of a strain of *Pseudomonas diminuta* (ATCC 19146) per square centimeter of membrane surface under a pressure of not less than 30 psi (2.0 bar). Such filter membranes are nominally rated 0.22 µm or 0.2 µm, depending on the manufacturer's practice.<sup>5</sup> This rating of filter membranes is also specified for reagents or media that have to be sterilized by filtration (see treatment of Isopropyl Myristate under Oils and Oily Solutions or Ointments and Creams in the chapter *Sterility Tests* (71)). Bacterial filter membranes (also known as analytical filter membranes), which are capable of retaining only larger microorganisms, are labeled with a nominal rating of 0.45 µm. No single authoritative method for rating 0.45-µm filters has been specified, and this rating depends on conventional practice among manufacturers; 0.45-µm filters are capable of retaining particular cultures of *Serratia marcescens* (ATCC 14756) or *Ps. diminuta*. Test pressures used vary from low (5 psi, 0.33 bar for *Serratia*, or 0.5 psi, 0.34 bar for *Ps. diminuta*) to high (50 psi, 3.4 bar). They are specified for sterility testing (see Membrane Filtration in the section Test for Sterility of the Product to be

Examined under *Sterility Tests* (71)) where less exhaustive microbial retention is required. There is a small probability of testing specimens contaminated solely with small microorganisms. Filter membranes with a very low nominal rating may be tested with a culture of *Acholeplasma laidlawii* or other strain of *Mycoplasma*, at a pressure of 7 psi (0.7 bar) and be nominally rated 0.1 µm. The nominal ratings based on microbial retention properties differ when rating is done by other means, e.g., by retention of latex spheres of various diameters. It is the user's responsibility to select a filter of correct rating for the particular purpose, depending on the nature of the product to be filtered. It is generally not feasible to repeat the tests of filtration capacity in the user's establishment. Microbial challenge tests are preferably performed under a manufacturer's conditions on each lot of manufactured filter membranes.

The user must determine whether filtration parameters employed in manufacturing will significantly influence microbial retention efficiency. Some of the other important concerns in the validation of the filtration process include product compatibility, sorption of drug, preservative or other additives, and initial effluent endotoxin content.

Since the effectiveness of the filtration process is also influenced by the microbial burden of the solution to be filtered, determining the microbiological quality of solutions prior to filtration is an important aspect of the validation of the filtration process, in addition to establishing the other parameters of the filtration procedure, such as pressures, flow rates, and filter unit characteristics. Hence, another method of describing filter-retaining capability is the use of the log reduction value (LRV). For instance, a 0.2-µm filter that can retain 107 microorganisms of a specified strain will have an LRV of not less than 7 under the stated conditions.

The process of sterilization of solutions by filtration has recently achieved new levels of proficiency, largely as a result of the development and proliferation of membrane filter technology. This class of filter media lends itself to more effective standardization and quality control and also gives the user greater opportunity to confirm the characteristics or properties of the filter assembly before and after use. The fact that membrane filters are thin polymeric films offers many advantages but also some disadvantages when compared to depth filters such as porcelain or sintered material. Since much of the membrane surface is a void or open space, the properly assembled and sterilized filter offers the advantage of a high flow rate. A disadvantage is that since the membrane is usually fragile, it is essential to determine that the assembly was properly made and that the membrane was not ruptured during assembly, sterilization, or use. The housings and filter assemblies that are chosen should first be validated for compatibility and integrity by the user. While it may be possible to mix assemblies and filter membranes produced by different manufacturers, the compatibility of these hybrid assemblies should first be validated. Additionally, there are other tests to be made by the manufacturer of the membrane filter, which are not usually repeated by the user. These include microbiological challenge tests. Results of these tests on each lot of manufactured filter membranes should be obtained from the manufacturer by users for their records.

Filtration for sterilization purposes is usually carried out with assemblies having membranes of nominal pore size rating of 0.2 µm or less, based on the validated challenge of not less than 10<sup>7</sup> *Pseudomonas diminuta* (ATCC No. 19146) suspension per square centimeter of filter surface area. Membrane filter media now available include cellulose acetate, cellulose nitrate, fluorocarbonate, acrylic polymers, polycarbonate, polyester, polyvinyl chloride, vinyl, nylon, polytef, and even metal membranes, and they may be reinforced or supported by an internal fabric. A membrane filter assembly should be tested for initial integrity prior to use, provided that such test does not impair the validity of the system, and should be tested after the filtration process is completed to demonstrate that the filter assembly maintained its integrity throughout the entire filtration procedure. Typical use tests are the bubble point test, the diffusive airflow test, the pressure hold test, and the forward flow test. These tests should be correlated with microorganism retention.



## Aseptic Processing

Although there is general agreement that sterilization of the final filled container as a dosage form or final packaged device is the preferred process for ensuring the minimal risk of microbial contamination in a lot, there is a substantial class of products that are not terminally sterilized but are prepared by a series of aseptic steps. These are designed to prevent the introduction of viable microorganisms into components, where sterile, or once an intermediate process has rendered the bulk product or its components free from viable microorganisms. This section provides a review of the principles involved in producing aseptically processed products with a minimal risk of microbial contamination in the finished lot of final dosage forms.

A product defined as aseptically processed is likely to consist of components that have been sterilized by one of the processes described earlier in this chapter. For example, the bulk product, if a filterable liquid, may have been sterilized by filtration. The final empty container components would probably be sterilized by heat, dry heat being employed for glass vials and an autoclave being employed for rubber closures. The areas of critical concern are the immediate microbial environment where these presterilized components are exposed during assembly to produce the finished dosage form and the aseptic filling operation.

The requirements for a properly designed, validated, and maintained filling or other aseptic processing facility are mainly directed to (1) an air environment free from viable microorganisms, of a proper design to permit effective maintenance of air supply units, and (2) the provision of trained operating personnel who are adequately equipped and gowned. The desired environment may be achieved through the high level of air filtration technology now available, which contributes to the delivery of air of the requisite microbiological quality.<sup>6</sup> The facilities include both primary (in the vicinity of the exposed article) and secondary (where the aseptic processing is carried out) barrier systems.

For a properly designed aseptic processing facility or aseptic filling area, consideration should be given to such features as nonporous and smooth surfaces, including walls and ceilings that can be sanitized frequently; gowning rooms with adequate space for personnel and storage of sterile garments; adequate separation of preparatory rooms for personnel from final aseptic processing rooms, with the availability if necessary of devices such as airlocks and air showers; proper pressure differentials between rooms, the most positive pressure being in the aseptic processing rooms or areas; the employment of laminar (unidirectional) airflow in the immediate vicinity of exposed product or components, and filtered air exposure thereto, with adequate air change frequency; appropriate humidity and temperature environmental controls; and a documented sanitization program. Proper training of personnel in hygienic and gowning techniques should be undertaken so that, for example, gowns, gloves, and other body coverings substantially cover exposed skin surfaces.

Certification and validation of the aseptic process and facility are achieved by establishing the efficiency of the filtration systems, by employing microbiological environmental monitoring procedures, and by processing of sterile culture medium as simulated product.

Monitoring of the aseptic facility should include periodic environmental filter examination as well as routine particulate and microbiological environmental monitoring and may include periodic sterile culture medium processing.

## STERILITY TESTING OF LOTS

It should be recognized that the referee sterility test might not detect microbial contamination if present in only a small percentage of the finished articles in the lot because the specified number of units to be taken imposes a significant statistical limitation on the utility of the test results. This inherent limitation, however, has to be accepted, because current knowledge offers no nondestructive alternatives for ascertaining the microbiological quality of every finished article in the lot, and it is not a feasible option to increase the number of specimens significantly.

The primary means of supporting the claim that a lot of finished articles purporting to be sterile meets the specifications consists of the documentation of the actual production and sterilization record of the lot and of the additional validation records that the sterilization process has the capability of totally inactivating the established product microbial burden or a more resistant challenge. Further, it should be demonstrated that any processing steps involving exposed product following the sterilization procedure are performed in an aseptic manner to prevent contamination. If data derived from the manufacturing process sterility assurance validation studies and from in-process controls are judged to provide greater assurance that the lot meets the required low probability of containing a contaminated unit (compared to sterility testing results from finished units drawn from that lot), any sterility test procedures adopted may be minimal, or dispensed with on a routine basis. However, assuming that all the above production criteria have been met, it may still be desirable to perform sterility testing on samples of the lot of finished articles. Such sterility testing is usually carried out directly after the lot is manufactured as a final product quality control test.<sup>7</sup> Sterility tests employed in this way in manufacturing control should not be confused with those described under [Sterility Tests](#) (71). The procedural details may be the same with regard to media, inocula and handling of specimens, but the number of units and/or incubation time(s) selected for testing may differ. The number should be chosen relative to the purpose to be served, i.e., according to whether greater or lesser reliance is placed on sterility testing in the context of all the measures for sterility assurance in manufacture. Also, longer times of incubation would make the test more sensitive to slow-growing microorganisms. In the growth promotion tests for media, such slow growers, particularly if isolated from the product microbial burden, should be included with the other test stains. Negative or satisfactory sterility test results serve only as further support of the existing evidence concerning the quality of the lot if all the pertinent production records of the lot are in order and the sterilizing or aseptic process is known to be effective. Unsatisfactory test results, however, in manufacturing quality control indicate a need for further action (see [Performance, Observation, and Interpretation](#)).

### Definition of a Lot and Selection of Specimens for Sterility Test Purposes

Articles may be terminally sterilized either in a chamber or by a continuous process. In the chamber process, a number of articles are sterilized simultaneously under controlled conditions—for example, in a steam autoclave—so that for the purpose of sterility testing, the lot is considered to be the contents of a single chamber. In the continuous process, the articles are sterilized individually and consecutively (for example, by exposure to electron-beam radiation), so that the lot is considered to be not larger than the total number of similar items subjected to uniform sterilization for a period of not more than 24 hours.

For aseptic fills, the term “filling operation” describes a group of final containers, identical in all respects, that have been aseptically filled with the same product from the same bulk within a period not longer than 24 consecutive hours without an interruption or a change that would affect the integrity of the filling assembly. The items tested should be representative of each filling assembly and should be selected at appropriate intervals throughout the entire filling operation. If more than three filling machines, each with either single or multiple filling stations, are used for filling a single lot, a minimum of 20 filled containers (not less than 10 per medium) should be tested for each filling machine, but the total number generally need not exceed 100 containers.

For small lots, in the case of either aseptic filling or terminal sterilization, if the number of final containers in the lot is between 20 and 200, about 10% of the containers should usually be tested. If the number of final containers in the lot is 20 or less, not fewer than 2 final containers should be tested.

## PERFORMANCE, OBSERVATION, AND INTERPRETATION

The facility for sterility testing should be such as to offer no greater a microbial challenge to the articles being tested than that of an aseptic processing production facility. The sterility testing procedure should be performed by individuals having a high level of aseptic technique proficiency. The test performance records of these individuals should be documented.

The extensive aseptic manipulations required to perform sterility testing may result in a probability of non-product-related contamination of the order of 10–3, a level similar to the overall efficiency of an aseptic operation and comparable to the microbial survivor probability of aseptically processed articles. This level of probability is significantly greater than that usually attributed to a terminal sterilization process, namely, 1 in 1 million or 10<sup>-6</sup> microbial survivor probability. Appropriate, known-to-be-sterile finished articles should be employed periodically as negative controls as a check on the reliability of the test procedure. Preferably, the technicians performing the test should be unaware that they are testing negative controls. Of these tests, a false-positive frequency not exceeding 2% is desirable.

For aseptically processed articles, these facts support the routine use of the test set forth under [Sterility Tests](#) (71) or a more elaborate one. The production and validation documentation should be acceptable and complete. For effectively terminally sterilized products, however, the lower microbial survivor probability may direct the use of a less extensive test than the compendial procedure specified under [Sterility Tests](#) (71), or even preclude altogether the necessity for performing one. This added reliability of sterility assurance of terminal sterilization depends upon a properly validated and documented sterilization process. Sterility testing alone is no substitute.

**Interpretation of Quality Control Tests**— The overall responsibility for the operation of the test unit and the interpretation of test results in relation to acceptance or rejection of a lot should be in the hands of those who have appropriate formal training in microbiology and have knowledge of industrial sterilization, aseptic processing, and the statistical concepts involved in sampling. These individuals should be knowledgeable also concerning the environmental control program in the test facility to ensure that the microbiological quality of the



air and critical work surfaces are consistently acceptable.

Quality control sterility tests (either according to the official referee test or modified tests) may be carried out in two separate stages in order to rule out false positive results. First Stage. Regardless of the sampling plan used, if no evidence of microbial growth is found, the results of the test may be taken as indicative of absence of intrinsic contamination of the lot.

If microbial growth is found, proceed to the Second Stage (unless the First Stage test can be invalidated). Evidence for invalidating a First Stage test in order to repeat it as a First Stage test may be obtained from a review of the testing environment and the relevant records thereto. Finding of microbial growth in negative controls need not be considered the sole grounds for invalidating a First Stage test. When proceeding to the Second Stage, particularly when depending on the results of the test for lot release, concurrently, initiate and document a complete review of all applicable production and control records. In this review, consideration should be paid to the following: (1) a check on monitoring records of the validated sterilization cycle applicable to the product, (2) sterility test history relating to the particular product for both finished and in-process samples, as well as sterilization records of supporting equipment, containers/closures, and sterile components, if any, and (3) environmental control data, including those obtained from media fills, exposure plates, filtering records, any sanitization records and microbial monitoring records of operators, gowns, gloves, and garbing practices.

Failing any lead from the above review, the current microbial profile of the product should be checked against the known historical profile for possible change. Records should be checked concomitantly for any changes in source of product components or in-processing procedures that might be contributory. Depending on the findings, and in extreme cases, consideration may have to be given to revalidation of the total manufacturing process. For the Second Stage, it is not possible to specify a particular number of specimens to be taken for testing. It is usual to select double the number specified for the First Stage under [Sterility Tests](#) 71, or other reasonable number. The minimum volumes tested from each specimen, the media, and the incubation periods are the same as those indicated for the First Stage.

If no microbial growth is found in the Second Stage, and the documented review of appropriate records and the indicated product investigation does not support the possibility of intrinsic contamination, the lot may meet the requirements of a test for sterility. If growth is found, the lot fails to meet the requirements of the test. As was indicated for the First Stage test, the Second Stage test may similarly be invalidated with appropriate evidence, and, if so done, repeated as a Second Stage test.

1 A number of guidelines dealing particularly with the development and validation of sterilization cycles and related topics have been published. These include, by the Parenteral Drug Association, Inc. (PDA), Validation of Steam Sterilization Cycles (Technical Monograph No. 1), Validation of Aseptic Filling for Solution Drug Products (Technical Monograph No. 2), and Validation of Dry Heat Processes Used for Sterilization and Depyrogenation (Technical Monograph No. 3); and by the Pharmaceutical Manufacturers Association (PMA), Validation of Sterilization of Large-Volume Parenterals—Current Concepts (Science and Technology Publication No. 25). Other series of technical publications on these subjects by the Health Industry Manufacturers Association (HIMA) include Validation of Sterilization Systems (Report No. 78-4.1), Sterilization Cycle Development (Report No. 78-4.2), Industrial Sterility: Medical Device Standards and Guidelines (Document #9, Vol. 1), and Operator Training . . . for Ethylene Oxide Sterilization, for Steam Sterilization Equipment, for Dry Heat Sterilization Equipment, and for Radiation Sterilization Equipment (Report Nos. 78-4.5 through 4.8). Recommended practice guidelines published by the Association for the Advancement of Medical Instrumentation (AAMI) include Guideline for Industrial Ethylene Oxide Sterilization of Medical Devices—Process Design, Validation, Routine Sterilization (No. OPEO-12/81) and Process Control Guidelines for the Radiation Sterilization of Medical Devices (No. RS-P 10/82). These detailed publications should be consulted for more extensive treatment of the principles and procedures described in this chapter.

2 An autoclave cycle, where specified in the compendia for media or reagents, is a period of 15 minutes at 121°, unless otherwise indicated.

3 See Ethylene Oxide, Encyclopedia of Industrial Chemical Analysis, 1971, 12, 317-340, John Wiley & Sons, Inc., and Use of Ethylene Oxide as a Sterilant in Medical Facilities, NIOSH Special Occupational Hazard Review with Control Recommendations, August 1977, U. S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, Division of Criteria Documentation and Standards Development, Priorities and Research Analysis Branch, Rockville, MD.

4 Detailed descriptions of these procedures have been published by the Association for the Advancement of Medical Instrumentation (AAMI) in the document entitled Process Control Guidelines for Radiation Sterilization of Medical Devices (No. RS-P 10/82).

5 Consult "Microbiological Evaluation of Filters for Sterilizing Liquids," Health Industry Manufacturers Association, Document No. 3, Vol. 4, 1982.

6 Available published standards for such controlled work areas include the following: (1) Federal Standard No. 209B, Clean Room and Work Station Requirements for a Controlled Environment, Apr. 24, 1973. (2) NASA Standard for Clean Room and Work Stations for Microbiologically Controlled Environment, publication NHB5340.2, Aug. 1967. (3) Contamination Control of Aerospace Facilities, U.S. Air Force, T.O. 00-25-203, 1 Dec. 1972, change 1-1, Oct. 1974.

7 Radioactive Pharmaceutical Products—Because of rapid radioactive decay, it is not feasible to delay the release of some radioactive pharmaceutical products in order to complete sterility tests on them. In such cases, results of sterility tests provide only retrospective confirmatory evidence for sterility assurance, which therefore depends on the primary means thereto established in the manufacturing and validation/certification procedures.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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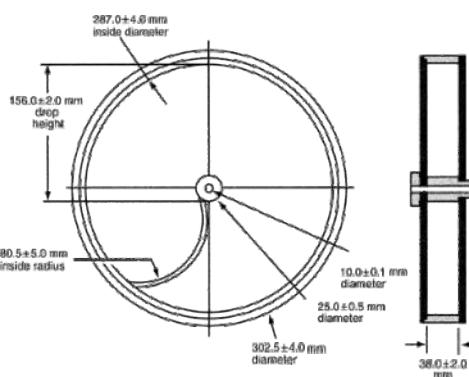
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#### 1216 TABLET FRIABILITY

This general information chapter has been harmonized with the corresponding texts of the European Pharmacopoeia and the Japanese Pharmacopoeia. The harmonized texts of these three pharmacopeias are therefore interchangeable, and the methods of the European Pharmacopoeia and/or the Japanese Pharmacopoeia may be used for demonstration of compliance instead of the present United States Pharmacopoeia general information chapter method. These pharmacopeias have undertaken not to make any unilateral change to this harmonized chapter.

This chapter provides guidelines for the friability determination of compressed, uncoated tablets. The test procedure presented in this chapter is generally applicable to most compressed tablets. Measurement of tablet friability supplements other physical strength measurements, such as tablet breaking force.

Use a drum, with an internal diameter between 283 and 291 mm and a depth between 36 and 40 mm, of transparent synthetic polymer with polished internal surfaces, and subject to minimum static build-up (see figure for a typical apparatus). One side of the drum is removable. The tablets are tumbled at each turn of the drum by a curved projection with an inside radius between 75.5 and 85.5 mm that extends from the middle of the drum to the outer wall. The outer diameter of the central ring is between 24.5 and 25.5 mm. The drum is attached to the horizontal axis of a device that rotates at 25 ±1 rpm. Thus, at each turn the tablets roll or slide and fall onto the drum wall or onto each other.



Tablet Friability Apparatus

For tablets with a unit weight equal to or less than 650 mg, take a sample of whole tablets corresponding as near as possible to 6.5 g. For tablets with a unit weight of more than 650 mg, take a sample of 10 whole tablets. The tablets should be carefully dedusted prior to testing. Accurately weigh the tablet sample, and place the tablets in the drum. Rotate the drum 100 times, and remove the tablets. Remove any loose dust from the tablets as before, and accurately weigh.

Generally, the test is run once. If obviously cracked, cleaved, or broken tablets are present in the tablet sample after tumbling, the sample fails the test. If the results are difficult to interpret or if the weight loss is greater than the targeted value, the test should be repeated twice and the mean of the three tests determined. A maximum mean weight loss from the three samples of not more than 1.0% is considered acceptable for most products.

If tablet size or shape causes irregular tumbling, adjust the drum base so that the base forms an angle of about 10° with the horizontal and the tablets no longer bind together when lying next to each other, which prevents them from falling freely.

Effervescent tablets and chewable tablets may have different specifications as far as friability is concerned. In the case of hygroscopic tablets, an appropriate humidity-controlled environment is required for testing.

Drums with dual scooping projections, or an apparatus with more than one drum, for the running of multiple samples at one time, are also permitted.

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\* The apparatus meeting these specifications is available from laboratory supply houses such as VanKel Technology Group, 13000 Weston Parkway, Cary, NC 27513, or from Erweka Instruments, Inc., 56 Quirk Road, Milford, CT 06460.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">William E. Brown</a> Senior Scientist 1-301-816-8380	(PDF05) Pharmaceutical Dosage Forms 05

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### 1217 TABLET BREAKING FORCE

#### INTRODUCTION

There are a variety of presentations for tablets as delivery systems for pharmaceutical agents, such as rapidly disintegrating, slowly disintegrating, eroding, chewable, and lozenge. Each of these presentations places a certain demand on the bonding, structure, and integrity of the compressed matrix. Tablets must be able to withstand the rigors of handling and transportation experienced in the manufacturing plant, in the drug distribution system, and in the field at the hands of the end users (patients/consumers). Manufacturing processes such as coating, packaging, and printing can involve considerable stresses, which the tablets must be able to withstand. For these reasons, the mechanical strength of tablets is of considerable importance and is routinely measured. Tablet strength serves both as a criterion by which to guide product development and as a quality control specification.

One commonly employed test of the ability of tablets to withstand mechanical stresses determines their resistance to chipping and surface abrasion by tumbling them in a rotating cylinder. The percentage weight loss after tumbling is referred to as the friability of the tablets. Standardized methods and equipment for testing friability have been provided in general chapter Tablet Friability (1216).

Another measure of the mechanical integrity of tablets is their breaking force, which is the force required to cause them to fail (i.e., break) in a specific plane. The tablets are generally placed between two platens, one of which moves to apply sufficient force to the tablet to cause fracture. For conventional, round (circular cross-section) tablets, loading occurs across their diameter (sometimes referred to as diametral loading), and fracture occurs in that plane.

The breaking force of tablets is commonly called hardness in the pharmaceutical literature; however, the use of this term is misleading. In material science, the term hardness refers to the resistance of a surface to penetration or indentation by a small probe. The term crushing strength is also frequently used to describe the resistance of tablets to the application of a compressive load. Although this term describes the true nature of the test more accurately than does hardness, it implies that tablets are actually crushed during the test, which often is not the case. Moreover, the term strength in this application can be questioned, because in the physical sciences that term is often used to describe a stress (e.g., tensile strength).

Thus, the term breaking force is preferred and will be used in the present discussion.

#### TABLET BREAKING FORCE DETERMINATIONS

Early measuring devices were typically hand operated. For example, the Monsanto (or Stokes) hardness tester was based on compressing tablets between two jaws via a spring gauge and screw. In the Pfizer hardness tester, the vertically mounted tablet was squeezed in a device that resembled a pair of pliers. In the Strong Cobb hardness tester, the breaking load was applied through the action of a small hydraulic pump that was first operated manually but was later motorized. Problems associated with these devices were related to operator variability in rates of loading and difficulties in proper setup and calibration. Modern testers employ mechanical drives, strain gauge-based load cells for force measurements, and electronic signal processing, and therefore are preferred. However, several important issues must be considered when using them for the analytical determination of breaking force; these are discussed below.

#### Platens

The platens should be parallel. Their faces should be polished smooth and precision-ground perpendicularly to the direction of movement. Perpendicularity must be preserved during platen movement, and the mechanism should be free of any bending or torsion displacements as the load is applied. The contact faces must be larger than the area of contact with the



tablet.

#### Rate and Uniformity of Loading

Either the rate of platen movement or the rate at which the compressive force is applied (i.e., the loading rate) should be constant. Maintaining a constant loading rate avoids the rapid buildup of compressive loads, which may lead to uncontrolled crushing or shear failure and greater variability in the measured breaking force. However, constant loading rate measurements may be too slow for real time monitoring of tablet production.

The rate at which the compressive load is applied can significantly affect results, because time-dependent processes may be involved in tablet failure (1). How a tablet matrix responds to differences in the loading rate depends on the mechanism of failure. At low strain rates, some materials may fail in a ductile manner, but brittle failure is more likely at faster strain rates. The transition from ductile to brittle failure is accompanied by an increase in the breaking force. Devices that simply crush tablets may produce deceptively reproducible data because they lack sensitivity.

The test must be run consistently with equipment which has been routinely calibrated. Changing from testing units of different designs or from different manufacturers will require comparison of data to ensure that the two units are subjecting the dosage form to similar stress in a similar manner. Currently available equipment provides a constant loading rate of 20 newtons (N) or less per second or a constant platen movement of 3.5 mm or less per second. Controlled and consistent breaking is an important test procedure attribute. To ensure comparability of results, testing must occur under identical conditions of loading rate or platen movement rate. Since there are certain advantages to each system of load application, both are found in practice. Because the particular testing situation and the type of tablet matrix being evaluated will pose different constraints, there is also no basis to declare an absolute preference for one system over the other. This general chapter proposes consideration of both approaches.

The different methods may lead to numerically different results for a particular tablet sample, requiring that the rate of load application or displacement must be specified along with the determined breaking force.

#### Dependence of Breaking Force on Tablet Geometry and Mass

Measurements of breaking force do not take into account the dimensions or shape of the tablet. Thicker tablets of the same material compressed under conditions identical to those of thinner tablets, with the same tooling shape and to the same peak force, will require greater breaking forces. Tablet orientation and failure should occur in a manner consistent with those used during the development of the dosage form. For direct comparisons (i.e., without any normalizations of the data), breaking force measurements should be performed on tablets having the same dimensions, geometry, and consistent orientation in test equipment.

#### Tablet Orientation

Tablet orientation in diametral compression of round tablets without any scoring is unequivocal. That is, the tablet is placed between the platens so that compression occurs across a diameter. However, tablets with a unique or complex shape may have no obvious orientation for breaking force determination. Because the breaking force may depend on the tablet's orientation in the tester, to ensure comparability of results, it is best to settle on a standard orientation, preferably one that is most readily and easily reproduced by operators. In general, tablets are tested either across the diameter or parallel to the longest axis. Scored tablets have two orientation possibilities. When they are oriented with their scores perpendicular to the platen faces, the likelihood that tensile failure will occur along the scored line increases. This provides information about the strength of the matrix at the weakest point in the structure. When scored tablets are oriented with their scores parallel to the platen faces, more general information about the strength of the matrix is derived.

Capsule-shaped tablets or scored tablets may best be broken in a three-point flexure test (2). A fitting, which is either installed on the platens or substituted for the platens, supports the tablet at its ends and permits the breaking load to be applied to the opposite face at the unsupported midpoint of the tablet. The fittings are often available from the same source that supplies the hardness tester.

#### Units, Resolution, and Calibration

Modern breaking force testers are usually calibrated in kiloponds or newtons. The relationship between these units of force (3) is 1 kilopond (kp) = 1 kilogram-force (kgf) = 9.80 N. The test results should be expressed in standard units of force which facilitate communication. Some breaking force testers also will provide a scale in Strong Cobb units (SCU), a carryover from the days when Strong Cobb hardness testers were in common usage. The conversion between SCU and N or kp must be viewed with caution, because the SCU is derived from a hydraulic device and is a pressure.

Generally, contemporary breaking force testers use modern electronic designs with digital readouts. Some units also have an integral printer or may be interfaced with a printer. Breaking forces should be readable to within 1 N.

Breaking force testers should be calibrated periodically. The force sensor as well as the mechanics of the apparatus needs to be considered. For the force sensor, the complete measuring range (or, at a minimum, the range used for measuring the test sample) should be calibrated to a precision of 1 N, using either the static or dynamic method. Static calibration generally employs traceable counterweights; at least three different points are checked to assess linearity. Dynamic calibration makes use of a traceable reference-load cell that is compressed between the platens. The functional calibration of a breaking force test apparatus should also confirm that the velocity and the constancy of velocity for load application or displacement are within prescribed tolerances throughout the range of platen movement.

#### Sample Size

In order to achieve sufficient statistical precision for the determination of average breaking force, a minimum of 6 tablet samples should be tested. The average breaking force alone may be adequate to fulfill the purpose of process or product quality control. In cases where breaking force may be particularly critical, the average plus individual breaking force values should be accessible.

#### TENSILE STRENGTH

The measurement of tensile strengths provides a more fundamental measure of the mechanical strength of the compacted material and takes into account the geometry of the tablet. If tablets fail in tension, the breaking force can be used to calculate the tensile strength. Unfortunately, this is practical only for simple shapes. If flat-faced round tablets (right circular cylinders) fail in tension, as indicated by a clean split into halves under diametral compression, the breaking force may be used to compute the tensile strength from the following equation (4), which applies only to cylindrical tablets:

$$\sigma_x = \frac{2F}{\pi DH}$$

in which  $\sigma_x$  is the tensile strength, F is the breaking force, D is the tablet diameter, and H is the tablet thickness. Because only tablets that fail in tension are counted, the data are based on tablets that fail in a consistent way. Thus, reproducibility of data should be enhanced when compared to conventional breaking-strength testing. Moreover, the data will be normalized with respect to tablet dimensions, because both diameter and thickness are included in the equation. The derivation of this equation may be found in standard texts (5, 6); it is based on elastic theory and the following assumptions:

1. The tablet is an isotropic body



2. Hooke's law is obeyed
3. The modulus of elasticity in compression and in tension is the same
4. Ideal point loading occurs

The derivation has been extended to convex-faced tablets (7, 8):

$$\sigma_x = \frac{10F}{\pi D^2} \left[ \frac{2.84H}{D} - \frac{0.126H}{W} + \frac{3.15W}{D} + 0.01 \right]^1$$

where  $\sigma_x$  is the tensile strength, F is the breaking force, D is the tablet diameter, H is the tablet thickness, and W is the central cylinder thickness (tablet wall height).

The slow and constant loading rate of modern motorized break force testers encourages tensile failure. However, ideal point loading may not occur, because of crushing and the induction of shear failure at the interface with the surface of the platens. The addition of padding to the platens helps prevent shear at contact points and promotes true tensile failure. On that basis, padding is strongly recommended when highly precise measurements are needed. Padding should be relatively thin so that any deviation from the assumption of true point-source force application will not be large. The padding should also collapse very easily so that its deformation does not become part of the force measured by the test apparatus.

In more routine settings involving measurements on a large number of samples, the addition of padding could contribute to inaccuracies in measurement as powder from previously tested samples becomes embedded in the collapsible matrix and thereby alters its properties. Unless provisions for frequent and routine replacement of the padding are made, it can be considered acceptable to ignore the use of padding material to maintain constancy of the test conditions.

Bending or flexure of tablets is another option for determining the tensile strength of tablets. Under ideal loading conditions, a breaking load applied to the unsupported midpoint of one face will result in the generation of pure tensile stress in the opposite face. If the tablets are right circular cylinders and are subjected to three-point flexure, the tensile strength may be estimated using the following equation (9):

$$\sigma_x = \frac{3FL}{2H^2D}$$

in which L is the distance between supports, and the other terms are as defined above. The assumptions are the same as those for calculating tensile strength from diametral compression. However, tensile strengths determined by flexure and diametral compression may not agree, because of likely nonideal loading and the induction of shear failure during testing.

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1221 TEASPOON

For household purposes, an American Standard Teaspoon has been established by the American National Standards Institute\* as containing  $4.93 \pm 0.24 \text{ mL}$ . In view of the almost universal practice of employing teaspoons ordinarily available in the household for the administration of medicine, the teaspoon may be regarded as representing 5 mL. Preparations intended for administration by teaspoon should be formulated on the basis of dosage in 5-mL units. Any dropper, syringe, medicine cup, special spoon, or other device used to administer liquids should deliver 5 mL wherever a teaspoon calibration is indicated. Under ideal conditions of use, the volume error incurred in measuring liquids for individual dose administration by means of such calibrated devices should be not greater than 10% of the indicated amount.

Household units are used often to inform the patient of the size of the dose. Fifteen milliliters should be considered 1 standard tablespoonful; 10 mL, 2 standard teaspoonfuls; and 5 mL, 1 standard teaspoonful. Doses of less than 5 mL are frequently stated as fractions of a teaspoonful or in drops.

Because of the difficulties involved in measuring liquids under normal conditions of use, patients should be cautioned that household spoons are not appropriate for measuring medicines. They should be directed to use the standard measures in the cooking-and-baking measuring spoon sets or, preferably, oral dosing devices that may be provided by the practitioner. It must be kept in mind that the actual volume of a spoonful of any given liquid is related to the latter's viscosity and surface tension, among other influencing factors. These factors can also cause variability in the true volumes contained in or delivered by medicine cups. Where accurate dosage is required, a calibrated syringe or dropper should be used.

\* American National Standards Institute, 1430 Broadway, New York, NY 10018.

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## INTRODUCTION

Parametric release is defined as the release of terminally sterilized batches or lots of sterile products based upon compliance with the defined critical parameters of sterilization without having to perform the requirements under [Sterility Tests \(71\)](#). Parametric release is a possibility when the mode of sterilization is very well understood, the physical parameters of processing are well defined, predictable, and measurable, and the lethality of the cycle has been microbiologically validated through the use of appropriate biological indicators or, in the case of ionizing radiation, the appropriate microbiological and dosimetric tests. The use of parametric release for sterilization processes requires prior FDA approval. It should be expected that the regulatory agencies evaluating submissions including the use of parametric product release would insist upon a well supported scientific rationale for the sterilization process and well documented validation data. The agencies would need assurance that any marketed sample of product will be sterile and if tested after release would pass the requirements for sterility as found in the general chapter [Sterility Tests \(71\)](#).

It is important to consider the limitations of the [Sterility Tests \(71\)](#) in the evaluation of terminally sterilized products. The sterility test described in general chapter [\(71\)](#) is limited in its sensitivity and is statistically ill-suited to the evaluation of terminally sterilized products given the exceedingly low probability of contaminated units. Therefore, once a sterilization process is fully validated and operates consistently, a combination of physical sterilization data such as accumulated lethality or dosimetry in combination with other methods, such as load monitors (e.g., biological indicators, thermochemical indicators, or physicochemical integrators), can provide more accurate information than the sterility test regarding the release of terminally sterilized product to the marketplace.

There are four modes of sterilization that theoretically and practically could qualify for parametric release: moist heat, dry heat, ethylene oxide, and ionizing radiation sterilization. This information chapter first will cover the general issues related to parametric release, regardless of the modes of sterilization, and then discuss some specific modes of sterilization. The chapter will not address the parametric release of terminally sterilized medical devices.

Terminally sterilized products represent the lowest risk category of sterile pharmaceutical products. Unlike products aseptically manufactured in a microbiologically controlled environment, terminally sterilized products are subjected to a sterilization process that imparts a measurable minimum sterility assurance level, or SAL. Because aseptic processing relies on exclusion of microbiological contamination and is not based upon lethality imparted on the product in its sealed container, it is not possible to estimate the SAL. It is important to note that in the case of aseptic processing, SAL can only be estimated from media fill contamination rates or other forms of risk assessment. In the case of terminal sterilization, it is possible to calculate a minimum SAL or Probability of Nonsterility (PNS) quite accurately. Therefore, the term SAL has different contextual meanings when used to describe aseptic rather than terminal processes, and it is important that this difference is fully understood by scientists and engineers working in the field of sterile product manufacturing and control.

The terms PNS and SAL are often used interchangeably.

Terminally sterilized products must have a probability of nonsterility (PNS) of not more than one in a million units produced. This is often stated as a PNS or SAL of 10<sup>-6</sup>, or the probability of product bioburden surviving the sterilization process in any single unit of product is less than one in one million. The proof that a terminally sterilized product complies with the 10<sup>-6</sup> PNS can be accomplished by several different sterilization cycle development approaches. The proper application of these methods requires extensive scientific knowledge regarding the sterilization method selected for use with a specific product.

The strategies used to validate a terminal sterilization process development fall into three categories:

1. Bioburden-based process.
2. Biological indicator/bioburden combined process.
3. Overkill process.

The bioburden-based process requires extensive knowledge of product bioburden. It should be noted that several radiation dose-setting procedures involve establishing radiation processes on the basis of bioburden count and radiation resistance. This method requires that at least a 10<sup>-6</sup> PNS be attained for bioburden by the sterilization process. This means that if the product bioburden action level is 10 microorganisms or one logarithm, at least seven logarithms of bioburden must be inactivated to assure a 10<sup>-6</sup> PNS. The bioburden-based method requires the user to develop suitable critical control points within the process to control the bioburden titer. Products that readily permit bioburden survival require more controlled manufacturing environments and more precise in-process control. This process is better suited for cycle development for clean or ultra-clean products containing a consistently low level of colony forming units (cfu) per product unit with a low frequency of spore-forming microorganisms. Also, this process may be necessary to permit terminal sterilization of a product that may potentially lose key qualities or attributes as a result of a more rigorous sterilization process.

The microbiologist may find that formal hazard analysis procedures, such as Hazard Analysis Critical Control Point (HACCP), are useful in establishing appropriate manufacturing control conditions and in-process control parameters.

The biological indicator/bioburden combined process is generally used when the manufacturer desires a sterilization process that demonstrates the inactivation of high numbers of biological indicator microorganisms known to be resistant to the process. While the manufacturer may have preferred utilizing an overkill process, potential loss of some product attributes may occur in an overkill process thereby necessitating the use of a biological indicator/bioburden combined process. This process requires knowledge of the bioburden load on and in the product, and a database relative to the sterilization resistance of the bioburden. The relative resistance of the selected biological indicator to that of the bioburden must be established on or in the product. Frequently, biological indicators bearing approximately 10<sup>6</sup> spores with D121-value > 1 minute are used in the development of such processes. Fractional exposure cycles are generally conducted to determine the relative sterilization resistance (or D value) between product inoculated with the biological indicator microorganism (s) and frequently encountered bioburden. This process is frequently used for sterilization cycle development by manufacturers of terminally sterilized parenteral products and for ethylene oxide sterilization of medical devices.

The overkill process is frequently used when the article being sterilized is completely inert to the sterilizing agent and sterilization cycle conditions without any concern for loss of product attributes or quality. When using this process, some bioburden knowledge should be available to ensure that the materials are not adulterated before sterilization. These data may include product bioburden count data and knowledge concerning the prevalence of spore formers. The database for this process need not be as extensive as bioburden data required for the bioburden process or the biological indicator/bioburden process. Generally, process-resistant biological indicators containing approximately 10<sup>6</sup> spores are used to establish the effectiveness of the sterilization process. However, a spore population of N0 can be chosen to confirm adequate process lethality. Overkill is generally defined as a process that would deliver a minimum of F0<sup>4</sup> of 12 minutes (see Critical Operating Parameters below) and is demonstrated biologically based upon the spore log reduction of calibrated biological indicators.

## GENERAL REVIEW

### Validation of Sterilization Process

Parametric release first requires that the chosen sterilization process be designed and validated to achieve a 10<sup>-6</sup> PNS. Validation of most sterilization processes includes the validation of physical parameters of the process and of its microbiological effectiveness through the use of biological indicators. However, the use of biological indicators for establishing or periodically validating gamma radiation sterilization processes is uncommon. Widely recognized biological indicator organisms are used in the validation of moist heat processes because they provide a means of comparing physically measured lethality data with biological lethality. There should be a reasonable correlation between physically measured lethality data (F0) and biological lethality as determined by the evaluation of the process with biological indicators.

The predictable effectiveness of bioburden-based terminal sterilization is based on the number and resistance of microorganisms on or in a product. For this reason, one component of parametric release is an active microbiology control program to monitor the count and sterilization resistance of product bioburden. Bioburden control and enumeration is of far less significance when the overkill process design is used. In many cases, overkill processes do not require extensive ongoing assessment of bioburden and require less in-process control of the manufacturing environment.

Sterilization Microbiology Control Program



The purpose of this control program is to ensure that the microbiological status of the product, prior to being terminally sterilized, has not significantly deviated from the established microbiological control level used for validation of the sterilization process. The microbiology control program includes the monitoring of the bioburden on or in the product and the monitoring of the microbiological status of any necessary containers, closures, or packaging materials. Also included is a program to evaluate the microbiological status of the environment where the product is processed. The control program is particularly important in cases where the terminal sterilization is not based on overkill, but rather on the bioburden or combined bioburden/biological indicator cycle development approach. In many cases, bioburden control and manufacturing environmental monitoring will not be required for overkill process designs, where the F0 of the process is at least 12 minutes. In other cases, even when overkill processes are employed, some limited monitoring will be needed. Monitoring of overkill processes for bioburden is generally limited to those products that support microbial growth. Of particular concern in this case is the potential for the product to be contaminated with microbial toxins or to be degraded by microorganisms.

The frequency of monitoring will depend on the variations of bioburden from potential sources. The number of microorganisms, their identification, as well as their resistance to the specified sterilization mode should be considered when parametric release of terminally sterilized product is established. Resistance to a specified sterilization mode by different species can influence sterilization effectiveness and the determination of sterilization process conditions when using the bioburden or combined bioburden/biological indicator method of cycle development. In the bioburden approach to process development, indicator organisms more resistant than typical bioburden may be used, although extreme differentials in resistance are not required. Information on the performance of biological indicators may be found in the general chapter [Biological Indicators—Resistance Performance Tests](#) 55.

#### Change Control System

Changes introduced to the sterilization processing equipment could result in a significant departure of the initially validated parametric release process. It is, therefore, essential that a change control system be instituted. A change control system is a formal system with appropriate standard operating procedures, which would include approval of changes in the sterilization processing equipment. This system would assess all the changes in relation to the critical parameters included in parametric release. The change control system also includes technical and management review and criteria for acceptance or revision of changes. If a change would significantly affect any critical parameter, each parameter would have to be revalidated in terms of sterility assurance of the pharmaceutical product to a minimum 10–6 PNS. Appropriate regulatory notification would also be part of the revalidation process.

#### Release Procedures

A quality assurance program should be established that describes in detail the batch or lot release steps for parametric release of sterilized products and the required documentation.

Although the assessment of the sterility assurance of products is primarily based on measurement of physical process parameters, a number of areas should be reviewed, documented, and approved for the parametric release of these products. These areas may include the following: a review of batch records; a review of the ongoing microbiological environmental control program results and presterilization bioburden; and a review of records of thermographic data, load monitors, and results of critical and noncritical data that may have been used to demonstrate process control. It is also important to ensure that the sterilizer is current relative to calibration, maintenance, and revalidation.

The implementation and practice of parametric release is not an intermittent program. Once such a program is implemented, release of the sterilized product is made in accordance with the requirements of the regulatory approved program. Product release by other means is not acceptable if the predefined critical operational parameters are not achieved.

#### MODES OF STERILIZATION

##### Moist Heat Sterilization

Moist heat sterilization of pharmaceutical products includes several types of sterilizing environments and sterilizing media. Saturated steam, hot water spray, and submerged hot water processes are all considered as moist heat sterilizing environments. Different processes may be used to sterilize products by moist heat, and they include batch-type sterilizers and continuous-type sterilizers.

##### critical operating parameters

A defined list of key process parameters and their respective operating limits are defined and established in the sterilization process specifications. Critical operating parameters are those that are absolutely essential to ensure product sterilization to a 10–6 PNS. Examples of critical operating parameters may include, but are not limited to, dwell time limits, minimum and maximum limits for process peak dwell temperature, average peak dwell temperature, and the results of the batch or lot release test that satisfies the requirements of CFR, Part 211 (e.g., a load monitor results from the laboratory). F0 may be used as a critical parameter only when temperature and time relationships are well defined. Other measured parameters may be considered secondary (or noncritical) parameters and may include maximum and minimum time to peak dwell, chamber pressure, and if applicable, chamber water level, sterilizing water time above defined temperature limits, and recirculating water pump pressure differential.

##### Ethylene Oxide Sterilization

The application of parametric release of pharmaceutical products sterilized by ethylene oxide is more difficult than parametric release of products sterilized by moist heat processes.

Critical parameters for ethylene oxide (ETO) sterilization are interrelated and more complex than moist heat processes.

##### critical operating parameters

Critical parameters may include the following: temperature, amount of relative humidity present, ethylene oxide concentration, overall exposure time, product and load density, and gas permeability factors.

Parametric release of pharmaceutical products can be achieved if an automated measurement system for the critical parameters is employed and sterilization loads are closely defined and validated relative to product types, densities, packaging materials, and overall load configurations. An example of the measurement of critical factors that may be considered for parametric release would be the use of calibrated ETO pressure recordings to provide an estimate of ETO concentration during the process hold time or the use of direct measurement of ETO concentration by IR or gas chromatography. Because of variances that might occur in the key parameters during sterilization, parametric release is not widely used for products sterilized by ETO.

However, to ensure parametric release, in addition to the attainment of process parameters of the ethylene oxide sterilization, biological indicators (and their sterility testing after sterilization processing) or the use of physicochemical integrators for the ethylene oxide sterilization are often used as load monitors (critical parameters).

##### Radiation Sterilization

Two radiation sterilizing processes have been used: gamma and electron beam sterilization (i.e., ionizing radiation). Some pharmaceutical products, either in bulk or in their finished formats, have been sterilized by radiation. In discussing the critical parameters of radiation sterilization necessary for parametric release, it is customary to refer to parametric release as dosimetric release. Dosimetric release is provided by the use of a chemical dosimeter that measures the delivery of a minimum specified radiation dosage, which has been shown to provide sterilization of the product to a minimum 10–6 PNS.

The use of a dosimeter in ionizing radiation sterilization measures delivery of a minimum absorbed radiation dose to a pre-established low dose zone in the irradiated product carrier. This will require mapping of the profile of absorbed ionizing radiation across the density ranges processed in the product carrier. The lowest specified radiation dosage for the process is correlated to predictable bioburden reduction levels by any one of the three documented methods.<sup>2</sup> An alternative method may be considered whereby extensive product bioburden count and radiation resistance data are available. Dose verification studies would be conducted to ensure that the worst case bioburden load, relative to resistance and numbers, can be inactivated at the lowest dose zone in the carrier system to provide at least a 10–6 PNS. This method would of course require an ongoing program of bioburden assessment. The target for the radiation cycle is a minimum 10–6 PNS relative to the product bioburden. Dosimetric release of a radiation-sterilized product depends on the delivery of at least a minimum dosage; thus, the critical operational parameters that govern the delivery of that dosage must be within specified limits. These operational critical parameters may include the following: a stacking configuration within the radiation carrier, bulk density of the product, speed of the conveyor or carrier system, distance to the radiation source, duration of product exposure, and appropriate defined adjustments for a decaying radiation source. Demonstration of consistency in the absorbed radiation dosage at areas of minimum and maximum zones of radiation absorption within the fully loaded carriers on a batch-to-batch basis is a necessary condition for dosimetric release of radiation-sterilized pharmaceutical products.



## SUMMARY

The conversion to parametric release in lieu of product sterility testing as described in general chapter [Sterility Tests](#) 71 requires prior FDA approval. Parametric release is advantageous for terminally sterilized products. The extensiveness of data required to establish parametric release, compared to the general chapter 71 procedures, which lack sensitivity to very low levels of microbial contamination, can result in a more accurate and reliable assessment of the probability of nonsterility of product lots.

1 F0 is defined as the calculated equivalent time (in minutes) of process lethality to time at 121.1°, assuming a Z value of 10.0° in the product being sterilized.

2 ANSI/AAMI/ISO 11137-1996, Sterilization of Health Care Products—Requirements for Validation and Routine Control—Radiation Sterilization, July 11, 1994.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

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## 1223 VALIDATION OF ALTERNATIVE MICROBIOLOGICAL METHODS

### INTRODUCTION

The purpose of this chapter is to provide guidance for validating methods for use as alternatives to the official compendial microbiological methods. For microbial recovery and identification, microbiological testing laboratories sometimes use alternative test methods to those described in the general chapters for a variety of reasons, such as economics, throughput, and convenience. Validation of these methods is required. Some guidance on validation of the use of alternate methods is provided in the Tests and Assays section in the General Notices and Requirements. This section also notes that in the event of a dispute, only the result obtained by the compendial test is conclusive.

Validation studies of alternate microbiological methods should take a large degree of variability into account. When conducting microbiological testing by conventional plate count, for example, one frequently encounters a range of results that is broader (%RSD 15 to 35) than ranges in commonly used chemical assays (%RSD 1 to 3). Many conventional microbiological methods are subject to sampling error, dilution error, plating error, incubation error, and operator error.

[Validation of Compendial Procedures](#) 1225 defines characteristics such as accuracy, precision, specificity, detection limit, quantification limit, linearity, range, ruggedness, and robustness in their application to analytical methods. These definitions are less appropriate for alternate microbiological method validation as "at least equivalent to the compendial method" given the comparative nature of the question (see the Tests and Assays—Procedures section in General Notices and Requirements). The critical question is whether or not the alternate method will yield results equivalent to, or better than, the results generated by the conventional method.

Other industry organizations have provided guidance for the validation of alternate microbiological methods. The suitability of a new or modified method should be demonstrated in a comparison study between the USP compendial method and the alternate method. The characteristics defined in this chapter may be used to establish this comparison.

### TYPES OF MICROBIOLOGICAL TESTS

It is critical to the validation effort to identify the portion of the test addressed by an alternate technology. For example, there is a variety of technologies available to detect the presence of viable cells. These techniques may have application in a variety of tests (e.g., bioburden, sterility test) but may not, in fact, replace the critical aspects of the test entirely. For example, a sterility test by membrane filtration may be performed according to the compendial procedure up to the point of combining the processed filter with the recovery media, and after that the presence of viable cells might then be demonstrated by use of some of the available technologies. Validation of this application would, therefore, require validation of the recovery system employed rather than the entire test.

There are three major types of determinations specific to microbiological tests. These include tests to determine whether microorganisms are present in a sample, tests to quantify the number of microorganisms (or to enumerate a specific subpopulation of the sample), and tests designed to identify microorganisms. This chapter does not address microbial identification.

#### Qualitative Tests for the Presence or Absence of Microorganisms

This type of test is characterized by the use of turbidity in a liquid growth medium as evidence of the presence of viable microorganisms in the test sample. The most common example of this test is the sterility test. Other examples of this type of testing are those tests designed to evaluate the presence or absence of a particular type of microorganism in a sample (e.g., coliforms in potable water and *E. coli* in oral dosage forms).

#### Quantitative Tests for Microorganisms

The plate count method is the most common example of this class of tests used to estimate the number of viable microorganisms present in a sample. The membrane filtration and Most Probable Number (MPN) multiple-tube methods are other examples of these tests. The latter was developed as a means to estimate the number of viable microorganisms present in a sample not amenable to direct plating or membrane filtration.

#### General Concerns

Validation of a microbiological method is the process by which it is experimentally established that the performance characteristics of the method meet the requirements for the intended application, in comparison to the traditional method. For example, it may not be necessary to fully validate the equivalence of a new quantitative method for use in the antimicrobial efficacy test by comparative studies, as the critical comparison is between the new method of enumeration and the plate count method (the current method for enumeration). As quantitative tests, by their nature, yield numerical data, they allow for the use of parametric statistical techniques. In contrast, qualitative microbial assays, e.g., the sterility test in the example above, may require analysis by nonparametric statistical methods. The validation of analytical methods for chemical assays follows well-established parameters as described in [Validation of Compendial Procedures](#) 1225. Validation of microbiological methods shares some of the same concerns, although consideration must be given to the unique nature of microbiological assays (see [Table 1](#)).

Table 1. Validation Parameters by Type of Microbiological Test

Parameter	Qualitative	Quantitative
	Tests	Tests
Accuracy	No	Yes
Precision	No	Yes
Specificity	Yes	Yes
Detection limit	Yes	Yes
Quantification limit	No	Yes
Linearity	No	Yes
Operational range	No	Yes



Robustness	Yes	Yes
Repeatability	Yes	Yes
Ruggedness	Yes	Yes

## VALIDATION OF QUALITATIVE TESTS FOR DEMONSTRATION OF Viable MICROORGANISMS IN A SAMPLE

## Specificity

The specificity of an alternate qualitative microbiological method is its ability to detect a range of microorganisms that may be present in the test article. This concern is adequately addressed by growth promotion of the media for qualitative methods that rely upon growth to demonstrate presence or absence of microorganisms. However, for those methods that do not require growth as an indicator of microbial presence, the specificity of the assay for microbes assures that extraneous matter in the test system does not interfere with the test.

## Limit of Detection

The limit of detection is the lowest number of microorganisms in a sample that can be detected under the stated experimental conditions. A microbiological limit test determines the presence or absence of microorganisms, e.g., absence of *Salmonella* spp. in 10 g. Due to the nature of microbiology, the limit of detection refers to the number of organisms present in the original sample before any dilution or incubation steps; it does not refer to the number of organisms present at the point of assay.

One method to demonstrate the limit of detection for a quantitative assay would be to evaluate the two methods (alternative and compendial) by inoculation with a low number of challenge microorganisms (not more than 5 cfu per unit) followed by a measurement of recovery. The level of inoculation should be adjusted until at least 50% of the samples show growth in the compendial test. It is necessary to repeat this determination several times, as the limit of detection of an assay is determined from a number of replicates (not less than

5). The ability of the two methods to detect the presence of low numbers of microorganisms can be demonstrated using the Chi square test. A second method to demonstrate equivalence between the two quantitative methods could be through the use of the Most Probable Number technique. In this method, a 5-tube design in a ten-fold dilution series could be used for both methods. These would then be challenged with equivalent inoculums (for example, a 10-1, 10-2, and 10-3 dilution from a stock suspension of approximately 50 cfu per mL to yield target inocula of 5, 0.5, and 0.05 cfu per tube) and the MPN of the original stock determined by each method. If the 95% confidence intervals overlapped, then the methods would be considered equivalent.

## Ruggedness

The ruggedness of a qualitative microbiological method is the degree of precision of test results obtained by analysis of the same samples under a variety of normal test conditions, such as different analysts, instruments, reagent lots, and laboratories. Ruggedness can be defined as the intrinsic resistance to the influences exerted by operational and environmental variables on the results of the microbiological method. Ruggedness is a validation parameter best suited to determination by the supplier of the test method who has easy access to multiple instruments and batches of components.

## Robustness

The robustness of a qualitative microbiological method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters, and provides an indication of its reliability during normal usage. Robustness is a validation parameter best suited to determination by the supplier of the test method. As there are no agreed upon standards for current methods, acceptance criteria are problematic and must be tailored to the specific technique. It is essential, however, that an estimate of the ruggedness of the alternate procedure be developed. The measure of robustness is not necessarily a comparison between the alternate method and the traditional, but rather a necessary component of validation of the alternate method so that the user knows the operating parameters of the method.

## VALIDATION OF QUANTITATIVE ESTIMATION OF Viable MICROORGANISMS IN A SAMPLE

As colony-forming units follow a Poisson distribution, the use of statistical tools appropriate to the Poisson rather than those used to analyze normal distributions is encouraged. If the user is more comfortable using tools geared towards normally distributed data, the use of a data transformation is frequently useful. Two techniques are available and convenient for microbiological data. Raw counts can be transformed to normally distributed data either by taking the log10 unit value for that count, or by taking the square root of count +1. The latter transformation is especially helpful if the data contain zero counts.

## Accuracy

The accuracy of this type of microbiological method is the closeness of the test results obtained by the alternate test method to the value obtained by the traditional method. It should be demonstrated across the operational range of the test. Accuracy is usually expressed as the percentage of recovery of microorganisms by the assay method.

Accuracy in a quantitative microbiological test may be shown by preparing a suspension of microorganisms at the upper end of the range of the test, that has been serially diluted down to the lower end of the range of the test. The operational range of the alternate method should overlap that of the traditional method. For example, if the alternate method is meant to replace the traditional plate count method for viable counts, then a reasonable range might be from 100 to 106 cfu per mL. At least 5 suspensions across the range of the test should be analyzed for each challenge organism. The alternate method should provide an estimate of viable microorganisms not less than 70% of the estimate provided by the traditional method, or the new method should be shown to recover at least as many organisms as the traditional method by appropriate statistical analysis, an example being an ANOVA analysis of the log10 unit transforms of the data points. Note that the possibility exists that an alternate method may recover an apparent higher number of microorganisms if it is not dependent on the growth of the microorganisms to form colonies or develop turbidity. This is determined in the Specificity evaluation.

## Precision

The precision of a quantitative microbiological method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of suspensions of laboratory microorganisms across the range of the test. The precision of a microbiological method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation). However, other appropriate measures may be applied.

One method to demonstrate precision uses a suspension of microorganisms at the upper end of the range of the test that has been serially diluted down to the lower end of the range of the test. At least 5 suspensions across the range of the test should be analyzed. For each suspension at least 10 replicates should be assayed in order to be able to calculate statistically significant estimates of the standard deviation or relative standard deviation (coefficient of variation). Generally, a RSD in the 15% to 35% range would be acceptable. Irrespective of the specific results, the alternate method should have a coefficient of variation that is not larger than that of the traditional method. For example, a plate count method might have the RSD ranges as shown in the following table.

Table 2. Expected RSD as a Function of cfu per Plate

cfu per Plate	Expected RSD
30-300	<15%
10-30	<25%
<10	<35%

## Specificity

The specificity of a quantitative microbiological method is its ability to detect a panel of microorganisms suitable to demonstrate that the method is fit for its intended purpose. This is demonstrated using the organisms appropriate for the purpose of the alternate method. It is important to challenge the alternate technology in a manner that would encourage false positive results (specific to that alternate technology) to demonstrate the suitability of the alternate method in comparison to the traditional method. This is especially important with those alternate methods that do not require growth for microbial enumeration (for example, any that do not require enrichment or can enumerate microorganisms into the range of 1-50 cells).



#### Limit of Quantification

The limit of quantification is the lowest number of microorganisms that can be accurately counted. As it is not possible to obtain a reliable sample containing a known number of microorganisms, it is essential that the limit of quantification of an assay is determined from a number of replicates ( $n > 5$ ) at each of at least 5 different points across the operational range of the assay. The limit of quantification should not be a number greater than that of the traditional method. Note that this may have an inherent limit due to the nature of bacterial enumeration and the Poisson distribution of bacterial counts (see [Validation of Microbial Recovery from Pharmacopeial Articles](#) (1225)). Therefore, the alternate method need only demonstrate that it is at least as sensitive as the traditional method to similar lower limits.

#### Linearity

The linearity of a quantitative microbiological test is its ability to produce results that are proportional to the concentration of microorganisms present in the sample within a given range. The linearity should be determined over the range of the test. A method to determine this would be to select at least 5 concentrations of each standard challenge microorganism and conduct at least 5 replicate readings of each concentration. An appropriate measure would be to calculate the square of the correlation coefficient,  $r^2$ , from a linear regression analysis of the data generated above. While the correlation coefficient does not provide an estimate of linearity, it is a convenient and commonly applied measure to approximate the relationship. The alternate method should not have an  $r^2$  value less than 0.95.

#### Limit of Detection

See Limit of Detection under Validation of Qualitative Tests for Demonstration of Viable Microorganisms in a Sample.

#### Range

The operational range of a quantitative microbiological method is the interval between the upper and lower levels of microorganisms that have been demonstrated to be determined with precision, accuracy, and linearity.

#### Ruggedness

See Ruggedness under Validation of Qualitative Tests for Demonstration of Viable Microorganisms in a Sample.

#### Robustness

See Robustness under Validation of Qualitative Tests for Demonstration of Viable Microorganisms in a Sample.

\* PDA Technical Report No. 33. The Evaluation, Validation and Implementation of New Microbiological Testing Methods. PDA Journal of Pharmaceutical Science & Technology. 54 Supplement TR#33 (3) 2000 and Official Methods Programs of AOAC International.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

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#### 1225 VALIDATION OF COMPENDIAL PROCEDURES

Test procedures for assessment of the quality levels of pharmaceutical articles are subject to various requirements. According to Section 501 of the Federal Food, Drug, and Cosmetic Act, assays and specifications in monographs of the United States Pharmacopeia and the National Formulary constitute legal standards. The Current Good Manufacturing Practice regulations [21 CFR 211.194(a)] require that test methods, which are used for assessing compliance of pharmaceutical articles with established specifications, must meet proper standards of accuracy and reliability. Also, according to these regulations [21 CFR 211.194(a)(2)], users of analytical methods described in USP-NF are not required to validate the accuracy and reliability of these methods, but merely verify their suitability under actual conditions of use. Recognizing the legal status of USP and NF standards, it is essential, therefore, that proposals for adoption of new or revised compendial analytical procedures be supported by sufficient laboratory data to document their validity.

The text of this information chapter harmonizes, to the extent possible, with the Tripartite International Conference on Harmonization (ICH) documents Validation of Analytical Procedures and the Methodology extension text, which are concerned with analytical procedures included as part of registration applications submitted within the EC, Japan, and the USA.

#### SUBMISSIONS TO THE COMPENDIA

Submissions to the compendia for new or revised analytical procedures should contain sufficient information to enable members of the USP Council of Experts and its Expert Committees to evaluate the relative merit of proposed procedures. In most cases, evaluations involve assessment of the clarity and completeness of the description of the analytical procedures, determination of the need for the procedures, and documentation that they have been appropriately validated. Information may vary depending upon the type of method involved. However, in most cases a submission will consist of the following sections.

**Rationale**— This section should identify the need for the procedure and describe the capability of the specific procedure proposed and why it is preferred over other types of determinations. For revised procedures, a comparison should be provided of limitations of the current compendial procedure and advantages offered by the proposed procedure.

**Proposed Analytical Procedure**— This section should contain a complete description of the analytical procedure sufficiently detailed to enable persons “skilled in the art” to replicate it. The write-up should include all important operational parameters and specific instructions such as preparation of reagents, performance of system suitability tests, description of blanks used, precautions, and explicit formulas for calculation of test results.

**Data Elements**— This section should provide thorough and complete documentation of the validation of the analytical procedure. It should include summaries of experimental data and calculations substantiating each of the applicable analytical performance characteristics. These characteristics are described in the following section.

#### VALIDATION

Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for the intended analytical applications. Typical analytical performance characteristics that should be considered in the validation of the types of procedures described in this document are listed in [Table 1](#). Because opinions may differ with respect to terminology and use, each of the performance characteristics is defined in the next section of this chapter, along with a delineation of a typical method or methods by which it may be measured.

Table 1. Typical Analytical Characteristics

Used in Method Validation
Accuracy
Precision
Specificity
Detection Limit



Quantitation Limit  
Linearity  
Range  
Robustness

In the case of compendial procedures, revalidation may be necessary in the following cases: a submission to the USP of a revised analytical procedure; or the use of an established general procedure with a new product or raw material (see below in Data Elements Required for Validation).

The ICH documents give guidance on the necessity for revalidation in the following circumstances: changes in the synthesis of the drug substance; changes in the composition of the drug product; and changes in the analytical procedure.

Analytical Performance Characteristics

accuracy

Definition— The accuracy of an analytical procedure is the closeness of test results obtained by that procedure to the true value. The accuracy of an analytical procedure should be established across its range.

Determination— In the case of the assay of a drug substance, accuracy may be determined by application of the analytical procedure to an analyte of known purity (e.g., a Reference Standard) or by comparison of the results of the procedure with those of a second, well-characterized procedure, the accuracy of which has been stated or defined.

In the case of the assay of a drug in a formulated product, accuracy may be determined by application of the analytical procedure to synthetic mixtures of the drug product components to which known amounts of analyte have been added within the range of the procedure. If it is not possible to obtain samples of all drug product components, it may be acceptable either to add known quantities of the analyte to the drug product (i.e., "to spike") or to compare results with those of a second, well-characterized procedure, the accuracy of which has been stated or defined.

In the case of quantitative analysis of impurities, accuracy should be assessed on samples (of drug substance or drug product) spiked with known amounts of impurities. Where it is not possible to obtain samples of certain impurities or degradation products, results should be compared with those obtained by an independent procedure. In the absence of other information, it may be necessary to calculate the amount of an impurity based on comparison of its response to that of the drug substance; the ratio of the responses of equal amounts of the impurity and the drug substance (relative response factor) should be used if known.

Accuracy is calculated as the percentage of recovery by the assay of the known added amount of analyte in the sample, or as the difference between the mean and the accepted true value, together with confidence intervals.

The ICH documents recommend that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels, covering the specified range (i.e., three concentrations and three replicates of each concentration).

Assessment of accuracy can be accomplished in a variety of ways, including evaluating the recovery of the analyte (percent recovery) across the range of the assay, or evaluating the linearity of the relationship between estimated and actual concentrations. The statistically preferred criterion is that the confidence interval for the slope be contained in an interval around 1.0, or alternatively, that the slope be close to 1.0. In either case, the interval or the definition of closeness should be specified in the validation protocol. The acceptance criterion will depend on the assay and its variability and on the product. Setting an acceptance criterion based on the lack of statistical significance of the test of the null hypothesis that the slope is 1.0 is not an acceptable approach.

precision

Definition— The precision of an analytical procedure is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample. The precision of an analytical procedure is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements. Precision may be a measure of either the degree of reproducibility or of repeatability of the analytical procedure under normal operating conditions. In this context, reproducibility refers to the use of the analytical procedure in different laboratories, as in a collaborative study. Intermediate precision (also known as ruggedness) expresses within-laboratory variation, as on different days, or with different analysts or equipment within the same laboratory. Repeatability refers to the use of the analytical procedure within a laboratory over a short period of time using the same analyst with the same equipment.

Determination— The precision of an analytical procedure is determined by assaying a sufficient number of aliquots of a homogeneous sample to be able to calculate statistically valid estimates of standard deviation or relative standard deviation (coefficient of variation). Assays in this context are independent analyses of samples that have been carried through the complete analytical procedure from sample preparation to final test result.

The ICH documents recommend that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure (i.e., three concentrations and three replicates of each concentration or using a minimum of six determinations at 100% of the test concentration).

specificity

Definition— The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedures.

[note—Other reputable international authorities (IUPAC, AOAC-I) have preferred the term "selectivity," reserving "specificity" for those procedures that are completely selective.] For the tests discussed below, the above definition has the following implications:

Identification Tests: ensure the identity of the analyte.

Purity Tests: ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte (e.g., related substances test, heavy metals limit, organic volatile impurities).

Assays: provide an exact result, which allows an accurate statement on the content or potency of the analyte in a sample.

Determination— In the case of qualitative analyses (identification tests), the ability to select between compounds of closely related structure that are likely to be present should be demonstrated. This should be confirmed by obtaining positive results (perhaps by comparison to a known reference material) from samples containing the analyte, coupled with negative results from samples that do not contain the analyte and by confirming that a positive response is not obtained from materials structurally similar to or closely related to the analyte.

In the case of analytical procedures for impurities, specificity may be established by spiking the drug substance or product with appropriate levels of impurities and demonstrating that these impurities are determined with appropriate accuracy and precision.

In the case of the assay, demonstration of specificity requires that it can be shown that the procedure is unaffected by the presence of impurities or excipients. In practice, this can be done by spiking the drug substance or product with appropriate levels of impurities or excipients and demonstrating that the assay result is unaffected by the presence of these extraneous materials.

If impurity or degradation product standards are unavailable, specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well-characterized procedure (e.g., a Pharmacopeial or other validated procedure). These comparisons should include samples stored under relevant stress conditions (e.g., light, heat, humidity, acid/base hydrolysis, oxidation). In the case of the assay, the results should be compared; in the case of chromatographic impurity tests, the impurity profiles should be compared.

The ICH documents state that when chromatographic procedures are used, representative chromatograms should be presented to demonstrate the degree of selectivity, and peaks should be appropriately labeled. Peak purity tests (e.g., using diode array or mass spectrometry) may be useful to show that the analyte chromatographic peak is not attributable to more than one component.

detection limit



**definition**— The detection limit is a characteristic of limit tests. It is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. Thus, limit tests merely substantiate that the amount of analyte is above or below a certain level. The detection limit is usually expressed as the concentration of analyte (e.g., percentage, parts per billion) in the sample.

**Determination**— For noninstrumental procedures, the detection limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

For instrumental procedures, the same approach may be used as for noninstrumental procedures. In the case of procedures submitted for consideration as official compendial procedures, it is almost never necessary to determine the actual detection limit. Rather, the detection limit is shown to be sufficiently low by the analysis of samples with known concentrations of analyte above and below the required detection level. For example, if it is required to detect an impurity at the level of 0.1%, it should be demonstrated that the procedure will reliably detect the impurity at that level.

In the case of instrumental analytical procedures that exhibit background noise, the ICH documents describe a common approach, which is to compare measured signals from samples with known low concentrations of analyte with those of blank samples. The minimum concentration at which the analyte can reliably be detected is established. Typically acceptable signal-to-noise ratios are 2:1 or 3:1. Other approaches depend on the determination of the slope of the calibration curve and the standard deviation of responses. Whatever method is used, the detection limit should be subsequently validated by the analysis of a suitable number of samples known to be near, or prepared at, the detection limit.

#### quantitation limit

**Definition**— The quantitation limit is a characteristic of quantitative assays for low levels of compounds in sample matrices, such as impurities in bulk drug substances and degradation products in finished pharmaceuticals. It is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. The quantitation limit is expressed as the concentration of analyte (e.g., percentage, parts per billion) in the sample.

**Determination**— For noninstrumental procedures, the quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be determined with acceptable accuracy and precision.

For instrumental procedures, the same approach may be used as for noninstrumental procedures. In the case of procedures submitted for consideration as official compendial procedures, it is almost never necessary to determine the actual quantitation limit. Rather, the quantitation limit is shown to be sufficiently low by the analysis of samples with known concentrations of analyte above and below the quantitation level. For example, if it is required that an analyte be assayed at the level of 0.1 mg per tablet, it should be demonstrated that the procedure will reliably quantitate the analyte at that level.

In the case of instrumental analytical procedures that exhibit background noise, the ICH documents describe a common approach, which is to compare measured signals from samples with known low concentrations of analyte with those of blank samples. The minimum concentration at which the analyte can reliably be quantified is established. A typically acceptable signal-to-noise ratio is 10:1. Other approaches depend on the determination of the slope of the calibration curve and the standard deviation of responses. Whatever approach is used, the quantitation limit should be subsequently validated by the analysis of a suitable number of samples known to be near, or prepared at, the quantitation limit.

#### linearity and range

**Definition of Linearity**— The linearity of an analytical procedure is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. Thus, in this section, "linearity" refers to the linearity of the relationship of concentration and assay measurement. In some cases, to attain linearity, the concentration and/or the measurement may be transformed. (Note that the weighting factors used in the regression analysis may change when a transformation is applied.) Possible transformations may include log, square root, or reciprocal, although other transformations are acceptable. If linearity is not attainable, a nonlinear model may be used. The goal is to have a model, whether linear or nonlinear, that describes closely the concentration-response relationship.

**Definition of Range**— The range of an analytical procedure is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated to be determined with a suitable level of precision, accuracy, and linearity using the procedure as written. The range is normally expressed in the same units as test results (e.g., percent, parts per million) obtained by the analytical procedure.

**Determination of Linearity and Range**— Linearity should be established across the range of the analytical procedure. It should be established initially by visual examination of a plot of signals as a function of analyte concentration of content. If there appears to be a linear relationship, test results should be established by appropriate statistical methods (e.g., by calculation of a regression line by the method of least squares). Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity. The correlation coefficient, y-intercept, slope of the regression line, and residual sum of squares should be submitted.

The range of the procedure is validated by verifying that the analytical procedure provides acceptable precision, accuracy, and linearity when applied to samples containing analyte at the extremes of the range as well as within the range.

ICH recommends that, for the establishment of linearity, a minimum of five concentrations normally be used. It is also recommended that the following minimum specified ranges should be considered:

Assay of a Drug Substance (or a finished product): from 80% to 120% of the test concentration.

Determination of an Impurity: from 50% to 120% of the acceptance criterion.

For Content Uniformity: a minimum of 70% to 130% of the test concentration, unless a wider or more appropriate range based on the nature of the dosage form (e.g., metered-dose inhalers) is justified.

For Dissolution Testing:  $\pm 20\%$  over the specified range (e.g., if the acceptance criteria for a controlled-release product cover a region from 20%, after 1 hour, and up to 90%, after 24 hours, the validated range would be 0% to 110% of the label claim).

#### robustness

**Definition**— The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in procedural parameters listed in the procedure documentation and provides an indication of its suitability during normal usage. Robustness may be determined during development of the analytical procedure.

#### system suitability

If measurements are susceptible to variations in analytical conditions, these should be suitably controlled, or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness and ruggedness should be that a series of system suitability parameters is established to ensure that the validity of the analytical procedure is maintained whenever used. Typical variations are the stability of analytical solutions, different equipment, and different analysts. In the case of liquid chromatography, typical variations are the pH of the mobile phase, the mobile phase composition, different lots or suppliers of columns, the temperature, and the flow rate. In the case of gas chromatography, typical variations are different lots or suppliers of columns, the temperature, and the flow rate.

System suitability tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being evaluated. They are especially important in the case of chromatographic procedures. Submissions to the USP should make note of the requirements under the System Suitability section in the general test chapter [Chromatography \(621\)](#).

#### Data Elements Required for Validation

Compendial test requirements vary from highly exacting analytical determinations to subjective evaluation of attributes. Considering this broad variety, it is only logical that different test procedures require different validation schemes. This chapter covers only the most common categories of tests for which validation data should be required. These categories are as follows:

**Category I**— Analytical procedures for quantitation of major components of bulk drug substances or active ingredients (including preservatives) in finished pharmaceutical products.

**Category II**— Analytical procedures for determination of impurities in bulk drug substances or degradation compounds in finished pharmaceutical products. These procedures include quantitative assays and limit tests.



Category III—Analytical procedures for determination of performance characteristics (e.g., dissolution, drug release).

Category IV—Identification tests.

For each category, different analytical information is needed. Listed in Table 2 are data elements that are normally required for each of these categories.

Table 2. Data Elements Required for Validation

Analytical Performance Characteristics	Category I	Category II		Category III	Category IV
		Quantitative	Limit Tests		
Accuracy	Yes	Yes	±	±	No
Precision	Yes	Yes	No	Yes	No
Specificity	Yes	Yes	Yes	±	Yes
Detection Limit	No	No	Yes	±	No
Quantitation Limit	No	Yes	No	±	No
Linearity	Yes	Yes	No	±	No
Range	Yes	Yes	±	±	No

\* May be required, depending on the nature of the specific test.

Already established general procedures (e.g., titrimetric determination of water, bacterial endotoxins) should be verified to establish their suitability for use, such as their accuracy (and absence of possible interference) when used for a new product or raw material.

The validity of an analytical procedure can be verified only by laboratory studies. Therefore, documentation of the successful completion of such studies is a basic requirement for determining whether a procedure is suitable for its intended application(s). Current compendial procedures are also subject to regulations that require demonstration of suitability under actual conditions of use (see [Verification of Compendial Procedures \(1226\)](#) for principles relative to the verification of compendial procedures). Appropriate documentation should accompany any proposal for new or revised compendial analytical procedures.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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## 1226 VERIFICATION OF COMPENDIAL PROCEDURES

The intent of this chapter is to provide general information on the verification of compendial procedures that are being performed for the first time to yield acceptable results utilizing the personnel, equipment, and reagents available. This chapter is not intended for retroactive application to already successfully established laboratory procedures. The chapter [Validation of Compendial Procedures \(1225\)](#) provides general information on characteristics that should be considered for various test categories and on the documentation that should accompany analytical procedures submitted for inclusion in USP—NF. Verification consists of assessing selected analytical performance characteristics, such as those that are described in chapter [\(1226\)](#), to generate appropriate, relevant data rather than repeating the validation process.

Users of compendial analytical procedures are not required to validate these procedures when first used in their laboratories, but documented evidence of suitability should be established under actual conditions of use. In the United States, this requirement is established in 21 CFR 211.194(a)(2) of the current Good Manufacturing Practice regulations, which states that "suitability of all testing methods used shall be verified under actual conditions of use."

Verification of microbiological procedures is not covered in this chapter because it is covered in USP general chapters [Antimicrobial Effectiveness \(51\)](#), [Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests \(61\)](#), [Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms \(62\)](#), [Sterility Tests \(71\)](#), and [Validation of Microbial Recovery from Pharmacopeial Articles \(1227\)](#).

### VERIFICATION PROCESS

Users should have the appropriate experience, knowledge, and training to understand and be able to perform the compendial procedures as written. Verification should be conducted by the user such that the results will provide confidence that the compendial procedure will perform suitably as intended.

If the verification of the compendial procedure is not successful, and assistance from USP staff has not resolved the problem, it may be concluded that the procedure may not be suitable for use with the article being tested in that laboratory. It may then be necessary to develop and validate an alternate procedure as allowed in the General Notices. The alternate procedure may be submitted to USP, along with the appropriate data, to support a proposal for inclusion or replacement of the current compendial procedure.

### VERIFICATION REQUIREMENTS

Verification requirements should be based on an assessment of the complexity of both the procedure and the material to which the procedure is applied. Although complete revalidation of a compendial method is not required to verify the suitability of the method under actual conditions of use, some of the analytical performance characteristics listed in chapter [\(1225\)](#), Table 2, may be used for the verification process. Only those characteristics that are considered to be appropriate for the verification of the particular method need to be evaluated. The degree and extent of the verification process may depend on the level of training and experience of the user, on the type of procedure and its associated equipment or instrumentation, on the specific procedural steps, and on which article(s) are being tested.

As an example, an assessment of specificity is a key parameter in verifying that a compendial procedure is suitable for use in assaying drug substances and drug products. For instance, acceptable specificity for a chromatographic method may be verified by conformance with system suitability resolution requirements (if specified in the method). However, drug substances from different suppliers may have different impurity profiles that are not addressed by the compendial test procedure. Similarly, the excipients in a drug product can vary widely among manufacturers and may have the potential to directly interfere with the procedure or cause the formation of impurities that are not addressed by the compendial procedure. In addition, drug products containing different excipients, antioxidants, buffers, or container extractives, may potentially interfere with the compendial procedure. In these cases, a more thorough assessment of specificity may be required to demonstrate suitability of the method for the particular drug substance or product. Other analytical performance characteristics such as an assessment of the limit of detection or quantitation and precision for impurities procedures may be useful to demonstrate the suitability of the compendial method under actual conditions of use.

Verification is not required for basic compendial test procedures that are routinely performed unless there is an indication that the compendial procedure is not appropriate for the article under test. Examples of basic compendial procedures include, but are not limited to, loss on drying, residue on ignition, various wet chemical procedures such as acid value, and simple instrumental methods such as pH measurements. However, for the application of already established routine procedures to compendial articles tested for the first time, it is



recommended that consideration be given to any new or different sample handling or solution preparation requirements.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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### 1227 VALIDATION OF MICROBIAL RECOVERY FROM PHARMACOPEIAL ARTICLES

This chapter provides guidelines for the validation of methods for the estimation of the number of viable microorganisms, for the detection of indicators or objectionable microorganisms, for the validation of microbiological methods used in antimicrobial effectiveness testing, and for the sterility testing of Pharmacopeial articles. It is generally understood that if a product possesses antimicrobial properties because of the presence of a specific preservative or because of its formulation, this antimicrobial property must be neutralized to recover viable microorganisms. This neutralization may be achieved by the use of a specific neutralizer, by dilution, by a combination of washing and dilution, or by any combination of these methods.

The tests under [Antimicrobial Effectiveness Testing](#), [Sterility Tests](#), [Microbial Enumeration Tests](#) and [Tests for Specified Microorganisms](#) require the validation of recovery methods. To ensure that the results of the tests are credible, neutralization of antimicrobial properties of the test solution is required before estimating the number of viable microorganisms.

#### INFLUENTIAL FACTORS

Several factors affect the measurement of a test solution's antimicrobial activity, and these must be considered in the validation design. They include the nature of the microorganisms used as challenge organisms, the preparation of the inoculum of challenge organisms, the specific conditions of the test, and the conditions of recovery. These factors also affect the validation of recovery methods for aqueous or nonaqueous products, irrespective of their antimicrobial properties; thus, all test methods should be validated with these factors in mind.

The nature of the challenge microorganism exerts a strong effect upon the response to the antimicrobial agent, and so upon the neutralization required for recovery. Represented among these organisms in compendial tests are Gram-positive bacteria, Gram-negative bacteria, yeasts, and molds. Each organism to be used in the test must be included in the validation.

The preparation of the inoculum of challenge microorganisms also affects the testing of products having antimicrobial properties. The growth and preparation of the challenge organism determines the physiological state of the cell. This state has a direct influence on the results of any test of antimicrobial efficacy. Microbial tests do not use individual cells; rather, populations of cells are harvested for study. The data generated from these studies are less variable if the cell populations are homogeneous. Liquid cultures or confluent growths on solid medium are best suited for reproducible culture preparation. The conditions of organism preparation and storage must be standardized for the neutralizer evaluation and should reflect the conditions of the antimicrobial assay.

The specific conditions of the test, including buffers used, water, light conditions, and temperature, must be reproduced in the validation study. All test conditions also should be standardized and performed in the validation study exactly as performed in the test.

The conditions of microbial recovery are among the most crucial in accurately estimating the number of microorganisms present in a test solution. The first consideration is the recovery medium used to support the growth of survivors. This concern is discussed in detail below. The second consideration is the incubation conditions. Optimal conditions for growth must be present to ensure complete growth and reproducible results.

#### METHODS OF NEUTRALIZING ANTIMICROBIAL PROPERTIES

Three common methods are used to neutralize antimicrobial properties of a product: (1) chemical inhibition, (2) dilution, and (3) filtration and washing.

##### Chemical Inhibition

[Table 1](#) shows known neutralizers for a variety of chemical antimicrobial agents and the reported toxicity of some chemical neutralizers to specific microorganisms. However, despite potential toxicity, the convenience and quick action of chemical inhibitors encourage their use. Chemical inhibition of bactericides is the preferred method for the antimicrobial efficacy test. The potential of chemical inhibitors should be considered in the membrane filtration and the direct transfer sterility tests. Antibiotics may not be susceptible to neutralization by chemical means, but rather by enzymatic treatment (e.g., penicillinase). These enzymes may be used where required.

Table 1. Some Common Neutralizers for Chemical Biocides

Neutralizer	Biocide Class	Potential Action of Biocides
Bisulfate	Glutaraldehyde, Mercurials	Non-Sporing Bacteria
Dilution	Phenolics, Alcohol, Aldehydes, Sorbate	—
Glycine	Aldehydes	Growing Cells
Lecithin	Quaternary Ammonium Compounds (QACs), Parabens, Bis-biguuanides	Bacteria
Mg <sup>2+</sup> or Ca <sup>2+</sup> ions	EDTA	—
Polysorbate	QACs, Iodine, Parabens	—
Thioglycolate	Mercurials	Staphylococci and Spores
Thiosulfate	Mercurials, Halogens, Aldehydes	Staphylococci

##### Dilution

A second approach to neutralizing antimicrobial properties of a product is by dilution, because the concentration of a chemical bactericide exerts a large effect on its potency. The relationship between concentration and antimicrobial effect differs among bactericidal agents but is constant for a particular antimicrobial agent. This relationship is exponential in nature, with the general formula:

$$C^{-\frac{1}{n}t} = k$$

in which C is the concentration; t is the time required to kill a standard inoculum; k is a constant; and the concentration exponent,  $\frac{1}{n}$ , is the slope of the plot of  $\log t$  versus  $\log C$ .

Antimicrobial agents with high  $\frac{1}{n}$  values are rapidly neutralized by dilution, whereas those with low  $\frac{1}{n}$  values are not good candidates for neutralization by dilution.

##### Membrane Filtration

An approach that is often used, especially in sterility testing, is neutralization by membrane filtration. This approach relies upon the physical retention of the microorganism on the membrane filter, with the antimicrobial agent passing through the filter into the filtrate. The filter is then incubated for recovery of viable microorganisms. However, filtration alone may not remove sufficient quantities of the bactericidal agent to allow growth of surviving microorganisms. Adherence of residual antimicrobial agents to the filter membrane may cause



growth inhibition. Filtration through a low-binding filter material, such as polyvinylidene difluoride, helps to minimize this growth inhibition. Additionally, the preservative may be diluted or flushed from the filter by rinsing with a benign fluid, such as diluting Fluid A (see Diluting and Rinsing Fluids for Membrane Filtration under [Sterility Tests](#) 71 for diluting fluid compositions). Chemical neutralizers in the rinsing fluid can ensure that any antimicrobial residue on the membrane does not interfere with the recovery of viable microorganisms.

#### VALIDATION OF NEUTRALIZATION METHODS—RECOVERY COMPARISONS

A validated method for neutralizing the antimicrobial properties of a product must meet two criteria: neutralizer efficacy and neutralizer toxicity. The validation study documents that the neutralization method employed is effective in inhibiting the antimicrobial properties of the product (neutralizer efficacy) without impairing the recovery of viable microorganisms (neutralizer toxicity). Validation protocols may meet these two criteria by comparing recovery results for treatment groups.

The first is the test group, in which the product is subjected to the neutralization method, then a low level of challenge microorganism [less than 100 colony-forming units (cfu)] is inoculated for recovery. The second is the peptone control group, in which the neutralization method is used with peptone, or diluting Fluid A (see [Sterility Tests](#) 71), as the test solution. The third is the viability group, in which the actual inoculum is used without exposure to the neutralization scheme. Similar recovery between the test group and the peptone group demonstrates adequate neutralizer efficacy; similar recovery between the peptone group and the viability group demonstrates adequate neutralizer toxicity.

In principle, the protocol must show that recovery of a low inoculum (less than 100 cfu) is not inhibited by the test sample and the neutralization method. Validation protocols may meet these two criteria by comparing recovery among three distinct test groups: (1) neutralized product with inoculum, (2) challenge inoculum control in buffered solution, and (3) inoculum in the absence of product or neutralizer. This can be established by directly comparing the result in the treated solution (1) to the inoculum (3) above. If the growth on the treated solution is not comparable to the growth on the inoculum group, it should be determined whether the neutralization method itself is toxic to the microorganisms.

##### Recovery on Agar Medium

In the tests under [Antimicrobial Effectiveness Testing](#) 51 and [Microbial Enumeration Tests](#) 61 and [Tests for Specified Microorganisms](#) 62, the number of viable challenge microorganisms in the product is estimated at various time intervals by calculating the concentration of cfu per mL by the plate count method. A design for validating neutralization would incorporate the treatment groups as described under Validation of Neutralization Methods—Recovery Comparisons. At least three independent replicates of the experiment should be performed, and each should demonstrate that the average number of cfu recovered from the challenge product is not less than 70% of that recovered from the inoculum control.

If a greater number of replicates is required in the validation study, the comparisons may be evaluated by transforming the numbers of cfu to their logarithmic values and analyzing the data statistically by the Student t test (pairwise comparisons) or by analysis of variance (ANOVA) (for comparing all groups). If ANOVA is used, and significant differences among the populations are determined, a test such as Dunnett's test may be used, with the peptone group used as the control group.

##### Recovery by Membrane Filtration

This validation follows the procedure described for Validation Test under [Sterility Tests](#) 71, with the exception of plating on solid medium to quantitate recovery. Three 100-mL rinses are assumed, but the volume and number of rinses are subject to validation. Each validation run should be performed independently at least three times.

In the test solution group, the product is filtered through the membrane filter, followed by two 100-mL portions of diluting-neutralizing fluid. After the second rinse has been filtered, a final 100-mL portion containing less than 100 cfu of the specific challenge microorganism is passed through the filter. This filter is then placed on the appropriate agar recovery medium and incubated for recovery.

The inoculum is directly plated onto the solid medium. It is possible that filtration will lead to reduced recovery of the challenge microorganism, either through inherent toxicity of the membrane or by adherence of the microorganism to the filtration vessel walls. A control group can be used to evaluate this component of membrane filtration validation. Diluting Fluid A is used as the dilution medium without exposing the filter to the product. After addition of the low-level inoculum to the final rinse, the filter is plated as above. Technique-specific loss of microorganisms can be estimated by comparing the recovery in the diluting Fluid A group to the inoculum count.

It is assumed in this discussion that the test sample can be filtered. If it is necessary to solubilize the test sample, the effects of the solubilization method on viable microorganisms must be determined. This situation can occur when testing ointments, suspensions, or other articles.

The method can be considered validated if the recovery rate in the three independent replicates is similar for the test solution and the diluting Fluid A control.

##### Recovery in Liquid Medium

It is assumed in Direct Inoculation of the Culture Medium in the section Test for Sterility of the Product to be Examined under [Sterility Tests](#) 71 that the recovery medium will allow for growth of all surviving microorganisms. The broth in that test must serve both to neutralize any antimicrobial properties of the test solution and to support the growth of the microorganisms. The treatment groups described under Validation of Neutralization Methods—Recovery Comparisons above can be used for validation of the recovery method, with the proportions of product and recovery medium varied to achieve adequate neutralization. The method can be considered validated if all groups show copious growth within 7 days for all microorganisms.

#### RECOVERY OF INJURED MICROORGANISMS

The validation studies described above use challenge microorganisms that have never been exposed to antimicrobial agents, and thus are not identical to organisms seen in antimicrobial effectiveness testing or when a sterility test is performed on a preserved product. If the use of alternative media is desired, the recovery of injured microorganisms should be addressed in the validation study. This may be done by directly comparing the recovery of each challenge microorganism on the preferred medium and on the alternative medium, after exposure to the product. This exposure should include at least two time periods showing survival of less than 100 cfu per mL, unless the rate of kill of the antimicrobial agent is such that no recovery is possible even if the microorganism is plated within minutes of exposure. This comparison should be performed at least three times. The alternative medium is validated if the recovery seen on that medium is no less than that seen on the preferred medium, within an error of 0.5 log units.

#### ESTIMATING THE NUMBER OF COLONY-FORMING UNITS

The accuracy of any estimate of viable cfu is affected by the number plated. As the number of viable cells plated increases, crowding effects decrease the accuracy of the count, reducing the estimate. As the number decreases, random error plays an increasing role in the estimate.

The accepted range for countable colonies on a standard agar plate is between 25 and 250 for most bacteria and *Candida albicans*. This range was established in the food industry for counting coliform bacteria in milk. This range is acceptable for compendial organisms, except for fungi. It is not optimal for counting all environmental isolates. The recommended counting range for *Aspergillus niger* is between 8 and 80 cfu per plate. The use of membrane filtration to recover challenge microorganisms, or the use of environmental isolates as challenge microorganisms in antimicrobial effectiveness testing, requires validation of the countable range. This validation may be performed by statistical comparison of estimated cfu from successive pairs in a dilution series. Prepare a suspension so that plating will provide approximately 1000 cfu per plate, and then dilute twofold to a theoretical concentration of approximately 1 cfu per plate. Plate all dilutions in the series in duplicate, and incubate for recovery under the conditions of the [Antimicrobial Effectiveness Testing](#) 51. Compare the estimates of cfu per mL from paired tubes in the dilution series by the formula:



$$\frac{|2L_{cfu} - H_{cfu}|}{\sqrt{2L_{cfu} + H_{cfu}}} \leq 1.96$$

in which  $L_{cfu}$  is the number of colonies on the plate with the lower count (greater dilution), and  $H_{cfu}$  is the number of colonies on the plate with the higher count (lesser dilution). The estimates of the cfu per mL provided by  $L_{cfu}$  and  $H_{cfu}$  should agree within the limits of the formula with a critical value of 1.96. The upper limit of plate counts is then defined as the number ( $H_{cfu}$ ) that reproducibly passes this test. This study should be independently repeated a sufficient number of times to establish an upper limit of cfu for the particular plating conditions.

There is a lower limit at which the ability of the antimicrobial effectiveness test to recover microorganisms becomes untenable. If the first plating is performed with 1 mL of a 10-1 dilution, cfu in the range of 1 to 10 per mL would not be seen. On this dilution plating, only the lower number of cfu may be reduced to 3, allowing as few survivors as 30 cfu per mL to be reported.

Lower counting thresholds for the greatest dilution plating in series must be justified. Numbers of colonies on a plate follow the Poisson distribution, so the variance of the mean value equals the mean value of counts. Therefore, as the mean number of cfu per plate becomes lower, the percentage error of the estimate increases (see [Table 2](#)). Three cfu per plate at the 10-1 dilution provide an estimate of 30 cfu per mL, with an error of 58% of the estimate.

Table 2. Error as a Percentage of Mean for Plate Counts

cfu per Plate	Standard Error	Error as % of Mean
30	5.48	18.3
29	5.39	18.6
28	5.29	18.9
27	5.20	19.2
26	5.10	19.6
25	5.00	20.0
24	4.90	20.4
23	4.80	20.9
22	4.69	21.3
21	4.58	21.8
20	4.47	22.4
19	4.36	22.9
18	4.24	23.6
17	4.12	24.3
16	4.00	25.0
15	3.87	25.8
14	3.74	26.7
13	3.61	27.7
12	3.46	28.9
11	3.32	30.2
10	3.16	31.6
9	3.00	33.3
8	2.83	35.4
7	2.65	37.8
6	2.45	40.8
5	2.24	44.7
4	2.00	50.0
3	1.73	57.7
2	1.41	70.7
1	1.00	100.0

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

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1230 WATER FOR HEALTH APPLICATIONS

#### WATER FOR HEMODIALYSIS

Chemical and microbial components that can be found in drinking water meeting U.S. Environmental Protection Agency National Primary Drinking Water Regulations (or equivalent) may have the potential to produce significant negative effects in patients undergoing hemodialysis. It is, therefore, necessary to subject the water to further treatment to reduce these components to acceptable levels. The Water for Hemodialysis monograph provides bacterial and chemical tests that are required to ensure patient safety. Additional testing is recommended as follows:



- Excess levels of aluminum, fluorides, and chlorides may be found seasonally in drinking water as a result of chemicals used in water treatment. These components should be monitored in Water for Hemodialysis being produced in accordance with established standard operating procedures. The maximum acceptable levels of these elements and compounds are listed in [Table 1](#).
- A comprehensive validation testing of the system producing Water for Hemodialysis should be performed, at least annually, to ensure that the water treatment equipment is functioning properly. The maximum acceptable levels of elements and compounds are listed in [Table 1](#). Routine testing is performed in accordance with the monograph.

Table 1. Maximum Allowable Chemical Levels in Water for Hemodialysis (water used to prepare dialysate and concentrates from powder at a dialysis facility and to reprocess dialyzers for multiple use)\*

Element or Compound	Maximum Concentration (mg/L)
Calcium	2 (0.1 mEq/L)
Magnesium	4 (0.3 mEq/L)
Potassium	8 (0.2 mEq/L)
Sodium	70 (3.0 mEq/L)
Antimony	0.006
Arsenic	0.005
Barium	0.10
Beryllium	0.0004
Cadmium	0.001
Chromium	0.014
Lead	0.005
Mercury	0.0002
Selenium	0.09
Silver	0.005
Aluminum	0.01
Chloramines	0.10
Free chlorine	0.50
Copper	0.10
Fluoride	0.20
Nitrate (as N)	2.00
Sulfate	100.00
Thallium	0.002
Zinc	0.10

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The chemical limits included in [Table 1](#) have been recognized by federal government agencies as standards for Water for Hemodialysis. Written standard operating procedures for water testing should be established by the physician in charge or the designated facility manager. The test frequency decision should be based upon historical data analysis, the quality of the source water as reported by the municipal water treatment facility or public health agency in the area, etc. Records should be maintained to document levels and any necessary action taken.

Chemical analysis of water components listed should be performed using methods referenced in the American Public Health Association's Standard Methods for the Examination of Water and Wastewater, 19th Edition,<sup>1</sup> those referenced in the U.S. Environmental Protection Agency's Methods for the Determination of Metals in Environmental Samples,<sup>2</sup> or equivalent methods as described in ANSI/AAMI RD 62:2001.

#### MICROBIAL CONSIDERATIONS

The [Water for Hemodialysis](#) monograph includes microbial limits of 100 cfu per mL and endotoxin limits of 2 USP Endotoxin Units per mL. Culture media should be Soybean–Casein Digest Agar Medium or equivalent, and colonies should be counted after incubation at a temperature range between 30° and 35°, for no less than 48 hours. Sampling the water should be done at the end of the water purification cascade at the point where the water enters the dialysis equipment. Samples should be assayed within 30 minutes of collection or immediately refrigerated and then assayed within 24 hours of collection. Quantification of bacterial endotoxins is performed using the Limulus Amebocyte Lysate (LAL) clotting method or any other LAL test found in the USP general test chapter [Bacterial Endotoxins Test \(85\)](#).

Because of the incubation time required to obtain definitive microbiological results, water systems should be microbiologically monitored to confirm that they continue to produce water of acceptable quality. "Alert" and "Action Levels" are therefore necessary for the monitoring and control of the system. An Alert Level constitutes a warning and does not require a corrective action. An Action Level indicates a drift from normal operating conditions and requires that corrective action be taken to bring the process back into the normal operating range. Exceeding an Alert or Action Level does not imply that water quality has been compromised. The recommended Action Level for a total viable microbial count in the product water is 50 cfu per mL, and the recommended Alert Level for bacterial endotoxins is 0.5 USP Endotoxin Unit per mL (also see Microbial Considerations under [Water for Pharmaceutical Purposes \(1231\)](#)).

1 American Public Health Association, Washington, DC 20005.

2 U.S. Environmental Protection Agency Publication EPA-600-R-94-111, Cincinnati, OH.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
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## INTRODUCTION

Water is widely used as a raw material, ingredient, and solvent in the processing, formulation, and manufacture of pharmaceutical products, active pharmaceutical ingredients (APIs) and intermediates, compendial articles, and analytical reagents. This general information chapter provides additional information about water, its quality attributes that are not included within a water monograph, processing techniques that can be used to improve water quality, and a description of minimum water quality standards that should be considered when selecting a water source.

This information chapter is not intended to replace existing regulations or guides that already exist to cover USA and International (ICH or WHO) GMP issues, engineering guides, or other regulatory (FDA, EPA, or WHO) guidances for water. The contents will help users to better understand pharmaceutical water issues and some of the microbiological and chemical concerns unique to water. This chapter is not an all-inclusive writing on pharmaceutical waters. It contains points that are basic information to be considered, when appropriate, for the processing, holding, and use of water. It is the user's responsibility to assure that pharmaceutical water and its production meet applicable governmental regulations, guidances, and the compendial specifications for the types of water used in compendial articles.

Control of the chemical purity of these waters is important and is the main purpose of the monographs in this compendium. Unlike other official articles, the bulk water monographs (Purified Water and Water for Injection) also limit how the article can be produced because of the belief that the nature and robustness of the purification process is directly related to the resulting purity. The chemical attributes listed in these monographs should be considered as a set of minimum specifications. More stringent specifications may be needed for some applications to ensure suitability for particular uses. Basic guidance on the appropriate applications of these waters is found in the monographs and is further explained in this chapter.

Control of the microbiological quality of water is important for many of its uses. All packaged forms of water that have monograph standards are required to be sterile because some of their intended uses require this attribute for health and safety reasons. USP has determined that a microbial specification for the bulk monographed waters is inappropriate and has not been included within the monographs for these waters. These waters can be used in a variety of applications, some requiring extreme microbiological control and others requiring none. The needed microbial specification for a given bulk water depends upon its use. A single specification for this difficult-to-control attribute would unnecessarily burden some water users with irrelevant specifications and testing. However, some applications may require even more careful microbial control to avoid the proliferation of microorganisms ubiquitous to water during the purification, storage, and distribution of this substance. A microbial specification would also be inappropriate when related to the "utility" or continuous supply nature of this raw material. Microbial specifications are typically assessed by test methods that take at least 48 to 72 hours to generate results. Because pharmaceutical waters are generally produced by continuous processes and used in products and manufacturing processes soon after generation, the water is likely to have been used well before definitive test results are available. Failure to meet a compendial specification would require investigating the impact and making a pass/fail decision on all product lots between the previous sampling's acceptable test result and a subsequent sampling's acceptable test result. The technical and logistical problems created by a delay in the result of such an analysis do not eliminate the user's need for microbial specifications. Therefore, such water systems need to be operated and maintained in a controlled manner that requires that the system be validated to provide assurance of operational stability and that its microbial attributes be quantitatively monitored against established alert and action levels that would provide an early indication of system control. The issues of water system validation and alert/action levels and specifications are included in this chapter.

## SOURCE OR FEED WATER CONSIDERATIONS

To ensure adherence to certain minimal chemical and microbiological quality standards, water used in the production of drug substances or as source or feed water for the preparation of the various types of purified waters must meet the requirements of the National Primary Drinking Water Regulations (NPDWR) (40 CFR 141) issued by the U.S. Environmental Protection Agency (EPA) or the drinking water regulations of the European Union or Japan, or the WHO drinking water guidelines. Limits on the types and quantities of certain organic and inorganic contaminants ensure that the water will contain only small, safe quantities of potentially objectionable chemical species. Therefore, water pretreatment systems will only be challenged to remove small quantities of these potentially difficult-to-remove chemicals. Also, control of objectionable chemical contaminants at the source-water stage eliminates the need to specifically test for some of them (e.g., trihalomethanes and heavy metals) after the water has been further purified.

Microbiological requirements of drinking water ensure the absence of coliforms, which, if determined to be of fecal origin, may indicate the potential presence of other potentially pathogenic microorganisms and viruses of fecal origin. Meeting these microbiological requirements does not rule out the presence of other microorganisms, which could be considered undesirable if found in a drug substance or formulated product.

To accomplish microbial control, Municipal Water Authorities add disinfectants to drinking water. Chlorine-containing and other oxidizing substances have been used for many decades for this purpose and have generally been considered to be relatively innocuous to humans. However, these oxidants can interact with naturally occurring organic matter to produce disinfection by-products (DBPs), such as trihalomethanes (THMs, including chloroform, bromodichloromethane, and dibromochloromethane) and haloacetic acids (HAAs, including dichloroacetic acid and trichloroacetic acid). The levels of DBPs produced vary with the level and type of disinfectant used and the levels and types of organic materials found in the water, which can vary seasonally.

Because high levels of DBPs are considered a health hazard in drinking water, Drinking Water Regulations mandate their control to generally accepted nonhazardous levels. However, depending on the unit operations used for further water purification, a small fraction of the DBPs in the starting water may carry over to the finished water. Therefore, the importance of having minimal levels of DBPs in the starting water, while achieving effective disinfection, is important.

DBP levels in drinking water can be minimized by using disinfectants such as ozone, chloramines, or chlorine dioxide. Like chlorine, their oxidative properties are sufficient to damage some pretreatment unit operations and must be removed early in the pretreatment process. The complete removal of some of these disinfectants can be problematic. For example, chloramines may degrade during the disinfection process or during pretreatment removal, thereby releasing ammonia, which in turn can carry over to the finished water. Pretreatment unit operations must be designed and operated to adequately remove the disinfectant, drinking water DBPs, and objectionable disinfectant degradants. A serious problem can occur if unit operations designed to remove chlorine were, without warning, challenged with chloramine-containing drinking water from a municipality that had been mandated to cease use of chlorine disinfection to comply with ever tightening EPA Drinking Water THM specifications. The dechlorination process might incompletely remove the chloramine, which could irreparably damage downstream unit operations, but also the release of ammonia during this process might carry through pretreatment and prevent the finished water from passing compendial conductivity specifications. The purification process must be reassessed if the drinking water disinfectant is changed, emphasizing the need for a good working relationship between the pharmaceutical water manufacturer and the drinking water provider.

## TYPES OF WATER

There are many different grades of water used for pharmaceutical purposes. Several are described in USP monographs that specify uses, acceptable methods of preparation, and quality attributes. These waters can be divided into two general types: bulk waters, which are typically produced on site where they are used; and packaged waters, which are produced, packaged, and sterilized to preserve microbial quality throughout their packaged shelf life. There are several specialized types of packaged waters, differing in their designated applications, packaging limitations, and other quality attributes.

There are also other types of water for which there are no monographs. These are all bulk waters, with names given for descriptive purposes only. Many of these waters are used in specific analytical methods. The associated text may not specify or imply certain quality attributes or modes of preparation. These nonmonographed waters may not necessarily adhere strictly to the stated or implied modes of preparation or attributes. Waters produced by other means or controlled by other test attributes may equally satisfy the intended uses for these waters. It is the user's responsibility to ensure that such waters, even if produced and controlled exactly as stated, be suitable for their intended use. Wherever the term "water" is used within this compendia without other descriptive adjectives or clauses, the intent is that water of no less purity than Purified Water be used.

What follows is a brief description of the various types of pharmaceutical waters and their significant uses or attributes. [Figure 1](#) may also be helpful in understanding some of the various types of waters.

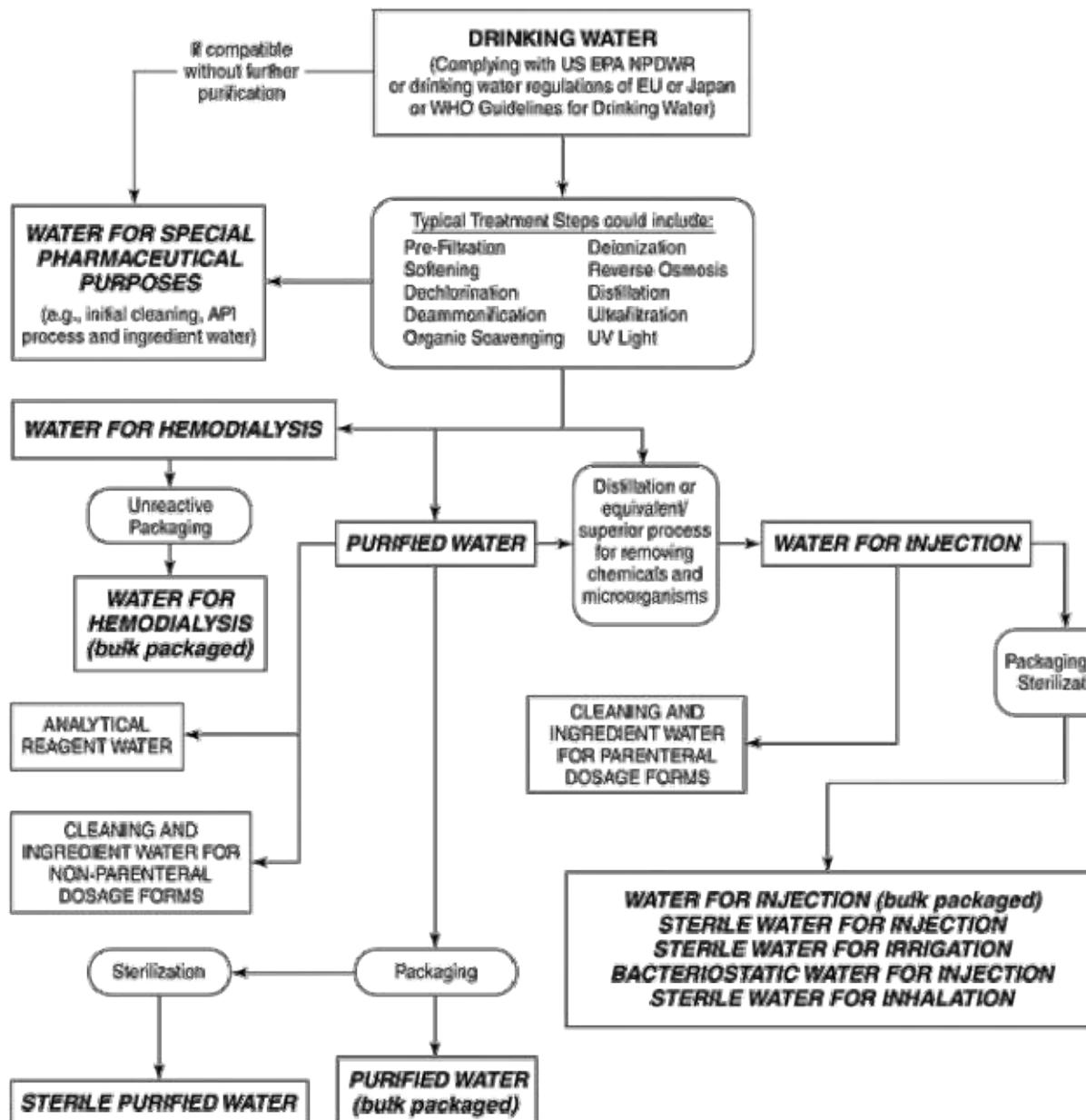


Figure 1. Water for pharmaceutical purposes.

Bulk Monographed Waters and Steam

The following waters are typically produced in large volume by a multiple-unit operation water system and distributed by a piping system for use at the same site. These particular pharmaceutical waters must meet the quality attributes as specified in the related monographs.

Purified Water—[Purified Water](#) (see USP monograph) is used as an excipient in the production of nonparenteral preparations and in other pharmaceutical applications, such as cleaning of certain equipment and nonparenteral product-contact components. Unless otherwise specified, Purified Water is also to be used for all tests and assays for which water is indicated (see General Notices and Requirements). Purified Water is also referenced throughout the USP-NF. Regardless of the font and letter case used in its spelling, water complying with the Purified Water monograph is intended. Purified Water must meet the requirements for ionic and organic chemical purity and must be protected from microbial contamination. The minimal quality of source or feed water for the production of Purified Water is Drinking Water. This source water may be purified using unit operations that include deionization, distillation, ion exchange, reverse osmosis, filtration, or other suitable purification procedures. Purified water systems must be validated to reliably and consistently produce and distribute water of acceptable chemical and microbiological quality. Purified water systems that function under ambient conditions are particularly susceptible to the establishment of tenacious biofilms of microorganisms, which can be the source of undesirable levels of viable microorganisms or endotoxins in the effluent water. These systems require frequent sanitization and microbiological monitoring to ensure water of appropriate microbiological quality at the points of use.

The Purified Water monograph also allows bulk packaging for commercial use elsewhere. When this is done, the required specifications are those of the packaged water Sterile Purified Water, except for Sterility and Labeling. There is a potential for microbial contamination and other quality changes of this bulk packaged nonsterile water to occur. Therefore, this form of Purified Water should be prepared and stored in such a fashion that limits microbial growth and/or simply used in a timely fashion before microbial proliferation renders it unsuitable for its intended use. Also depending on the material used for packaging, there could be extractable compounds leaching into the water from the packaging. Though this article may meet its required chemical attributes, such extractables may render the water an inappropriate choice for some applications. It is the user's responsibility to assure fitness for use of this packaged article when used in manufacturing, clinical, or analytical applications where the pure bulk form of the water is indicated.

Water for Injection—[Water for Injection](#) (see USP monograph) is used as an excipient in the production of parenteral and other preparations where product endotoxin content must be controlled, and in other pharmaceutical applications, such as cleaning of certain equipment and parenteral product-contact components. The minimum quality of source or feed water for the generation of [Water for Injection](#) is Drinking Water as defined by the U.S. EPA, EU, Japan, or the WHO. This source water may be pre-treated to render it suitable for subsequent distillation (or whatever other validated process is used according to the monograph). The finished water must meet all of the chemical requirements for Purified Water as well as an additional bacterial endotoxin specification. Since endotoxins are produced by the kinds of microorganisms that are prone to inhabit water, the equipment and procedures used by the system to purify, store, and distribute Water for Injection must be designed to minimize or prevent microbial contamination as well as remove incoming endotoxins from the



starting water. Water for Injection systems must be validated to reliably and consistently produce and distribute this quality of water.

The Water for Injection monograph also allows it to be packed in bulk for commercial use. Required specifications include the test for Bacterial endotoxins, and those of the packaged water Sterile Purified Water, except for Labeling. Bulk packaged Water for Injection is required to be sterile, thus eliminating microbial contamination quality changes. However, packaging extractables may render this water an inappropriate choice for some applications. It is the user's responsibility to ensure fitness for use of this packaged article when used in manufacturing, clinical, or analytical applications where the purer bulk form of the water is indicated.

Water for Hemodialysis— [Water for Hemodialysis](#) (see USP monograph) is used for hemodialysis applications, primarily the dilution of hemodialysis concentrate solutions. It is produced and used on-site and is made from EPA Drinking Water which has been further purified to reduce chemical and microbiological components. It may be packaged and stored in unreactive containers that preclude bacterial entry. The term "unreactive containers" implies that the container, especially its water contact surfaces, are not changed in any way by the water, such as by leaching of container-related compounds into the water or by any chemical reaction or corrosion caused by the water. The water contains no added antimicrobials and is not intended for injection. Its attributes include specifications for Water conductivity, Total organic carbon (or oxidizable substances), Microbial limits, and Bacterial endotoxins. The water conductivity and total organic carbon attributes are identical to those established for Purified Water and Water for Injection; however, instead of total organic carbon, the organic content may alternatively be measured by the test for Oxidizable substances. The Microbial limits attribute for this water is unique among the "bulk" water monographs, but is justified on the basis of this water's specific application that has microbial content requirements related to its safe use. The Bacterial endotoxins attribute is likewise established at a level related to its safe use.

Pure Steam— [Pure Steam](#) (see USP monograph) is also sometimes referred to as "clean steam". It is used where the steam or its condensate would directly contact official articles or article-contact surfaces such as during their preparation, sterilization, or cleaning where no subsequent processing step is used to remove any codeposited impurity residues. These Pure Steam applications include but are not limited to porous load sterilization processes, to product or cleaning solutions heated by direct steam injection, or in humidification of processes where steam injection is used to control the humidity inside processing vessels where the official articles or their in-process forms are exposed. The primary intent of using this quality of steam is to ensure that official articles or article-contact surfaces exposed to it are not contaminated by residues within the steam.

Pure Steam is prepared from suitably pretreated source water analogously to either the pretreatment used for Purified Water or Water for Injection. The water is vaporized with suitable mist elimination, and distributed under pressure. The sources of undesirable contaminants within Pure Steam could arise from entrained source water droplets, anti-corrosion steam additives, or residues from the steam production and distribution system itself. The attributes in the [Pure Steam](#) monograph should detect most of the contaminants that could arise from these sources. If the official article exposed to potential Pure Steam residues is intended for parenteral use or other applications where the pyrogenic content must be controlled,

the Pure Steam must additionally meet the specification for [Bacterial Endotoxins](#) (85).

These purity attributes are measured on the condensate of the article, rather than the article itself. This, of course, imparts great importance to the cleanliness of the Pure Steam condensate generation and collection process because it must not adversely impact the quality of the resulting condensed fluid.

Other steam attributes not detailed in the monograph, in particular, the presence of even small quantities of noncondensable gases or the existence of a superheated or dry state, may also be important for applications such as sterilization. The large release of energy (latent heat of condensation) as water changes from the gaseous to the liquid state is the key to steam's sterilization efficacy and its efficiency, in general, as a heat transfer agent. If this phase change (condensation) is not allowed to happen because the steam is extremely hot and in a persistent super heated, dry state, then its usefulness could be seriously compromised. Noncondensable gases in steam tend to stratify or collect in certain areas of a steam sterilization chamber or its load. These surfaces would thereby be at least partially insulated from the steam condensation phenomenon, preventing them from experiencing the full energy of the sterilizing conditions. Therefore, control of these kinds of steam attributes, in addition to its chemical purity, may also be important for certain Pure Steam applications.

However, because these additional attributes are use-specific, they are not mentioned in the Pure Steam monograph.

Note that less pure "plant steam" may be used for steam sterilization of nonproduct contact nonporous loads, for general cleaning of nonproduct contact equipment, as a nonproduct contact heat exchange medium, and in all compatible applications involved in bulk pharmaceutical chemical and API manufacture.

#### Packaged Monographed Waters

The following monographed waters are packaged forms of either Purified Water or Water for Injection that have been sterilized to preserve their microbiological properties. These waters may have specific intended uses as indicated by their names and may also have restrictions on packaging configurations related to those uses. In general, these packaged waters may be used in lieu of the bulk form of water from which they were derived. However, the user should take into consideration that the packaging and sterilization processes used for the articles may leach materials from the packaging material into the water over its shelf life, rendering it less pure than the original water placed into the package. The chemical attributes of these waters are still defined primarily by the wet chemistry methods and specifications similar to those formerly used for the bulk pharmaceutical waters prior to their replacement with water conductivity and total organic carbon (TOC). It is the user's responsibility to ensure fitness for use of this article when used in manufacturing, clinical, or analytical applications where the purer bulk form of the water is indicated.

Sterile Purified Water— [Sterile Purified Water](#) (see USP monograph) is Purified Water, packaged and rendered sterile. It is used in the preparation of nonparenteral compendial dosage forms or in analytical applications requiring Purified Water where access to a validated Purified Water system is not practical, where only a relatively small quantity is needed, where sterile Purified Water is required, or where bulk packaged Purified Water is not suitably microbiologically controlled.

Sterile Water for Injection— [Sterile Water for Injection](#) (see USP monograph) is Water for Injection packaged and rendered sterile. It is used for extemporaneous prescription compounding and as a sterile diluent for parenteral products. It may also be used for other applications where bulk Water for Injection or Purified Water is indicated but where access to a validated water system is either not practical or where only a relatively small quantity is needed. Sterile Water for Injection is packaged in single-dose containers not larger than 1 L in size.

Bacteriostatic Water for Injection— [Bacteriostatic Water for Injection](#) (see USP monograph) is sterile Water for Injection to which has been added one or more suitable antimicrobial preservatives. It is intended to be used as a diluent in the preparation of parenteral products, most typically for multi-dose products that require repeated content withdrawals. It may be packaged in single-dose or multiple-dose containers not larger than 30 mL.

Sterile Water for Irrigation— [Sterile Water for Irrigation](#) (see USP monograph) is Water for Injection packaged and sterilized in single-dose containers of larger than 1 L in size that allows rapid delivery of its contents. It need not meet the requirement under small-volume injections in the general test chapter [Particulate Matter in Injections](#) (788). It may also be used in other applications which do not have particulate matter specifications, where bulk Water for Injection or Purified Water is indicated but where access to a validated water system is not practical, or where somewhat larger quantities than are provided as Sterile Water for Injection are needed.

Sterile Water for Inhalation— [Sterile Water for Inhalation](#) (see USP monograph) is Water for Injection that is packaged and rendered sterile and is intended for use in inhalators and in the preparation of inhalation solutions. It carries a less stringent specification for bacterial endotoxins than Sterile Water for Injection, and therefore, is not suitable for parenteral applications.

#### Nonmonographed Manufacturing Waters

In addition to the bulk monographed waters described above, nonmonographed waters can also be used in pharmaceutical processing steps such as cleaning, synthetic steps, or a starting material for further purification. The following is a description of several of these nonmonographed waters as cited in various locations within this compendia.

Drinking Water— This type of water can be referred to as Potable Water (meaning drinkable or fit to drink), National Primary Drinking Water, Primary Drinking Water, or National Drinking Water. Except where a singular drinking water specification is stated (such as the NPDWR [U.S. Environmental Protection Agency's National Primary Drinking Water Regulations as cited in 40 CFR Part 141]), this water must comply with the quality attributes of either the NPDWR, or the drinking water regulations of the European Union or Japan, or the WHO Drinking Water Guidelines. It may be derived from a variety of sources including a public water utility, a private water supply (e.g., a well), or a combination of these sources. Drinking Water may be used in the early stages of cleaning pharmaceutical manufacturing equipment and product-contact components. Drinking Water is also the minimum quality of water that should be used for the preparation of official substances and other bulk pharmaceutical ingredients. Where compatible with the processes, the allowed contaminant levels in Drinking Water are generally considered safe for use for official substances and other drug substances. Where required by the processing of the materials to achieve their required final purity, higher qualities of water may be needed for these manufacturing steps, perhaps even as pure as Water for Injection or Purified Water. Such higher purity waters, however, might require only selected attributes to be of higher purity than Drinking Water (see [Figure 2](#) below). Drinking Water is the prescribed source or feed water for the production of bulk



monographed pharmaceutical waters. The use of Drinking Water specifications establishes a reasonable set of maximum allowable levels of chemical and microbiological contaminants with which a water purification system will be challenged. As seasonal variations in the quality attributes of the Drinking Water supply can occur, due consideration to its synthetic and cleaning uses must be given. The processing steps in the production of pharmaceutical waters must be designed to accommodate this variability.



\* Drinking Water is water complying with US EPA NPDWR or drinking water regulations of EU or Japan or WHO drinking water guidelines.

\*\* Water for sterile API's or dosage forms must first be rendered sterile if there is not a subsequent sterilization step in the process where used.

\*\*\* See guidance in this chapter where waters other than Purified Water are required by some USP tests and assays.

Note: All water systems should be validated with whatever microbial control is needed to suit the intended purposes of the water.

Figure 2. Selection of water for pharmaceutical purposes.

Hot Purified Water— This water is used in the preparation instructions for USP–NF articles and is clearly intended to be Purified Water that has been heated to an unspecified temperature in order to enhance solubilization of other ingredients. There is no upper temperature limit for the water (other than being less than 100°), but for each monograph there is an implied lower limit below which the desired solubilization effect would not occur.

#### Nonmonographed Analytical Waters

Both General Notices and Requirements and the introductory section to Reagents, Indicators, and Solutions clearly state that where the term "water," without qualification or other specification, is indicated for use in analyses, the quality of water shall be Purified Water. However, numerous such qualifications do exist. Some of these qualifications involve methods of preparation, ranging from specifying the primary purification step to specifying additional purification. Other qualifications call for specific attributes to be met that might otherwise interfere with analytical processes. In most of these latter cases, the required attribute is not specifically tested. Rather, a further "purification process" is specified that ostensibly allows the water to adequately meet this required attribute.

However, preparation instructions for many reagents were carried forward from the innovator's laboratories to the originally introduced monograph for a particular USP–NF article or general test chapter. The quality of the reagent water described in these tests may reflect the water quality designation of the innovator's laboratory. These specific water designations may have originated without the innovator's awareness of the requirement for Purified Water in USP–NF tests. Regardless of the original reason for the creation of these numerous special analytical waters, it is possible that the attributes of these special waters could now be met by the basic preparation steps and current specifications of Purified Water. In some



cases, however, some of the cited post-processing steps are still necessary to reliably achieve the required attributes.

Users are not obligated to employ specific and perhaps archaically generated forms of analytical water where alternatives with equal or better quality, availability, or analytical performance may exist. The consistency and reliability for producing these alternative analytical waters should be verified as producing the desired attributes. In addition, any alternative analytical water must be evaluated on an application-by-application basis by the user to ensure its suitability. Following is a summary of the various types of nonmonographed analytical waters that are cited in the USP-NF.

**Distilled Water**— This water is produced by vaporizing liquid water and condensing it in a purer state. It is used primarily as a solvent for reagent preparation, but it is also specified in the execution of other aspects of tests, such as for rinsing an analyte, transferring a test material as a slurry, as a calibration standard or analytical blank, and for test apparatus cleaning. It is also cited as the starting water to be used for making High Purity Water. Because none of the cited uses of this water imply a need for a particular purity attribute that can only be derived by distillation, water meeting the requirements for Purified Water derived by other means of purification could be equally suitable where Distilled Water is specified.

**Freshly Distilled Water**— Also called “recently distilled water”, it is produced in a similar fashion to Distilled Water and should be used shortly after its generation. This implies the need to avoid endotoxin contamination as well as any other adventitious forms of contamination from the air or containers that could arise with prolonged storage. It is used for preparing solutions for subcutaneous test animal injections as well as for a reagent solvent in tests for which there appears to be no particularly high water purity needed that could be ascribable to being “freshly distilled”. In the “test-animal” use, the term “freshly distilled” and its testing use imply a chemical, endotoxin, and microbiological purity that could be equally satisfied by Water for Injection (though no reference is made to these chemical, endotoxin, or microbial attributes or specific protection from recontamination). For nonanimal uses, water meeting the requirements for Purified Water derived by other means of purification and/or storage periods could be equally suitable where “recently distilled water” or Freshly Distilled Water is specified.

**Deionized Water**— This water is produced by an ion-exchange process in which the contaminating ions are replaced with either H<sup>+</sup> or OH<sup>-</sup> ions. Similarly to Distilled Water, Deionized Water is used primarily as a solvent for reagent preparation, but it is also specified in the execution of other aspects of tests, such as for transferring an analyte within a test procedure, as a calibration standard or analytical blank, and for test apparatus cleaning. Also, none of the cited uses of this water imply any needed purity attribute that can only be achieved by deionization. Therefore, water meeting the requirements for Purified Water that is derived by other means of purification could be equally suitable where Deionized Water is specified.

**Freshly Deionized Water**— This water is prepared in a similar fashion to Deionized Water, though as the name suggests, it is to be used shortly after its production. This implies the need to avoid any adventitious contamination that could occur upon storage. This water is indicated for use as a reagent solvent as well as for cleaning. Due to the nature of the testing, Purified Water could be a reasonable alternative for these applications.

**Deionized Distilled Water**— This water is produced by deionizing (see Deionized Water) Distilled Water. This water is used as a reagent in a liquid chromatography test that requires a high purity. Because of the importance of this high purity, water that barely meets the requirements for Purified Water may not be acceptable. High Purity Water (see below) could be a reasonable alternative for this water.

**Filtered Distilled or Deionized Water**— This water is essentially Purified Water produced by distillation or deionization that has been filtered through a 1.2-μm rated membrane. This water is used in particulate matter testing where the presence of particles in the water could bias the test results (see [Particulate Matter in Injections](#) (788)). Because the chemical water purity needed for this test could also be afforded by water purification processes other than distillation or deionization, filtered water meeting the requirements for Purified Water but produced by means other than distillation or deionization could be equally suitable.

**Filtered Water**— This water is Purified Water that has been filtered to remove particles that could interfere with the analysis where the water is used. Where used for preparing samples for particulate matter testing (see [Particulate Matter in Injections](#) (788)), though unspecified in monographs, water filtration should be through a 1.2-μm filter to be consistent with the general test chapter. Where used as a chromatography reagent, monograph-specified filter ratings range from 0.5 μm to unspecified.

**High Purity Water**— The preparation of this water is defined in [Containers—Glass](#) (660). It is water that is prepared by deionizing previously distilled water, and then filtering it through a 0.45-μm rated membrane. This water must have an in-line conductivity of not greater than 0.15 μS/cm (6.67 Megohm-cm) at 25°. For the sake of purity comparison, the analogous Stage 1 and 2 conductivity requirements for Purified Water at the same temperature are 1.3 μS/cm and 2.1 μS/cm, respectively. The preparation specified in [Containers—Glass](#) (660) uses materials that are highly efficient deionizers and that do not contribute copper ions or organics to the water, assuring a very high quality water. If the water of this purity contacts the atmosphere even briefly as it is being used or drawn from its purification system, its conductivity will immediately degrade, by as much as about 1.0 μS/cm, as atmospheric carbon dioxide dissolves in the water and equilibrates to bicarbonate ions. Therefore, if the analytical use requires that water purity remains as high as possible, its use should be protected from atmospheric exposure. This water is used as a reagent, as a solvent for reagent preparation, and for test apparatus cleaning where less pure waters would not perform acceptably. However, if a user's routinely available purified water is filtered and meets or exceeds the conductivity specifications of High Purity Water, it could be used in lieu of High Purity Water.

**Ammonia-Free Water**— Functionally, this water must have a negligible ammonia concentration to avoid interference in tests sensitive to ammonia. It has been equated with High Purity Water that has a significantly tighter Stage 1 conductivity specification than Purified Water because of the latter's allowance for a minimal level of ammonium among other ions. However, if the user's Purified Water were filtered and met or exceeded the conductivity specifications of High Purity Water, it would contain negligible ammonia or other ions and could be used in lieu of High Purity Water.

**Carbon Dioxide-Free Water**— The introductory portion of the Reagents, Indicators, and Solutions section defines this water as Purified Water that has been vigorously boiled for at least 5 minutes, then cooled and protected from absorption of atmospheric carbon dioxide. Because the absorption of carbon dioxide tends to drive down the water pH, most of the uses of Carbon Dioxide-Free Water are either associated as a solvent in pH-related or pH-sensitive determinations or as a solvent in carbonate-sensitive reagents or determinations. Another use of this water is for certain optical rotation and color and clarity of solution tests. Though it is possible that this water is indicated for these tests simply because of its purity, it is also possible that the pH effects of carbon dioxide containing water could interfere with the results of these tests. A third plausible reason that this water is indicated is that outgassing air bubbles might interfere with these photometric-type tests. The boiled water preparation approach will also greatly reduce the concentrations of many other dissolved gases along with carbon dioxide. Therefore, in some of the applications for Carbon Dioxide-Free Water, it could be the inadvertent deaeration effect that actually renders this water suitable. In addition to boiling, deionization is perhaps an even more efficient process for removing dissolved carbon dioxide (by drawing the dissolved gas equilibrium toward the ionized state with subsequent removal by the ion-exchange resins). If the starting Purified Water is prepared by an efficient deionization process and protected after deionization from exposure to atmospheric air, water that is carbon dioxide-free can be effectively made without the application of heat. However this deionization process does not deaerate the water, so if Purified Water prepared by deionization is considered as a substitute water in a test requiring Carbon Dioxide-Free Water, the user must verify that it is not actually water akin to Deaerated Water (discussed below) that is needed for the test. As indicated in High Purity Water, even brief contact with the atmosphere can allow small amounts of carbon dioxide to dissolve, ionize, and significantly degrade the conductivity and lower the pH. If the analytical use requires the water to remain as pH-neutral and as carbon dioxide-free as possible, even the analysis should be protected from atmospheric exposure. However, in most applications, atmospheric exposure during testing does not significantly affect its suitability in the test.

**Ammonia- and Carbon Dioxide-Free Water**— As implied by the name, this water should be prepared by approaches compatible with those mentioned for both Ammonia-Free Water and Carbon Dioxide-Free Water. Because the carbon dioxide-free attribute requires post-production protection from the atmosphere, it is appropriate to first render the water ammonia-free using the High Purity Water process followed by the boiling and carbon dioxide-protected cooling process. The High Purity Water deionization process for creating Ammonia-Free

Water will also remove the ions generated from dissolved carbon dioxide and ultimately, by forced equilibration to the ionized state, all the dissolved carbon dioxide. Therefore, depending on its use, an acceptable procedure for making Ammonia- and Carbon Dioxide-Free Water could be to transfer and collect High Purity Water in a carbon dioxide intrusion-protected container.

**Deaerated Water**— This water is Purified Water that has been treated to reduce the content of dissolved air by “suitable means”. In the Reagents section, approaches for boiling, cooling (similar to Carbon Dioxide-Free Water but without the atmospheric carbon dioxide protection), and sonication are given as applicable for test uses other than dissolution and drug release testing. Though Deaerated Water is not mentioned by name in [Dissolution](#) (711), suggested methods for deaerating dissolution media (which may be water) include warming to 41°, vacuum filtering through a 0.45-μm rated membrane, and vigorously stirring the filtrate while maintaining the vacuum. This chapter specifically indicates that other validated approaches may be used. In other monographs that also do not mention Deaerated Water by name, degassing of water and other reagents is accomplished by sparging with helium. Deaerated Water is used in both dissolution testing as well as liquid chromatography applications where outgassing could either interfere with the analysis itself or cause erroneous results due to inaccurate volumetric withdrawals. Applications where ambient temperature water is used for reagent preparation, but the tests are performed at elevated



...peratures, are candidates for outgassing effects. If outgassing could interfere with test performance, including chromatographic flow, colorimetric or photometric measurements, volumetric accuracy, then Deraerated Water should probably be used, whether called for in the analysis or not. The above deaeration approaches might not render the water "gas-free". At best, they reduce the dissolved gas concentrations so that outgassing caused by temperature changes is not likely.

Recently Boiled Water— This water may include recently or freshly boiled water (with or without mention of cooling in the title), but cooling prior to use is clearly intended. Occasionally it is necessary to use when hot. Recently Boiled Water is specified because it is used in a pH-related test or carbonate-sensitive reagent, in an oxygen-sensitive test or reagent, or in a test where outgassing could interfere with the analysis, such as specific gravity or an appearance test.

Oxygen-Free Water— The preparation of this water is not specifically described in the compendia. Neither is there an oxygen specification or analysis mentioned. However, all uses involve analyses of materials that could be sensitive to oxidation by atmospheric oxygen. Procedures for the removal of dissolved oxygen from solvents, though not necessarily water, are mentioned in [Polarography](#) (801) and [Spectrophotometry and Light-Scattering](#) (851). These procedures involve simple sparging of the liquid with an inert gas such as nitrogen or helium followed by inert gas blanketing to prevent oxygen reabsorption. The sparging times cited range from 5 to 15 minutes to an unspecified period. Some Purified Water and Water for Injection systems produce water that is maintained in a hot state and that is inert gas blanketed during its preparation and storage and distribution. Though oxygen is poorly soluble in hot water, such water may not be oxygen-free. Whatever procedure used for removing oxygen should be verified as reliably producing water that is fit for use.

LAL Reagent Water— This water is also referred to as endotoxin-free water. This is usually Water for Injection, which may have been sterilized. It is free from a level of endotoxin that would yield any detectable reaction or interference with the Limulus amebocyte lysate reagent used in the [Bacterial Endotoxins Test](#) (85).

Organic-Free Water— This water is defined by Residual Solvents (467) as producing no significantly interfering gas chromatography peaks. Referenced monographs specify using this water as the solvent for the preparation of standard and test solutions for the Residual solvents test.

Lead-Free Water— This water is used as a transferring diluent for an analyte in a [Lead](#) (251) test. Though no specific instructions are given for its preparation, it must not contain any detectable lead. Purified Water should be a suitable substitute for this water.

Chloride-Free Water— This water is specified as the solvent for use in an assay that contains a reactant that precipitates in the presence of chloride. Though no specific preparation instructions are given for this water, its rather obvious attribute is having a very low chloride level in order to be unreactive with this chloride sensitive reactant. Purified Water could be used for this water but should be tested to assure it is unreactive.

Hot Water— The uses of this water include solvents for achieving or enhancing reagent solubilization, restoring the original volume of boiled or hot solutions, rinsing insoluble analytes free of hot water soluble impurities, solvents for reagent recrystallization, apparatus cleaning, and as a solubility attribute for various USP-NF articles. In only one monograph is the temperature of "hot" water specified; so in all the other cases, the water temperature is less important, but should be high enough to achieve the desirable effect. In all cases, the chemical quality of the water is implied to be that of Purified Water.

#### VALIDATION AND QUALIFICATION OF WATER PURIFICATION, STORAGE, AND DISTRIBUTION SYSTEMS

Establishing the dependability of pharmaceutical water purification, storage, and distribution systems requires an appropriate period of monitoring and observation. Ordinarily, few problems are encountered in maintaining the chemical purity of Purified Water and Water for Injection. Nevertheless, the advent of using conductivity and TOC to define chemical purity has allowed the user to more quantitatively assess the water's chemical purity and its variability as a function of routine pretreatment system maintenance and regeneration. Even the presence of such unit operations as heat exchangers and use point hoses can compromise the chemical quality of water within and delivered from an otherwise well-controlled water system. Therefore, an assessment of the consistency of the water's chemical purity over time must be part of the validation program. However, even with the most well controlled chemical quality, it is often more difficult to consistently meet established microbiological quality criteria owing to phenomena occurring during and after chemical purification. A typical program involves intensive daily sampling and testing of major process points for at least one month after operational criteria have been established for each unit operation, point of use, and sampling point.

An overlooked aspect of water system validation is the delivery of the water to its actual location of use. If this transfer process from the distribution system outlets to the water use locations (usually with hoses) is defined as outside the water system, then this transfer process still needs to be validated to not adversely affect the quality of the water to the extent it becomes unfit for use. Because routine microbial monitoring is performed for the same transfer process and components (e.g., hoses and heat exchangers) as that of routine water use (see Sampling Considerations), there is some logic to include this water transfer process within the distribution system validation.

Validation is the process whereby substantiation to a high level of assurance that a specific process will consistently produce a product conforming to an established set of quality attributes is acquired and documented. Prior to and during the very early stages of validation, the critical process parameters and their operating ranges are established. A validation program qualifies and documents the design, installation, operation, and performance of equipment. It begins when the system is defined and moves through several stages: installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ). A graphical representation of a typical water system validation life cycle is shown in [Figure 3.](#)

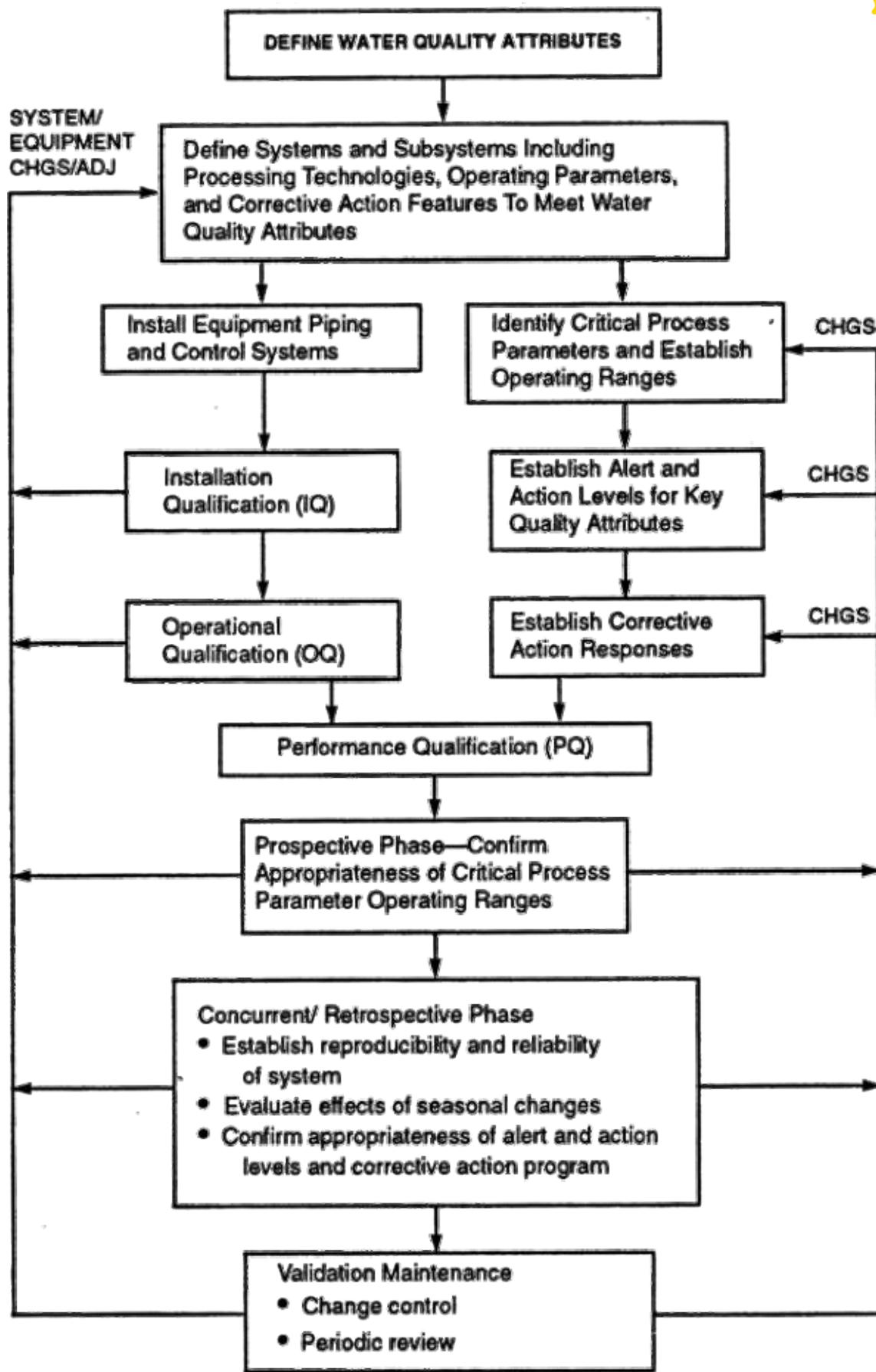


Fig. 3. Water system validation life cycle.

A validation plan for a water system typically includes the following steps: (1) establishing standards for quality attributes of the finished water and the source water; (2) defining suitable unit operations and their operating parameters for achieving the desired finished water quality attributes from the available source water; (3) selecting piping, equipment, controls, and monitoring technologies; (4) developing an IQ stage consisting of instrument calibrations, inspections to verify that the drawings accurately depict the final configuration of the water system and, where necessary, special tests to verify that the installation meets the design requirements; (5) developing an OQ stage consisting of tests and inspections to



Verify that the equipment, system alerts, and controls are operating reliably and that appropriate alert and action levels are established (This phase of qualification may overlap with aspects of the next step.); and (6) developing a prospective PQ stage to confirm the appropriateness of critical process parameter operating ranges (During this phase of validation, alert and action levels for key quality attributes and operating parameters are verified.); (7) assuring the adequacy of ongoing control procedures, e.g., sanitization frequency; (8) supplementing a validation maintenance program (also called continuous validation life cycle) that includes a mechanism to control changes to the water system and establishes and carries out scheduled preventive maintenance including recalibration of instruments (In addition, validation maintenance includes a monitoring program for critical process parameters and a corrective action program.); (9) instituting a schedule for periodic review of the system performance and requalification, and (10) completing protocols and documenting Steps 1 through 9.

#### PURIFIED WATER AND WATER FOR INJECTION SYSTEMS

The design, installation, and operation of systems to produce Purified Water and Water for Injection include similar components, control techniques, and procedures. The quality attributes of both waters differ only in the presence of a bacterial endotoxin requirement for Water for Injection and in their methods of preparation, at least at the last stage of preparation. The similarities in the quality attributes provide considerable common ground in the design of water systems to meet either requirement. The critical difference is the degree of control of the system and the final purification steps needed to ensure bacterial and bacterial endotoxin removal.

Production of pharmaceutical water employs sequential unit operations (processing steps) that address specific water quality attributes and protect the operation of subsequent treatment steps. A typical evaluation process to select an appropriate water quality for a particular pharmaceutical purpose is shown in the decision tree in Figure 2. This diagram may be used to assist in defining requirements for specific water uses and in the selection of unit operations. The final unit operation used to produce Water for Injection is limited to distillation or other processes equivalent or superior to distillation in the removal of chemical impurities as well as microorganisms and their components. Distillation has a long history of reliable performance and can be validated as a unit operation for the production of Water for Injection, but other technologies or combinations of technologies can be validated as being equivalently effective. Other technologies, such as ultrafiltration following other chemical purification process, may be suitable in the production of Water for Injection if they can be shown through validation to be as effective and reliable as distillation. The advent of new materials for older technologies, such as reverse osmosis and ultrafiltration, that allow intermittent or continuous operation at elevated, microbial temperatures, show promise for a valid use in producing Water for Injection.

The validation plan should be designed to establish the suitability of the system and to provide a thorough understanding of the purification mechanism, range of operating conditions, required pretreatment, and the most likely modes of failure. It is also necessary to demonstrate the effectiveness of the monitoring scheme and to establish the documentation and qualification requirements for the system's validation maintenance. Trials conducted in a pilot installation can be valuable in defining the operating parameters and the expected water quality and in identifying failure modes. However, qualification of the specific unit operation can only be performed as part of the validation of the installed operational system. The selection of specific unit operations and design characteristics for a water system should take into account the quality of the feed water, the technology chosen for subsequent processing steps, the extent and complexity of the water distribution system, and the appropriate compendial requirements. For example, in the design of a system for Water for Injection, the final process (distillation or whatever other validated process is used according to the monograph) must have effective bacterial endotoxin reduction capability and must be validated.

#### UNIT OPERATIONS CONCERN

The following is a brief description of selected unit operations and the operation and validation concerns associated with them. Not all unit operations are discussed, nor are all potential problems addressed. The purpose is to highlight issues that focus on the design, installation, operation, maintenance, and monitoring parameters that facilitate water system validation.

##### Prefiltration

The purpose of prefiltration—also referred to as initial, coarse, or depth filtration—is to remove solid contaminants down to a size of 7 to 10  $\mu\text{m}$  from the incoming source water supply and protect downstream system components from particulates that can inhibit equipment performance and shorten their effective life. This coarse filtration technology utilizes primarily sieving effects for particle capture and a depth of filtration medium that has a high "dirt load" capacity. Such filtration units are available in a wide range of designs and for various applications. Removal efficiencies and capacities differ significantly, from granular bed filters such as multimedia or sand for larger water systems, to depth cartridges for smaller water systems. Unit and system configurations vary widely in type of filtering media and location in the process. Granular or cartridge prefilters are often situated at or near the head of the water pretreatment system prior to unit operations designed to remove the source water disinfectants. This location, however, does not preclude the need for periodic microbial control because biofilm can still proliferate, although at a slower rate in the presence of source water disinfectants. Design and operational issues that may impact performance of depth filters include channeling of the filtering media, blockage from silt, microbial growth, and filtering-media loss during improper backwashing. Control measures involve pressure and flow monitoring during use and backwashing, sanitizing, and replacing filtering media. An important design concern is sizing of the filter to prevent channeling or media loss resulting from inappropriate water flow rates as well as proper sizing to minimize excessively frequent or infrequent backwashing or cartridge filter replacement.

##### Activated Carbon

Granular activated carbon beds adsorb low molecular weight organic material and oxidizing additives, such as chlorine and chloramine compounds, removing them from the water. They are used to achieve certain quality attributes and to protect against reaction with downstream stainless steel surfaces, resins, and membranes. The chief operating concerns regarding activated carbon beds include the propensity to support bacteria growth, the potential for hydraulic channeling, the organic adsorption capacity, appropriate water flow rates and contact time, the inability to be regenerated in situ, and the shedding of bacteria, endotoxins, organic chemicals, and fine carbon particles. Control measures may involve monitoring water flow rates and differential pressures, sanitizing with hot water or steam, backwashing, testing for adsorption capacity, and frequent replacement of the carbon bed. If the activated carbon bed is intended for organic reduction, it may also be appropriate to monitor influent and effluent TOC. It is important to note that the use of steam for carbon bed sanitization is often incompletely effective due to steam channeling rather than even permeation through the bed. This phenomenon can usually be avoided by using hot water sanitization. It is also important to note that microbial biofilm development on the surface of the granular carbon particles (as well as on other particles such as found in deionizer beds and even multimedia beds) can cause adjacent bed granules to "stick" together. When large masses of granules are agglomerated in this fashion, normal backwashing and bed fluidization flow parameters may not be sufficient to disperse them, leading to ineffective removal of trapped debris, loose biofilm, and penetration of microbial controlling conditions (as well as regenerant chemicals as in the case of agglomerated deionizer resins). Alternative technologies to activated carbon beds can be used in order to avoid their microbial problems, such as disinfectant-neutralizing chemical additives and regenerable organic scavenging devices. However, these alternatives do not function by the same mechanisms as activated carbon, may not be as effective at removing disinfectants and some organics, and have a different set of operating concerns and control measures that may be nearly as troublesome as activated carbon beds.

##### Additives

Chemical additives are used in water systems (a) to control microorganisms by use of sanitants such as chlorine compounds and ozone, (b) to enhance the removal of suspended solids by use of flocculating agents, (c) to remove chlorine compounds, (d) to avoid scaling on reverse osmosis membranes, and (e) to adjust pH for more effective removal of carbonate and ammonia compounds by reverse osmosis. These additives do not constitute "added substances" as long as they are either removed by subsequent processing steps or are otherwise absent from the finished water. Control of additives to ensure a continuously effective concentration and subsequent monitoring to ensure their removal should be designed into the system and included in the monitoring program.

##### Organic Scavengers

Organic scavenging devices use macroreticular weakly basic anion-exchange resins capable of removing organic material and endotoxins from the water. They can be regenerated with appropriate biocidal caustic brine solutions. Operating concerns are associated with organic scavenging capacity, particulate, chemical and microbiological fouling of the reactive resin surface, flow rate, regeneration frequency, and shedding of resin fragments. Control measures include TOC testing of influent and effluent, backwashing, monitoring hydraulic performance, and using downstream filters to remove resin fines.

##### Softeners

Water softeners may be located either upstream or downstream of disinfectant removal units. They utilize sodium-based cation-exchange resins to remove water-hardness ions, such



calcium and magnesium, that could foul or interfere with the performance of downstream processing equipment such as reverse osmosis membranes, deionization devices, and distillation units. Water softeners can also be used to remove other lower affinity cations, such as the ammonium ion, that may be released from chloramine disinfectants commonly used in drinking water and which might otherwise carryover through other downstream unit operations. If ammonium removal is one of its purposes, the softener must be located downstream of the disinfectant removal operation, which itself may liberate ammonium from neutralized chloramine disinfectants. Water softener resin beds are regenerated with concentrated sodium chloride solution (brine). Concerns include microorganism proliferation, channeling caused by biofilm agglomeration of resin particles, appropriate water flow rates and contact time, ion-exchange capacity, organic and particulate resin fouling, organic leaching from new resins, fracture of the resin beads, resin degradation by excessively chlorinated water, and contamination from the brine solution used for regeneration. Control measures involve recirculation of water during periods of low water use, periodic sanitization of the resin and brine system, use of microbial control devices (e.g., UV light and chlorine), locating the unit upstream of the disinfectant removal step (if used only for softening), appropriate regeneration frequency, effluent chemical monitoring (e.g., hardness ions and possibly ammonium), and downstream filtration to remove resin fines. If a softener is used for ammonium removal from chloramine-containing source water, then capacity, contact time, resin surface fouling, pH, and regeneration frequency are very important.

#### Deionization

Deionization (DI), and continuous electrodeionization (CEDI) are effective methods of improving the chemical quality attributes of water by removing cations and anions. DI systems have charged resins that require periodic regeneration with an acid and base. Typically, cationic resins are regenerated with either hydrochloric or sulfuric acid, which replace the captured positive ions with hydrogen ions. Anionic resins are regenerated with sodium or potassium hydroxide, which replace captured negative ions with hydroxide ions. Because free endotoxin is negatively charged, there is some removal of endotoxin achieved by the anionic resin. Both regenerant chemicals are biocidal and offer a measure of microbial control. The system can be designed so that the cation and anion resins are in separate or "twin" beds or they can be mixed together to form a mixed bed. Twin beds are easily regenerated but deionize water less efficiently than mixed beds, which have a considerably more complex regeneration process. Rechargeable resin canisters can also be used for this purpose.

The CEDI system uses a combination of mixed resin, selectively permeable membranes, and an electric charge, providing continuous flow (product and waste concentrate) and continuous regeneration. Water enters both the resin section and the waste (concentrate) section. As it passes through the resin, it is deionized to become product water. The resin acts as a conductor enabling the electrical potential to drive the captured cations and anions through the resin and appropriate membranes for concentration and removal in the waste water stream. The electrical potential also separates the water in the resin (product) section into hydrogen and hydroxide ions. This permits continuous regeneration of the resin without the need for regenerant additives. However, unlike conventional deionization, CEDI units must start with water that is already partially purified because they generally cannot produce

Purified Water quality when starting with the heavier ion load of unpurified source water.

Concerns for all forms of deionization units include microbial and endotoxin control, chemical additive impact on resins and membranes, and loss, degradation, and fouling of resin. Issues of concern specific to DI units include regeneration frequency and completeness, channeling, caused by biofilm agglomeration of resin particles, organic leaching from new resins, complete resin separation for mixed bed regeneration, and mixing air contamination (mixed beds). Control measures vary but typically include recirculation loops, effluent microbial control by UV light, conductivity monitoring, resin testing, microporous filtration of mixing air, microbial monitoring, frequent regeneration to minimize and control microorganism growth, sizing the equipment for suitable water flow and contact time, and use of elevated temperatures. Internal distributor and regeneration piping for mixed bed units should be configured to ensure that regeneration chemicals contact all internal bed and piping surfaces and resins. Rechargeable canisters can be the source of contamination and should be carefully monitored. Full knowledge of previous resin use, minimum storage time between regeneration and use, and appropriate sanitizing procedures are critical factors ensuring proper performance.

#### Reverse Osmosis

Reverse osmosis (RO) units employ semipermeable membranes. The "pores" of RO membranes are actually intersegmental spaces among the polymer molecules. They are big enough for permeation of water molecules, but too small to permit passage of hydrated chemical ions. However, many factors including pH, temperature, and differential pressure across the membrane affect the selectivity of this permeation. With the proper controls, RO membranes can achieve chemical, microbial, and endotoxin quality improvement. The process streams consist of supply water, product water (permeate), and wastewater (reject). Depending on source water, pretreatment and system configuration variations and chemical additives may be necessary to achieve desired performance and reliability.

A major factor affecting RO performance is the permeate recovery rate, that is, the amount of the water passing through the membrane compared to the amount rejected. This is influenced by the several factors, but most significantly by the pump pressure. Recoveries of 75% are typical, and can accomplish a 1 to 2 log purification of most impurities. For most feed waters, this is usually not enough to meet Purified Water conductivity specifications. A second pass of this permeate water through another RO stage usually achieves the necessary permeate purity if other factors such as pH and temperature have been appropriately adjusted and the ammonia from chlorinated source water has been previously removed. Increasing recoveries with higher pressures in order to reduce the volume of reject water will lead to reduced permeate purity. If increased pressures are needed over time to achieve the same permeate flow, this is an indication of partial membrane blockage that needs to be corrected before it becomes irreversibly fouled, and expensive membrane replacement is the only option.

Other concerns associated with the design and operation of RO units include membrane materials that are extremely sensitive to sanitizing agents and to particulate, chemical, and microbial membrane fouling; membrane and seal integrity; the passage of dissolved gases, such as carbon dioxide and ammonia; and the volume of wastewater, particularly where water discharge is tightly regulated by local authorities. Failure of membrane or seal integrity will result in product water contamination. Methods of control involve suitable pretreatment of the influent water stream, appropriate membrane material selection, integrity challenges, membrane design and heat tolerance, periodic sanitization, and monitoring of differential pressures, conductivity, microbial levels, and TOC.

The development of RO units that can tolerate sanitizing water temperatures as well as operate efficiently and continuously at elevated temperatures has added greatly to their microbial control and to the avoidance of biofouling. RO units can be used alone or in combination with DI and CEDI units as well as ultrafiltration for operational and quality enhancements.

#### Ultrafiltration

Ultrafiltration is a technology most often employed in pharmaceutical water systems for removing endotoxins from a water stream. It can also use semipermeable membranes, but unlike RO, these typically use polysulfone membranes whose intersegmental "pores" have been purposefully exaggerated during their manufacture by preventing the polymer molecules from reaching their smaller equilibrium proximities to each other. Depending on the level of equilibrium control during their fabrication, membranes with differing molecular weight "cutoffs" can be created such that molecules with molecular weights above these cutoffs ratings are rejected and cannot penetrate the filtration matrix.

Ceramic ultrafilters are another molecular sieving technology. Ceramic ultrafilters are self supporting and extremely durable, backwashable, chemically cleanable, and steam sterilizable. However, they may require higher operating pressures than membrane type ultrafilters.

All ultrafiltration devices work primarily by a molecular sieving principle. Ultrafilters with molecular weight cutoff ratings in the range of 10,000 to 20,000 Da are typically used in water systems for removing endotoxins. This technology may be appropriate as an intermediate or final purification step. Similar to RO, successful performance is dependent upon pretreatment of the water by upstream unit operations.

Issues of concern for ultrafilters include compatibility of membrane material with heat and sanitizing agents, membrane integrity, fouling by particles and microorganisms, and seal integrity. Control measures involve filtration medium selection, sanitization, flow design (dead end vs. tangential), integrity challenges, regular cartridge changes, elevated feed water temperature, and monitoring TOC and differential pressure. Additional flexibility in operation is possible based on the way ultrafiltration units are arranged such as in a parallel or series configurations. Care should be taken to avoid stagnant water conditions that could promote microorganism growth in back-up or standby units.

#### Charge-Modified Filtration

Charge-modified filters are usually microbially retentive filters that are treated during their manufacture to have a positive charge on their surfaces. Microbial retentive filtration will be described in a subsequent section, but the significant feature of these membranes is their electrostatic surface charge. Such charged filters can reduce endotoxin levels in the fluids passing through them by their adsorption (owing to endotoxin's negative charge) onto the membrane surfaces. Though ultrafilters are more often employed as a unit operation for endotoxin removal in water systems, charge-modified filters may also have a place in endotoxin removal particularly where available upstream pressures are not sufficient for ultrafiltration and for a single, relatively short term use. Charge-modified filters may be difficult to validate for long-term or large-volume endotoxin retention. Even though their purified standard endotoxin retention can be well characterized, their retention capacity for "natural" endotoxins is difficult to gauge. Nevertheless, utility could be demonstrated and validated



short-term, single-use filters at points of use in water systems that are not designed for endotoxin control or where only an endotoxin "polishing" (removal of only slight or occasional endotoxin levels) is needed. Control and validation concerns include volume and duration of use, flow rate, water conductivity and purity, and constancy and concentration of endotoxin levels being removed. All of these factors may have to be evaluated and challenged prior to using this approach, making this a difficult-to-validate application. Even so, there may still be a possible need for additional backup endotoxin testing both upstream and downstream of the filter.

#### Microbial-Retentive Filtration

Microbial-retentive membrane filters have experienced an evolution of understanding in the past decade that has caused previously held theoretical retention mechanisms to be reconsidered. These filters have a larger effective "pore size" than ultrafilters and are intended to prevent the passage of microorganisms and similarly sized particles without unduly restricting flow. This type of filtration is widely employed within water systems for filtering the bacteria out of both water and compressed gases as well as for vent filters on tanks and stills and other unit operations. However, the properties of the water system microorganisms seem to challenge a filter's microbial retention from water with phenomena absent from other aseptic filtration applications, such as filter sterilizing of pharmaceutical formulations prior to packaging. In the latter application, sterilizing grade filters are generally considered to have an assigned rating of 0.2 or 0.22  $\mu\text{m}$ . This rather arbitrary rating is associated with filters that have the ability to retain a high level challenge of a specially prepared inoculum of *Brevundimonas* (formerly *Pseudomonas*) *diminuta*. This is a small microorganism originally isolated decades ago from a product that had been "filter sterilized" using a 0.45- $\mu\text{m}$  rated filter. Further study revealed that a percentage of cells of this microorganism could reproducibly penetrate the 0.45- $\mu\text{m}$  sterilizing filters. Through historic correlation of *B. diminuta* retaining tighter filters, thought to be twice as good as 0.45- $\mu\text{m}$  filter, assigned ratings of 0.2 or 0.22  $\mu\text{m}$  with their successful use in product solution filter sterilization, both this filter rating and the associated high level *B. diminuta* challenge have become the current benchmarks for sterilizing filtration. New evidence now suggests that for microbial-retentive filters used for pharmaceutical water, *B. diminuta* may not be the best model microorganism.

An archaic understanding of microbial retentive filtration would lead one to equate a filter's rating with the false impression of a simple sieve or screen that absolutely retains particles sized at or above the filter's rating. A current understanding of the mechanisms involved in microbial retention and the variables that can affect those mechanisms has yielded a far more complex interaction of phenomena than previously understood. A combination of simple sieve retention and surface adsorption are now known to contribute to microbial retention.

The following all interact to create some unusual and surprising retention phenomena for water system microorganisms: the variability in the range and average pore sizes created by the various membrane fabrication processes, the variability of the surface chemistry and three-dimensional structure related to the different polymers used in these filter matrices, and the size and surface properties of the microorganism intended to be retained by the filters. *B. diminuta* may not be the best challenge microorganisms for demonstrating bacterial retention for 0.2- to 0.22- $\mu\text{m}$  rated filters for use in water systems because it appears to be more easily retained by these filters than some water system flora. The well-documented appearance of water system microorganisms on the downstream sides of some 0.2- to 0.22- $\mu\text{m}$  rated filters after a relatively short period of use seems to support that some penetration phenomena are at work. Unknown for certain is if this downstream appearance is caused by a "blow-through" or some other pass-through phenomenon as a result of tiny cells or less cell "stickiness", or by a "growth through" phenomenon as a result of cells hypothetically replicating their way through the pores to the downstream side. Whatever is the penetration mechanism, 0.2- to 0.22- $\mu\text{m}$  rated membranes may not be the best choice for some water system uses.

Microbial retention success in water systems has been reported with the use of some manufacturers' filters arbitrarily rated as 0.1  $\mu\text{m}$ . There is general agreement that for a given manufacturer, their 0.1- $\mu\text{m}$  rated filters are tighter than their 0.2- to 0.22- $\mu\text{m}$  rated filters. However, comparably rated filters from different manufacturers in water filtration applications may not perform equivalently owing to the different filter fabrication processes and the nonstandardized microbial retention challenge processes currently used for defining the 0.1- $\mu\text{m}$  filter rating. It should be noted that use of 0.1- $\mu\text{m}$  rated membranes generally results in a sacrifice in flow rate compared to 0.2- to 0.22- $\mu\text{m}$  membranes, so whatever membranes are chosen for a water system application, the user must verify that the membranes are suitable for their intended application, use period, and use process, including flow rate.

For microbial retentive gas filtrations, the same sieving and adsorptive retention phenomena are at work as in liquid filtration, but the adsorptive phenomenon is enhanced by additional electrostatic interactions between particles and filter matrix. These electrostatic interactions are so strong that particle retention for a given filter rating is significantly more efficient in gas filtration than in water or product solution filtrations. These additional adsorptive interactions render filters rated at 0.2 to 0.22  $\mu\text{m}$  unquestionably suitable for microbial retentive gas filtrations. When microbially retentive filters are used in these applications, the membrane surface is typically hydrophobic (non-wettable by water). A significant area of concern for gas filtration is blockage of tank vents by condensed water vapor, which can cause mechanical damage to the tank. Control measures include electrical or steam tracing and a self-draining orientation of vent filter housings to prevent accumulation of vapor condensate. However, a continuously high filter temperature will take an oxidative toll on polypropylene components of the filter, so sterilization of the unit prior to initial use, and periodically thereafter, as well as regular visual inspections, integrity tests, and changes are recommended control methods.

In water applications, microbial retentive filters may be used downstream of unit operations that tend to release microorganisms or upstream of unit operations that are sensitive to microorganisms. Microbial retentive filters may also be used to filter water feeding the distribution system. It should be noted that regulatory authorities allow the use of microbial retentive filters within distribution systems or even at use points if they have been properly validated and are appropriately maintained. A point-of-use filter should only be intended to "polish" the microbial quality of an otherwise well-maintained system and not to serve as the primary microbial control device. The efficacy of system microbial control measures can only be assessed by sampling the water upstream of the filters. As an added measure of protection, in-line UV lamps, appropriately sized for the flow rate (see Sanitization), may be used just upstream of microbial retentive filters to inactivate microorganisms prior to their capture by the filter. This tandem approach tends to greatly delay potential microbial penetration phenomena and can substantially extend filter service life.

#### Ultraviolet Light

The use of low-pressure UV lights that emit a 254-nm wavelength for microbial control is discussed under Sanitization, but the application of UV light in chemical purification is also emerging. This 254-nm wavelength is also useful in the destruction of ozone. With intense emissions at wavelengths around 185 nm (as well as at 254 nm), medium pressure UV lights have demonstrated utility in the destruction of the chlorine containing disinfectants used in source water as well as for interim stages of water pretreatment. High intensities of this wavelength alone or in combination with other oxidizing sanitants, such as hydrogen peroxide, have been used to lower TOC levels in recirculating distribution systems. The organics are typically converted to carbon dioxide, which equilibrates to bicarbonate, and incompletely oxidized carboxylic acids, both of which can easily be removed by polishing ion-exchange resins. Areas of concern include adequate UV intensity and residence time, gradual loss of UV emissivity with bulb age, gradual formation of UV-absorbing film at the water contact surface, incomplete photodegradation during unforeseen source water hyperchlorination, release of ammonia from chloramine photodegradation, unapparent UV bulb failure, and conductivity degradation in distribution systems using 185-nm UV lights. Control measures include regular inspection or emissivity alarms to detect bulb failures or film occlusions, regular UV bulb sleeve cleaning and wiping, downstream chlorine detectors, downstream polishing deionizers, and regular (approximately yearly) bulb replacement.

#### Distillation

Distillation units provide chemical and microbial purification via thermal vaporization, mist elimination, and water vapor condensation. A variety of designs is available including single effect, multiple effect, and vapor compression. The latter two configurations are normally used in larger systems because of their generating capacity and efficiency. Distilled water systems require different feed water controls than required by membrane systems. For distillation, due consideration must be given to prior removal of hardness and silica impurities that may foul or corrode the heat transfer surfaces as well as prior removal of those impurities that could volatize and condense along with the water vapor. In spite of general perceptions, even the best distillation process cannot afford absolute removal of contaminating ions and endotoxin. Most stills are recognized as being able to accomplish at least a 3 to 4 log reduction in these impurity concentrations. Areas of concern include carry-over of volatile organic impurities such as trihalomethanes (see Source and Feed Water Considerations) and gaseous impurities such as ammonia and carbon dioxide, faulty mist elimination, evaporator flooding, inadequate blowdown, stagnant water in condensers and evaporators, pump and compressor seal design, pinhole evaporator and condenser leaks, and conductivity (quality) variations during start-up and operation.

Methods of control may involve preliminary decarbonation steps to remove both dissolved carbon dioxide and other volatile or noncondensable impurities; reliable mist elimination to minimize feedwater droplet entrainment; visual or automated high water level indication to detect boiler flooding and boil over; use of sanitary pumps and compressors to minimize microbial and lubricant contamination of feedwater and condensate; proper drainage during inactive periods to minimize microbial growth and accumulation of associated endotoxin in boiler water; blow down control to limit the impurity concentration effect in the boiler to manageable levels; on-line conductivity sensing with automated diversion to waste to prevent unacceptable water upon still startup or still malfunction from getting into the finished water distribution system; and periodic integrity testing for pinhole leaks to routinely assure condensate is not compromised by nonvolatized source water contaminants.

#### Storage Tanks



Storage tanks are included in water distribution systems to optimize processing equipment capacity. Storage also allows for routine maintenance within the pretreatment train while maintaining continuous supply to meet manufacturing needs. Design and operation considerations are needed to prevent or minimize the development of biofilm, to minimize corrosion, to aid in the use of chemical sanitization of the tanks, and to safeguard mechanical integrity. These considerations may include using closed tanks with smooth interiors, the ability to spray the tank headspace using sprayballs on recirculating loop returns, and the use of heated, jacketed/insulated tanks. This minimizes corrosion and biofilm development and aids in thermal and chemical sanitization. Storage tanks require venting to compensate for the dynamics of changing water levels. This can be accomplished with a properly oriented and heat-traced filter housing fitted with a hydrophobic microbial retentive membrane filter affixed to an atmospheric vent. Alternatively, an automatic membrane-filtered compressed gas blanketing system may be used. In both cases, rupture disks equipped with a rupture alarm device should be used as a further safeguard for the mechanical integrity of the tank. Areas of concern include microbial growth or corrosion due to irregular or incomplete sanitization and microbial contamination from unalarmed rupture disk failures caused by condensate-occluded vent filters.

#### Distribution Systems

Distribution system configuration should allow for the continuous flow of water in the piping by means of recirculation. Use of nonrecirculating, dead-end, or one-way systems or system segments should be avoided whenever possible. If not possible, these systems should be periodically flushed and more closely monitored. Experience has shown that continuously recirculated systems are easier to maintain. Pumps should be designed to deliver fully turbulent flow conditions to facilitate thorough heat distribution (for hot water sanitized systems) as well as thorough chemical sanitant distribution. Turbulent flow also appear to either retard the development of biofilms or reduce the tendency of those biofilms to shed bacteria into the water. If redundant pumps are used, they should be configured and used to avoid microbial contamination of the system.

Components and distribution lines should be sloped and fitted with drain points so that the system can be completely drained. In stainless steel distribution systems where the water is circulated at a high temperature, dead legs and low-flow conditions should be avoided, and valved tie-in points should have length-to-diameter ratios of six or less. If constructed of heat tolerant plastic, this ratio should be even less to avoid cool points where biofilm development could occur. In ambient temperature distribution systems, particular care should be exercised to avoid or minimize dead leg ratios of any size and provide for complete drainage. If the system is intended to be steam sanitized, careful sloping and low-point drainage is crucial to condensate removal and sanitization success. If drainage of components or distribution lines is intended as a microbial control strategy, they should also be configured to be completely dried using dry compressed air (or nitrogen if appropriate employee safety measures are used). Drained but still moist surfaces will still support microbial proliferation. Water exiting from the distribution system should not be returned to the system without first passing through all or a portion of the purification train.

The distribution design should include the placement of sampling valves in the storage tank and at other locations, such as in the return line of the recirculating water system. Where feasible, the primary sampling sites for water should be the valves that deliver water to the points of use. Direct connections to processes or auxiliary equipment should be designed to prevent reverse flow into the controlled water system. Hoses and heat exchangers that are attached to points of use in order to deliver water for a particular use must not chemically or microbiologically degrade the water quality. The distribution system should permit sanitization for microorganism control. The system may be continuously operated at sanitizing conditions or sanitized periodically.

#### INSTALLATION, MATERIALS OF CONSTRUCTION, AND COMPONENT SELECTION

Installation techniques are important because they can affect the mechanical, corrosive, and sanitary integrity of the system. Valve installation attitude should promote gravity drainage. Pipe supports should provide appropriate slopes for drainage and should be designed to support the piping adequately under worst-case thermal and flow conditions. The methods of connecting system components including units of operation, tanks, and distribution piping require careful attention to preclude potential problems. Stainless steel welds should provide reliable joints that are internally smooth and corrosion-free. Low-carbon stainless steel, compatible wire filler, where necessary, inert gas, automatic welding machines, and regular inspection and documentation help to ensure acceptable weld quality. Follow-up cleaning and passivation are important for removing contamination and corrosion products and to re-establish the passive corrosion resistant surface. Plastic materials can be fused (welded) in some cases and also require smooth, uniform internal surfaces. Adhesive glues and solvents should be avoided due to the potential for voids and extractables. Mechanical methods of joining, such as flange fittings, require care to avoid the creation of offsets, gaps, penetrations, and voids. Control measures include good alignment, properly sized gaskets, appropriate spacing, uniform sealing force, and the avoidance of threaded fittings.

Materials of construction should be selected to be compatible with control measures such as sanitizing, cleaning, and passivating. Temperature rating is a critical factor in choosing appropriate materials because surfaces may be required to handle elevated operating and sanitization temperatures. Should chemicals or additives be used to clean, control, or sanitize the system, materials resistant to these chemicals or additives must be utilized. Materials should be capable of handling turbulent flow and elevated velocities without wear of the corrosion-resistant film such as the passive chromium oxide surface of stainless steel. The finish on metallic materials such as stainless steel, whether it is a refined mill finish, polished to a specific grit, or an electropolished treatment, should complement system design and provide satisfactory corrosion and microbial activity resistance as well as chemical sanitizability. Auxiliary equipment and fittings that require seals, gaskets, diaphragms, filter media, and membranes should exclude materials that permit the possibility of extractables, shedding, and microbial activity. Insulating materials exposed to stainless steel surfaces should be free of chlorides to avoid the phenomenon of stress corrosion cracking that can lead to system contamination and the destruction of tanks and critical system components.

Specifications are important to ensure proper selection of materials and to serve as a reference for system qualification and maintenance. Information such as mill reports for stainless steel and reports of composition, ratings, and material handling capabilities for nonmetallic substances should be reviewed for suitability and retained for reference. Component (auxiliary equipment) selection should be made with assurance that it does not create a source of contamination intrusion. Heat exchangers should be constructed to prevent leakage of heat transfer medium to the pharmaceutical water and, for heat exchanger designs where prevention may fail, there should be a means to detect leakage. Pumps should be of sanitary design with seals that prevent contamination of the water. Valves should have smooth internal surfaces with the seat and closing device exposed to the flushing action of water, such as occurs in diaphragm valves. Valves with pocket areas or closing devices (e.g., ball, plug, gate, globe) that move into and out of the flow area should be avoided.

#### SANITIZATION

Microbial control in water systems is achieved primarily through sanitization practices. Systems can be sanitized using either thermal or chemical means. Thermal approaches to system sanitization include periodic or continuously circulating hot water and the use of steam. Temperatures of at least 80° are most commonly used for this purpose, but continuously recirculating water of at least 65° has also been used effectively in insulated stainless steel distribution systems when attention is paid to uniformity and distribution of such self-sanitizing temperatures. These techniques are limited to systems that are compatible with the higher temperatures needed to achieve sanitization. Although thermal methods control biofilm development by either continuously inhibiting their growth or, in intermittent applications, by killing the microorganisms within biofilms, they are not effective in removing established biofilms. Killed but intact biofilms can become a nutrient source for rapid biofilm regrowth after the sanitizing conditions are removed or halted. In such cases, a combination of routine thermal and periodic supplementation with chemical sanitization might be more effective. The more frequent the thermal sanitization, the more likely biofilm development and regrowth can be eliminated. Chemical methods, where compatible, can be used on a wider variety of construction materials. These methods typically employ oxidizing agents such as halogenated compounds, hydrogen peroxide, ozone, peracetic acid, or combinations thereof. Halogenated compounds are effective sanitizers but are difficult to flush from the system and may leave biofilms intact. Compounds such as hydrogen peroxide, ozone, and peracetic acid oxidize bacteria and biofilms by forming reactive peroxides and free radicals (notably hydroxyl radicals). The short half-life of ozone in particular, and its limitation on achievable concentrations require that it be added continuously during the sanitization process. Hydrogen peroxide and ozone rapidly degrade to water and oxygen; peracetic acid degrades to acetic acid in the presence of UV light. In fact, ozone's ease of degradation to oxygen using 254-nm UV lights at use points allow it to be most effectively used on a continuous basis to provide continuously sanitizing conditions.

In-line UV light at a wavelength of 254 nm can also be used to continuously "sanitize" water circulating in the system, but these devices must be properly sized for the water flow. Such devices inactivate a high percentage (but not 100%) of microorganisms that flow through the device but cannot be used to directly control existing biofilm upstream or downstream of the device. However, when coupled with conventional thermal or chemical sanitization technologies or located immediately upstream of a microbially retentive filter, it is most effective and can prolong the interval between system sanitizations.

It is important to note that microorganisms in a well-developed biofilm can be extremely difficult to kill, even by aggressive oxidizing biocides. The less developed and therefore thinner the biofilm, the more effective the biocidal action. Therefore, optimal biocide control is achieved by frequent biocide use that does not allow significant biofilm development between treatments.

Sanitization steps require validation to demonstrate the capability of reducing and holding microbial contamination at acceptable levels. Validation of thermal methods should include a



heat distribution study to demonstrate that sanitization temperatures are achieved throughout the system, including the body of use point valves. Validation of chemical methods require demonstrating adequate chemical concentrations throughout the system, exposure to all wetted surfaces, including the body of use point valves, and complete removal of the sanitant from the system at the completion of treatment. Methods validation for the detection and quantification of residues of the sanitant or its objectionable degradants is an essential part of the validation program. The frequency of sanitization should be supported by, if not triggered by, the results of system microbial monitoring. Conclusions derived from trend analysis of the microbiological data should be used as the alert mechanism for maintenance. The frequency of sanitization should be established in such a way that the system operates in a state of microbiological control and does not routinely exceed alert levels (see Alert and Action Levels and Specifications).

#### OPERATION, MAINTENANCE, AND CONTROL

A preventive maintenance program should be established to ensure that the water system remains in a state of control. The program should include (1) procedures for operating the system, (2) monitoring programs for critical quality attributes and operating conditions including calibration of critical instruments, (3) schedule for periodic sanitization, (4) preventive maintenance of components, and (5) control of changes to the mechanical system and to operating conditions.

**Operating Procedures**— Procedures for operating the water system and performing routine maintenance and corrective action should be written, and they should also define the point when action is required. The procedures should be well documented, detail the function of each job, assign who is responsible for performing the work, and describe how the job is to be conducted. The effectiveness of these procedures should be assessed during water system validation.

**Monitoring Program**— Critical quality attributes and operating parameters should be documented and monitored. The program may include a combination of in-line sensors or automated instruments (e.g., for TOC, conductivity, hardness, and chlorine), automated or manual documentation of operational parameters (such as flow rates or pressure drop across a carbon bed, filter, or RO unit), and laboratory tests (e.g., total microbial counts). The frequency of sampling, the requirement for evaluating test results, and the necessity for initiating corrective action should be included.

**Sanitization**— Depending on system design and the selected units of operation, routine periodic sanitization may be necessary to maintain the system in a state of microbial control. Technologies for sanitization are described above.

**Preventive Maintenance**— A preventive maintenance program should be in effect. The program should establish what preventive maintenance is to be performed, the frequency of maintenance work, and how the work should be documented.

**Change Control**— The mechanical configuration and operating conditions must be controlled. Proposed changes should be evaluated for their impact on the whole system. The need to requalify the system after changes are made should be determined. Following a decision to modify a water system, the affected drawings, manuals, and procedures should be revised.

#### SAMPLING CONSIDERATIONS

Water systems should be monitored at a frequency that is sufficient to ensure that the system is in control and continues to produce water of acceptable quality. Samples should be taken from representative locations within the processing and distribution system. Established sampling frequencies should be based on system validation data and should cover critical areas including unit operation sites. The sampling plan should take into consideration the desired attributes of the water being sampled. For example, systems for Water for Injection because of their more critical microbiological requirements, may require a more rigorous sampling frequency.

Analyses of water samples often serve two purposes: in-process control assessments and final quality control assessments. In-process control analyses are usually focused on the attributes of the water within the system. Quality control is primarily concerned with the attributes of the water delivered by the system to its various uses. The latter usually employs some sort of transfer device, often a flexible hose, to bridge the gap between the distribution system use-point valve and the actual location of water use. The issue of sample collection location and sampling procedure is often hotly debated because of the typically mixed use of the data generated from the samples, for both in-process control and quality control. In these single sample and mixed data use situations, the worst-case scenario should be utilized. In other words, samples should be collected from use points using the same delivery devices, such as hoses, and procedures, such as preliminary hose or outlet flushing, as are employed by production from those use points. Where use points per se cannot be sampled, such as hard-piped connections to equipment, special sampling ports may be used. In all cases, the sample must represent as closely as possible the quality of the water used in production. If a point of use filter is employed, sampling of the water prior to and after the filter is needed because the filter will mask the microbial control achieved by the normal operating procedures of the system.

Samples containing chemical sanitizing agents require neutralization prior to microbiological analysis. Samples for microbiological analysis should be tested immediately, or suitably refrigerated to preserve the original microbial attributes until analysis can begin. Samples of flowing water are only indicative of the concentration of planktonic (free floating) microorganisms present in the system. Biofilm microorganisms (those attached to water system surfaces) are usually present in greater numbers and are the source of the planktonic population recovered from grab samples. Microorganisms in biofilms represent a continuous source of contamination and are difficult to directly sample and quantify. Consequently, the planktonic population is usually used as an indicator of system contamination levels and is the basis for system Alert and Action Levels. The consistent appearance of elevated planktonic levels is usually an indication of advanced biofilm development in need of remedial control. System control and sanitization are key in controlling biofilm formation and the consequent planktonic population.

Sampling for chemical analyses is also done for in-process control and for quality control purposes. However, unlike microbial analyses, chemical analyses can be and often are performed using on-line instrumentation. Such on-line testing has unequivocal in-process control purposes because it is not performed on the water delivered from the system. However, unlike microbial attributes, chemical attributes are usually not significantly degraded by hoses. Therefore, through verification testing, it may be possible to show that the chemical attributes detected by the on-line instrumentation (in-process testing) are equivalent to those detected at the ends of the use point hoses (quality control testing). This again creates a single sample and mixed data use scenario. It is far better to operate the instrumentation in a continuous mode, generating large volumes of in-process data, but only using a defined small sampling of that data for QC purposes. Examples of acceptable approaches include using highest values for a given period, highest time-weighted average for a given period (from fixed or rolling sub-periods), or values at a fixed daily time. Each approach has advantages and disadvantages relative to calculation complexity and reflection of continuous quality, so the user must decide which approach is most suitable or justifiable.

#### CHEMICAL CONSIDERATIONS

The chemical attributes of Purified Water and Water for Injection were specified by a series of chemistry tests for various specific and nonspecific attributes with the intent of detecting chemical species indicative of incomplete or inadequate purification. While these methods could have been considered barely adequate to control the quality of these waters, they nevertheless stood the test of time. This was partly because the operation of water systems was, and still is, based on on-line conductivity measurements and specifications generally thought to preclude the failure of these archaic chemistry attribute tests.

USP moved away from these chemical attribute tests to contemporary analytical technologies for the bulk waters Purified Water and Water for Injection. The intent was to upgrade the analytical technologies without tightening the quality requirements. The two contemporary analytical technologies employed were TOC and conductivity. The TOC test replaced the test for Oxidizable substances that primarily targeted organic contaminants. A multistaged Conductivity test which detects ionic (mostly inorganic) contaminants replaced, with the exception of the test for Heavy metals, all of the inorganic chemical tests (i.e., Ammonia, Calcium, Carbon dioxide, Chloride, Sulfate).

Replacing the heavy metals attribute was considered unnecessary because (a) the source water specifications (found in the NPDWR) for individual Heavy metals were tighter than the approximate limit of detection of the Heavy metals test for USP XXII Water for Injection and Purified Water (approximately 0.1 ppm), (b) contemporary water system construction materials do not leach heavy metal contaminants, and (c) test results for this attribute have uniformly been negative—there has not been a confirmed occurrence of a singular test failure (failure of only the Heavy metals test with all other attributes passing) since the current heavy metal drinking water standards have been in place. Nevertheless, since the presence of heavy metals in Purified Water or Water for Injection could have dire consequences, its absence should at least be documented during new water system commissioning and validation or through prior test results records.

Total solids and pH are the only tests not covered by conductivity testing. The test for Total solids was considered redundant because the nonselective tests of conductivity and TOC could detect most chemical species other than silica, which could remain undetected in its colloidal form. Colloidal silica in Purified Water and Water for Injection is easily removed by



silica water pretreatment steps and even if present in the water, constitutes no medical or functional hazard except under extreme and rare situations. In such extreme situations, other attribute extremes are also likely to be detected. It is, however, the user's responsibility to ensure fitness for use. If silica is a significant component in the source water, and the purification unit operations could be operated or fail and selectively allow silica to be released into the finished water (in the absence of co-contaminants detectable by conductivity), then either silica-specific or a total solids type testing should be utilized to monitor and control this rare problem.

The pH attribute was eventually recognized to be redundant to the conductivity test (which included pH as an aspect of the test and specification); therefore, pH was dropped as a separate attribute test.

The rationale used by USP to establish its conductivity specification took into consideration the conductivity contributed by the two least conductive former attributes of Chloride and Ammonia, thereby precluding their failure had those wet chemistry tests been performed. In essence, the Stage 3 conductivity specifications (see [Water Conductivity \(645\)](#)) were established from the sum of the conductivities of the limit concentrations of chloride ions (from pH 5.0 to 6.2) and ammonia ions (from pH 6.3 to 7.0), plus the unavoidable contribution of other conductivity-contributing ions from water ( $H^+$  and  $OH^-$ ), dissolved atmospheric  $CO_2$  (as  $HCO_3^-$ ), and an electro-balancing quantity of either  $Na^+$  or  $Cl^-$ , depending on the pH-induced ionic imbalance (see [Table 1](#)). The Stage 2 conductivity specification is the lowest value on this table, 2.1  $\mu S/cm$ . The Stage 1 specifications, designed primarily for on-line measurements, were derived essentially by summing the lowest values in the contributing ion columns for each of a series of tables similar to [Table 1](#), created for each 5° increment between 0° and 100°. For example purposes, the italicized values in [Table 1](#), the conductivity data table for 25°, were summed to yield a conservative value of 1.3  $\mu S/cm$ , the Stage 1 specification for a nontemperature compensated, nonatmosphere equilibrated water sample that actual had a measured temperature of 25° to 29°. Each 5° increment table was similarly treated to yield the individual values listed in the table of Stage 1 specifications (see [Water Conductivity \(645\)](#)).

Table 1. Contributing Ion Conductivities of the Chloride–Ammonia Model as a Function of pH (in atmosphere-equilibrated water at 25°)

pH	Conductivity ( $\mu S/cm$ )							Stage 3 Limit
	$H^+$	$OH^-$	$HCO_3^-$	$Cl^-$	$Na^+$	$NH_4^+$	Combined Conductivities	
5.0	3.49	0	0.02	1.01	0.19	0	4.71	4.7
5.1	2.77	0	0.02	1.01	0.29	0	4.09	4.1
5.2	2.20	0	0.03	1.01	0.38	0	3.62	3.6
5.3	1.75	0	0.04	1.01	0.46	0	3.26	3.3
5.4	1.39	0	0.05	1.01	0.52	0	2.97	3.0
5.5	1.10	0	0.06	1.01	0.58	0	2.75	2.8
5.6	0.88	0	0.08	1.01	0.63	0	2.60	2.6
5.7	0.70	0	0.10	1.01	0.68	0	2.49	2.5
5.8	0.55	0	0.12	1.01	0.73	0	2.41	2.4
5.9	0.44	0	0.16	1.01	0.78	0	2.39	2.4
6.0	0.35	0	0.20	1.01	0.84	0	2.40	2.4
6.1	0.28	0	0.25	1.01	0.90	0	2.44	2.4
6.2	0.22	0	0.31	1.01	0.99	0	2.53	2.5
6.3	0.18	0	0.39	0.63	0	1.22	2.42	2.4
6.4	0.14	0.01	0.49	0.45	0	1.22	2.31	2.3
6.5	0.11	0.01	0.62	0.22	0	1.22	2.18	2.2
6.6	0.09	0.01	0.78	0	0.04	1.22	2.14	2.1
6.7	0.07	0.01	0.99	0	0.27	1.22	2.56	2.6
6.8	0.06	0.01	1.24	0	0.56	1.22	3.09	3.1
6.9	0.04	0.02	1.56	0	0.93	1.22	3.77	3.8
7.0	0.03	0.02	1.97	0	1.39	1.22	4.63	4.6

As stated above, this rather radical change to utilizing a conductivity attribute as well as the inclusion of a TOC attribute allowed for on-line measurements. This was a major philosophical change and allowed major savings to be realized by industry. The TOC and conductivity tests can also be performed "off-line" in the laboratories using collected samples, though sample collection tends to introduce opportunities for adventitious contamination that can cause false high readings. The collection of on-line data is not, however, without challenges. The continuous readings tend to create voluminous amounts of data where before only a single data point was available. As stated under Sampling Considerations, continuous in-process data is excellent for understanding how a water system performs during all of its various usage and maintenance events in real time, but is too much data for QC purposes. Therefore, a justifiable fraction or averaging of the data can be used that is still representative of the overall water quality being used.

Packaged waters present a particular dilemma relative to the attributes of conductivity and TOC. The package itself is the source of chemicals (inorganics and organics) that leach over time into the water and can easily be detected. The irony of organic leaching from plastic packaging is that when the Oxidizable substances test was the only "organic contaminant" test for both bulk and packaged waters, that test's insensitivity to those organic leachables rendered their presence in packaged water at high concentrations (many times the TOC specification for bulk water) virtually undetectable. Similarly, glass containers can also leach inorganics, such as sodium, which are easily detected by conductivity, but are undetected by the wet chemistry tests for water (other than pH or Total solids). Most of these leachables are considered harmless by current perceptions and standards at the rather significant concentrations present. Nevertheless, they effectively degrade the quality of the high-purity waters placed into these packaging system. Some packaging materials contain more leachables than others and may not be as suitable for holding water and maintaining its purity.

The attributes of conductivity and TOC tend to reveal more about the packaging leachables than they do about the water's original purity. These "allowed" leachables could render the packaged versions of originally equivalent bulk water essentially unsuitable for many uses where the bulk waters are perfectly adequate.

#### MICROBIAL CONSIDERATIONS

The major exogenous source of microbial contamination of bulk pharmaceutical water is source or feed water. Feed water quality must, at a minimum, meet the quality attributes of Drinking Water for which the level of coliforms are regulated. A wide variety of other microorganisms, chiefly Gram-negative bacteria, may be present in the incoming water. These microorganisms may compromise subsequent purification steps. Examples of other potential exogenous sources of microbial contamination include unprotected vents, faulty air filters, ruptured rupture disks, backflow from contaminated outlets, unsanitized distribution system "openings" including routine component replacements, inspections, repairs, and expansions, inadequate drain and air-breaks, and replacement activated carbon, deionizer resins, and regenerant chemicals. In these situations, the exogenous contaminants may not be normal aquatic bacteria but rather microorganisms of soil or even human origin. The detection of nonaquatic microorganisms may be an indication of a system component failure, which should trigger investigations that will remediate their source. Sufficient care should be given to system design and maintenance in order to minimize microbial contamination from these exogenous sources.

Unit operations can be a major source of endogenous microbial contamination. Microorganisms present in feed water may adsorb to carbon bed, deionizer resins, filter membranes, and other unit operation surfaces and initiate the formation of a biofilm. In a high-purity water system, biofilm is an adaptive response by certain microorganisms to survive in this low nutrient environment. Downstream colonization can occur when microorganisms are shed from existing biofilm-colonized surfaces and carried to other areas of the water system.



Microorganisms may also attach to suspended particles such as carbon bed fines or fractured resin particles. When the microorganisms become planktonic, they serve as a source of contamination to subsequent purification equipment (compromising its functionality) and to distribution systems.

Another source of endogenous microbial contamination is the distribution system itself. Microorganisms can colonize pipe surfaces, rough welds, badly aligned flanges, valves, and unidentified dead legs, where they proliferate, forming a biofilm. The smoothness and composition of the surface may affect the rate of initial microbial adsorption, but once adsorbed, biofilm development, unless otherwise inhibited by sanitizing conditions, will occur regardless of the surface. Once formed, the biofilm becomes a continuous source of microbial contamination.

#### ENDOTOXIN CONSIDERATIONS

Endotoxins are lipopolysaccharides found in and shed from the cell envelope that is external to the cell wall of Gram-negative bacteria. Gram-negative bacteria that form biofilms can become a source of endotoxins in pharmaceutical waters. Endotoxins may occur as clusters of lipopolysaccharide molecules associated with living microorganisms, fragments of dead microorganisms or the polysaccharide slime surrounding biofilm bacteria, or as free molecules. The free form of endotoxins may be released from cell surfaces of the bacteria that colonize the water system, or from the feed water that may enter the water system. Because of the multiplicity of endotoxin sources in a water system, endotoxin quantitation in a water system is not a good indicator of the level of biofilm abundance within a water system.

Endotoxin levels may be minimized by controlling the introduction of free endotoxins and microorganisms in the feed water and minimizing microbial proliferation in the system. This may be accomplished through the normal exclusion or removal action afforded by various unit operations within the treatment system as well as through system sanitization. Other control methods include the use of ultrafilters or charge-modified filters, either in-line or at the point of use. The presence of endotoxins may be monitored as described in the general

test chapter [Bacterial Endotoxins Test \(85\)](#).

#### MICROBIAL ENUMERATION CONSIDERATIONS

The objective of a water system microbiological monitoring program is to provide sufficient information to control and assess the microbiological quality of the water produced. Product quality requirements should dictate water quality specifications. An appropriate level of control may be maintained by using data trending techniques and, if necessary, limiting specific contraindicated microorganisms. Consequently, it may not be necessary to detect all of the microorganisms species present in a given sample. The monitoring program and methodology should indicate adverse trends and detect microorganisms that are potentially harmful to the finished product, process, or consumer. Final selection of method variables should be based on the individual requirements of the system being monitored.

It should be recognized that there is no single method that is capable of detecting all of the potential microbial contaminants of a water system. The methods used for microbial monitoring should be capable of isolating the numbers and types of organisms that have been deemed significant relative to in-process system control and product impact for each individual system. Several criteria should be considered when selecting a method to monitor the microbial content of a pharmaceutical water system. These include method sensitivity, range of organisms types or species recovered, sample processing throughput, incubation period, cost, and methodological complexity. An alternative consideration to the use of the classical "culture" approaches is a sophisticated instrumental or rapid test method that may yield more timely results. However, care must be exercised in selecting such an alternative approach to ensure that it has both sensitivity and correlation to classical culture approaches, which are generally considered the accepted standards for microbial enumeration.

Consideration should also be given to the timeliness of microbial enumeration testing after sample collection. The number of detectable planktonic bacteria in a sample collected in a scrupulously clean sample container will usually drop as time passes. The planktonic bacteria within the sample will tend to either die or to irretrievably adsorb to the container walls reducing the number of viable planktonic bacteria that can be withdrawn from the sample for testing. The opposite effect can also occur if the sample container is not scrupulously clean and contains a low concentration of some microbial nutrient that could promote microbial growth within the sample container. Because the number of recoverable bacteria in a sample can change positively or negatively over time after sample collection, it is best to test the samples as soon as possible after being collected. If it is not possible to test the sample within about 2 hours of collection, the sample should be held at refrigerated temperatures ( $2^{\circ}$  to  $8^{\circ}$ ) for a maximum of about 12 hours to maintain the microbial attributes until analysis. In situations where even this is not possible (such as when using off-site contract laboratories), testing of these refrigerated samples should be performed within 48 hours after sample collection. In the delayed testing scenario, the recovered microbial levels may not be the same as would have been recovered had the testing been performed shortly after sample collection. Therefore, studies should be performed to determine the existence and acceptability of potential microbial enumeration aberrations caused by protracted testing delays.

#### The Classical Culture Approach

Classical culture approaches for microbial testing of water include but are not limited to pour plates, spread plates, membrane filtration, and most probable number (MPN) tests. These methods are generally easy to perform, are less expensive, and provide excellent sample processing throughput. Method sensitivity can be increased via the use of larger sample sizes. This strategy is used in the membrane filtration method. Culture approaches are further defined by the type of medium used in combination with the incubation temperature and duration. This combination should be selected according to the monitoring needs presented by a specific water system as well as its ability to recover the microorganisms of interest: those that could have a detrimental effect on the product or process uses as well as those that reflect the microbial control status of the system.

There are two basic forms of media available for traditional microbiological analysis: "high nutrient" and "low nutrient". High-nutrient media such as plate count agar (TGYA) and m-HPC agar (formerly m-SPC agar), are intended as general media for the isolation and enumeration of heterotrophic or "copiotrophic" bacteria. Low-nutrient media such as R2A agar and NWRI agar (HPCA), may be beneficial for isolating slow growing "oligotrophic" bacteria and bacteria that require lower levels of nutrients to grow optimally. Often some facultative oligotrophic bacteria are able to grow on high nutrient media and some facultative copiotrophic bacteria are able to grow on low-nutrient media, but this overlap is not complete. Low-nutrient and high-nutrient cultural approaches may be concurrently used, especially during the validation of a water system, as well as periodically thereafter. This concurrent testing could determine if any additional numbers or types of bacteria can be preferentially recovered by one of the approaches. If so, the impact of these additional isolates on system control and the end uses of the water could be assessed. Also, the efficacy of system controls and sanitization on these additional isolates could be assessed.

Duration and temperature of incubation are also critical aspects of a microbiological test method. Classical methodologies using high nutrient media are typically incubated at  $30^{\circ}$  to  $35^{\circ}$  for 48 to 72 hours. Because of the flora in certain water systems, incubation at lower temperatures (e.g.,  $20^{\circ}$  to  $25^{\circ}$ ) for longer periods (e.g., 5 to 7 days) can recover higher microbial counts when compared to classical methods. Low-nutrient media are designed for these lower temperature and longer incubation conditions (sometimes as long as 14 days to maximize recovery of very slow growing oligotrophs or sanitant injured microorganisms), but even high-nutrient media can sometimes increase their recovery with these longer and cooler incubation conditions. Whether or not a particular system needs to be monitored using high- or low-nutrient media with higher or lower incubation temperatures or shorter or longer incubation times should be determined during or prior to system validation and periodically reassessed as the microbial flora of a new water system gradually establish a steady state relative to its routine maintenance and sanitization procedures. The establishment of a "steady state" can take months or even years and can be perturbed by a change in use patterns, a change in routine and preventative maintenance or sanitization procedures, and frequencies, or any type of system intrusion, such as for component replacement, removal, or addition. The decision to use longer incubation periods should be made after balancing the need for timely information and the type of corrective actions required when an alert or action level is exceeded with the ability to recover the microorganisms of interest.

The advantages gained by incubating for longer times, namely recovery of injured microorganisms, slow growers, or more fastidious microorganisms, should be balanced against the need to have a timely investigation and to take corrective action, as well as the ability of these microorganisms to detrimentally affect products or processes. In no case, however, should incubation at  $30^{\circ}$  to  $35^{\circ}$  be less than 48 hours or less than 96 hours at  $20^{\circ}$  to  $25^{\circ}$ .

Normally, the microorganisms that can thrive in extreme environments are best cultivated in the laboratory using conditions simulating the extreme environments from which they were taken. Therefore, thermophilic bacteria might be able to exist in the extreme environment of hot pharmaceutical water systems, and if so, could only be recovered and cultivated in the laboratory if similar thermal conditions were provided. Thermophilic aquatic microorganisms do exist in nature, but they typically derive their energy for growth from harnessing the energy from sunlight, from oxidation/reduction reactions of elements such as sulfur or iron, or indirectly from other microorganisms that do derive their energy from these processes. Such chemical/nutritional conditions do not exist in high purity water systems, whether ambient or hot. Therefore, it is generally considered pointless to search for thermophiles from hot pharmaceutical water systems owing to their inability to grow there.

The microorganisms that inhabit hot systems tend to be found in much cooler locations within these systems, for example, within use-point heat exchangers or transfer hoses. If this



occurs, the kinds of microorganisms recovered are usually of the same types that might be expected from ambient water systems. Therefore, the mesophilic microbial cultivation conditions described later in this chapter are usually adequate for their recovery.

#### "Instrumental" Approaches

Examples of instrumental approaches include microscopic visual counting techniques (e.g., epifluorescence and immunofluorescence) and similar automated laser scanning approaches and radiometric, impedometric, and biochemically based methodologies. These methods all possess a variety of advantages and disadvantages. Advantages could be their precision and accuracy or their speed of test result availability as compared to the classical cultural approach. In general, instrument approaches often have a shorter lead time for obtaining results, which could facilitate timely system control. This advantage, however, is often counterbalanced by limited sample processing throughput due to extended sample collection time, costly and/or labor-intensive sample processing, or other instrument and sensitivity limitations.

Furthermore, instrumental approaches are typically destructive, precluding subsequent isolate manipulation for characterization purposes. Generally, some form of microbial isolate characterization, if not full identification, may be a required element of water system monitoring. Consequently, culturing approaches have traditionally been preferred over instrumental approaches because they offer a balance of desirable test attributes and post-test capabilities.

#### Suggested Methodologies

The following general methods were originally derived from Standard Methods for the Examination of Water and Wastewater, 17th Edition, American Public Health Association, Washington, DC 20005. Even though this publication has undergone several revisions since its first citation in this chapter, the methods are still considered appropriate for establishing trends in the number of colony-forming units observed in the routine microbiological monitoring of pharmaceutical waters. It is recognized, however, that other combinations of media and incubation time and temperature may occasionally or even consistently result in higher numbers of colony-forming units being observed and/or different species being recovered.

The extended incubation periods that are usually required by some of the alternative methods available offer disadvantages that may outweigh the advantages of the higher counts that may be obtained. The somewhat higher baseline counts that might be observed using alternate cultural conditions would not necessarily have greater utility in detecting an excursion or a trend. In addition, some alternate cultural conditions using low-nutrient media tend to lead to the development of microbial colonies that are much less differentiated in colonial appearance, an attribute that microbiologists rely on when selecting representative microbial types for further characterization. It is also ironical that the nature of some of the slow growers and the extended incubation times needed for their development into visible colonies may also lead to those colonies being largely nonviable, which limits their further characterization and precludes their subculture and identification.

Methodologies that can be suggested as generally satisfactory for monitoring pharmaceutical water systems are as follows. However, it must be noted that these are not referee methods nor are they necessarily optimal for recovering microorganisms from all water systems. The users should determine through experimentation with various approaches which methodologies are best for monitoring their water systems for in-process control and quality control purposes as well as for recovering any contraindicated species they may have specified.

Drinking Water:	pour plate method or membrane filtration method <sup>1</sup>
	Sample Volume—1.0 mL minimum <sup>2</sup> Growth Medium—Plate Count Agar <sup>3</sup> Incubation Time—48 to 72 hours minimum Incubation Temperature—30° to 35°
Purified Water:	pour plate or membrane filtration method <sup>4</sup>
	Sample Volume—1.0 mL minimum <sup>2</sup> Growth Medium—Plate Count Agar <sup>3</sup> Incubation Time—48 to 72 hours minimum Incubation Temperature—30° to 35°

Water for Injection:	membrane filtration method <sup>1</sup>
	Sample Volume—100 mL minimum <sup>2</sup> Growth Medium—Plate Count Agar <sup>3</sup> Incubation Time—48 to 72 hours minimum Incubation Temperature—30° C to 35° C

<sup>1</sup> A membrane filter with a rating of 0.45 µm is generally considered preferable even though the cellular width of some of the bacteria in the sample may be narrower than this. The efficiency of the filtration process still allows the retention of a very high percentage of these smaller cells and is adequate for this application. Filters with smaller ratings may be used if desired, but for a variety of reasons the ability of the retained cells to develop into visible colonies may be compromised, so count accuracy must be verified by a reference approach.

<sup>2</sup> When colony counts are low to undetectable using the indicated minimum sample volume, it is generally recognized that a larger sample volume should be tested in order to gain better assurance that the resulting colony count is more statistically representative. The sample volume to consider testing is dependent on the user's need to know (which is related to the established alert and action levels and the water system's microbial control capabilities) and the statistical reliability of the resulting colony count. In order to test a larger sample volume, it may be necessary to change testing techniques, e.g., changing from a pour plate to a membrane filtration approach. Nevertheless, in a very low to nil count scenario, a maximum sample volume of around 250 to 300 mL is usually considered a reasonable balance of sample collecting and processing ease and increased statistical reliability. However, when sample volumes larger than about 2 mL are needed, they can only be processed using the membrane filtration method.

<sup>3</sup> Also known as Standard Methods Agar, Standard Methods Plate Count Agar, or TGYA, this medium contains tryptone (pancreatic digest of casein), glucose and yeast extract.

#### IDENTIFICATION OF MICROORGANISMS

Identifying the isolates recovered from water monitoring methods may be important in instances where specific waterborne microorganisms may be detrimental to the products or processes in which the water is used. Microorganism information such as this may also be useful when identifying the source of microbial contamination in a product or process. Often a limited group of microorganisms is routinely recovered from a water system. After repeated recovery and characterization, an experienced microbiologist may become proficient at their identification based on only a few recognizable traits such as colonial morphology and staining characteristics. This may allow for a reduction in the number of identifications to representative colony types, or, with proper analyst qualification, may even allow testing short cuts to be taken for these microbial identifications.

#### ALERT AND ACTION LEVELS AND SPECIFICATIONS

Though the use of alert and action levels is most often associated with microbial data, they can be associated with any attribute. In pharmaceutical water systems, almost every quality attribute, other than microbial quality, can be very rapidly determined with near-real time results. These short-delay data can give immediate system performance feedback, serving as ongoing process control indicators. However, because some attributes may not continuously be monitored or have a long delay in data availability (like microbial monitoring data), properly established Alert and Action Levels can serve as an early warning or indication of a potentially approaching quality shift occurring between or at the next periodic monitoring. In a validated water system, process controls should yield relatively constant and more than adequate values for these monitored attributes such that their Alert and Action Levels are infrequently broached.

As process control indicators, alert and action levels are designed to allow remedial action to occur that will prevent a system from deviating completely out of control and producing water unfit for its intended use. This "intended use" minimum quality is sometimes referred to as a "specification" or "limit". In the opening paragraphs of this chapter, rationale was presented for no microbial specifications being included within the body of the bulk water (Purified Water and Water for Injection) monographs. This does not mean that the user should not have microbial specifications for these waters. To the contrary, in most situations such specifications should be established by the user. The microbial specification should reflect



maximum microbial level at which the water is still fit for use without compromising the quality needs of the process or product where the water is used. Because water from a given system may have many uses, the most stringent of these uses should be used to establish this specification.

Where appropriate, a microbial specification could be qualitative as well as quantitative. In other words, the number of total microorganisms may be as important as the number of a specific microorganism or even the absence of a specific microorganism. Microorganisms that are known to be problematic could include opportunistic or overt pathogens, nonpathogenic indicators of potentially undetected pathogens, or microorganisms known to compromise a process or product, such as by being resistant to a preservative or able to proliferate in or degrade a product. These microorganisms comprise an often ill-defined group referred to as "objectionable microorganisms". Because objectionable is a term relative to the water's use, the list of microorganisms in such a group should be tailored to those species with the potential to be present and problematic. Their negative impact is most often demonstrated when they are present in high numbers, but depending on the species, an allowable level may exist, below which they may not be considered objectionable.

As stated above, alert and action levels for a given process control attribute are used to help maintain system control and avoid exceeding the pass/fail specification for that attribute. Alert and action levels may be both quantitative and qualitative. They may involve levels of total microbial counts or recoveries of specific microorganisms. Alert levels are events or levels that, when they occur or are exceeded, indicate that a process may have drifted from its normal operating condition. Alert level excursions constitute a warning and do not necessarily require a corrective action. However, alert level excursions usually lead to the alerting of personnel involved in water system operation as well as QA. Alert level excursions may also lead to additional monitoring with more intense scrutiny of resulting and neighboring data as well as other process indicators. Action levels are events or higher levels that, when they occur or are exceeded, indicate that a process is probably drifting from its normal operating range. Examples of kinds of action level "events" include exceeding alert levels repeatedly; or in multiple simultaneous locations, a single occurrence of exceeding a higher microbial level; or the individual or repeated recovery of specific objectionable microorganisms. Exceeding an action level should lead to immediate notification of both QA and personnel involved in water system operations so that corrective actions can immediately be taken to bring the process back into its normal operating range. Such remedial actions should also include efforts to understand and eliminate or at least reduce the incidence of a future occurrence. A root cause investigation may be necessary to devise an effective preventative action strategy. Depending on the nature of the action level excursion, it may also be necessary to evaluate its impact on the water uses during that time. Impact evaluations may include delineation of affected batches and additional or more extensive product testing. It may also involve experimental product challenges.

Alert and action levels should be derived from an evaluation of historic monitoring data called a trend analysis. Other guidelines on approaches that may be used, ranging from "inspectional" to statistical evaluation of the historical data have been published. The ultimate goal is to understand the normal variability of the data during what is considered a typical operational period. Then, trigger points or levels can be established that will signal when future data may be approaching (alert level) or exceeding (action level) the boundaries of that "normal variability". Such alert and action levels are based on the control capability of the system as it was being maintained and controlled during that historic period of typical control.

In new water systems where there is very limited or no historic data from which to derive data trends, it is common to simply establish initial alert and action levels based on a combination of equipment design capabilities but below the process and product specifications where water is used. It is also common, especially for ambient water systems, to microbiologically "mature" over the first year of use. By the end of this period, a relatively steady state microbial population (microorganism types and levels) will have been allowed or promoted to develop as a result of the collective effects of routine system maintenance and operation, including the frequency of unit operation rebeddings, backwashings, regenerations, and sanitizations. This microbial population will typically be higher than was seen when the water system was new, so it should be expected that the data trends (and the resulting alert and action levels) will increase over this "maturation" period and eventually level off.

A water system should be designed so that performance-based alert and action levels are well below water specifications. With poorly designed or maintained water systems, the system owner may find that initial new system microbial levels were acceptable for the water uses and specifications, but the mature levels are not. This is a serious situation, which if not correctable with more frequent system maintenance and sanitization, may require expensive water system renovation or even replacement. Therefore, it cannot be overemphasized that water systems should be designed for ease of microbial control, so that when monitored against alert and action levels, and maintained accordingly, the water continuously meets all applicable specifications.

An action level should not be established at a level equivalent to the specification. This leaves no room for remedial system maintenance that could avoid a specification excursion. Exceeding a specification is a far more serious event than an action level excursion. A specification excursion may trigger an extensive finished product impact investigation, substantial remedial actions within the water system that may include a complete shutdown, and possibly even product rejection.

Another scenario to be avoided is the establishment of an arbitrarily high and usually nonperformance based action level. Such unrealistic action levels deprive users of meaningful indicator values that could trigger remedial system maintenance. Unrealistically high action levels allow systems to grow well out of control before action is taken, when their intent should be to catch a system imbalance before it goes wildly out of control.

Because alert and action levels should be based on actual system performance, and the system performance data are generated by a given test method, it follows that those alert and action levels should be valid only for test results generated by the same test method. It is invalid to apply alert and action level criteria to test results generated by a different test method. The two test methods may not equivalently recover microorganisms from the same water samples. Similarly invalid is the use of trend data to derive alert and action levels for one water system, but applying those alert and action levels to a different water system. Alert and action levels are water system and test method specific.

Nevertheless, there are certain maximum microbial levels above which action levels should never be established. Water systems with these levels should unarguably be considered out of control. Using the microbial enumeration methodologies suggested above, generally considered maximum action levels are 100 cfu per mL for Purified Water and 10 cfu per 100 mL for Water for Injection. However, if a given water system controls microorganisms much more tightly than these levels, appropriate alert and action levels should be established from these tighter control levels so that they can truly indicate when water systems may be starting to trend out of control. These in-process microbial control parameters should be established well below the user-defined microbial specifications that delineate the water's fitness for use.

Special consideration is needed for establishing maximum microbial action levels for Drinking Water because the water is often delivered to the facility in a condition over which the user has little control. High microbial levels in Drinking Water may be indicative of a municipal water system upset, broken water main, or inadequate disinfection, and therefore, potential contamination with objectionable microorganisms. Using the suggested microbial enumeration methodology, a reasonable maximum action level for Drinking Water is 500 cfu per mL. Considering the potential concern for objectionable microorganisms raised by such high microbial levels in the feedwater, informing the municipality of the problem so they may begin corrective actions should be an immediate first step. In-house remedial actions may or may not also be needed, but could include performing additional coliform testing on the incoming water and pretreating the water with either additional chlorination or UV light irradiation or filtration or a combination of approaches.

#### Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Gary E. Ritchie, M.Sc.</a> Scientific Fellow 1-301-816-8353	(PW05) Pharmaceutical Waters 05

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#### 1241 WATER—SOLID INTERACTIONS IN PHARMACEUTICAL SYSTEMS

Pharmaceutical solids as raw materials or as dosage forms most often come in contact with water during processing and storage. This may occur (1) during crystallization, lyophilization, wet granulation, or spray drying, and (2) because of exposure upon handling and storage to an atmosphere containing water vapor or exposure to other materials in a dosage form that contains water capable of distributing to other ingredients. Some properties known to be altered by the association of solids with water include rates of chemical degradation in the "solid-state," crystal growth and dissolution, dispersibility and wetting, powder flow, lubricity, powder compatibility, and compact hardness.

Water can associate with solids in two ways. It can interact only at the surface (adsorption), and it can penetrate the bulk solid structure (absorption). When both adsorption and absorption occur, the term sorption is often used. Adsorption is particularly critical in affecting the properties of solids when the specific surface area is large. Large values of specific surface area are seen with solids having very small particles, as well as with solids having a high degree of intraparticle porosity.

Absorption is characterized by an association of water per g of solid that is much greater than that which can form a monomolecular layer on the available surface, and an amount that



Generally independent of the specific surface area. Most crystalline solids will not absorb water into their bulk structures because of the close packing and high degree of order of the crystal lattice. Indeed, it has been shown that the degree of absorption into solids exhibiting partial crystallinity and partial amorphous structure is often inversely proportional to the degree of crystallinity. With some crystalline solids, however, crystal hydrates may form. These hydrates may exhibit a stoichiometric relationship, in terms of water molecules bound per solid molecule, or they may be nonstoichiometric. Upon dehydration, crystal hydrates may (1) retain their original crystal structure, (2) lose their crystallinity and become amorphous, or (3) transform to a new anhydrous or less hydrated crystal form.

Amorphous or partially amorphous solids are capable of taking up significant amounts of water when there is sufficient molecular disorder in the solid to permit penetration and dissolution of the water molecule. Such behavior is observed with most amorphous polymers and with small-molecular-weight solids rendered amorphous during preparation, e.g., lyophilization, or after milling. The introduction of defects into highly crystalline solids will also produce this behavior. The greater the chemical affinity of water for the solid, the greater the total amount that can be absorbed. When water is absorbed by amorphous solids, the bulk properties of the solid can be significantly altered. It is well established, for example, that amorphous solids, depending on the temperature, can exist in at least one of two states, "glassy" or "fluid"; the temperature at which one state transforms into the other is the glass transition temperature,  $T_g$ . Water absorbed into the bulk solid structure, by virtue of its effect on the free volume of the solid, can act as an efficient plasticizer and reduce the value of  $T_g$ . Since the rheological properties of "fluid" and "glassy" states are quite different, i.e., the "fluid" state exhibits much less viscosity as one goes increasingly above the glass transition temperature, it is not surprising that a number of important bulk properties dependent on the rheology of the solid are affected by moisture content. Since amorphous solids are metastable relative to the crystalline form of the material, with small-molecular-weight materials, it is possible for absorbed moisture to initiate reversion of the solid to the crystalline form, particularly if the solid is transformed by the sorbed water to a fluid state. This is the basis of "cake collapse," often observed during the lyophilization process. An additional phenomenon noted specifically with water-soluble solids is their tendency to deliquesce, i.e., to dissolve in their own sorbed water, at relative humidities,  $RH_i$ , in excess of the relative humidity of a saturated solution of the solid,  $RH_o$ . Deliquescence arises because of the high water solubility of the solid and the significant effect it has on the colligative properties of water. It is a dynamic process that continues to occur as long as  $RH_i$  is greater than  $RH_o$ .

Although precautions can be taken when water is perceived to be a problem, i.e., eliminate all moisture, reduce contact with the atmosphere, or control the relative humidity of the atmosphere, such precautions generally add expense to the process with no guarantee that during the life of the product further problems associated with moisture will be avoided. It is also important to recognize that in many situations a certain level of water in a solid is required for proper performance, e.g., powder compaction. It is essential for both reasons, therefore, that as much as possible be known about the effects of moisture on solids before strategies are developed for their handling, storage, and use. Some of the more critical pieces of required information concerning water-solid interactions are (1) total amount of water present, (2) the extent to which adsorption and absorption occur, (3) whether or not crystal hydrates form, (4) specific surface area of the solid, as well as such properties as degree of crystallinity, degree of porosity, and glass transition and melting temperatures, (5) site of water interaction, the extent of binding, and the degree of molecular mobility, (6) effects of temperature and relative humidity, (7) various factors that might influence the rate at which water vapor can be taken up by a solid, and (8) for water-soluble solids capable of being solubilized by the sorbed water, under what conditions dissolution will take place.

**Determination of Sorption-Desorption Isotherms**— The tendency to take up water vapor is best assessed by measuring sorption or desorption as a function of relative humidity, at constant temperature, and under conditions where sorption or desorption is essentially occurring independently of time, i.e., equilibrium. Relative humidity,  $RH$ , is defined as:

$$RH = (P_c) / (P_o) \times 100$$

where  $P_c$  is the pressure of water vapor in the system and  $P_o$  is the vapor pressure of pure water under the same conditions. The ratio  $P_c / P_o$  is referred to as the relative pressure. It is usually varied by the use of saturated salt solutions in a closed system. Sorption or water uptake is best assessed starting with dried samples and subjecting them to a known relative humidity. Desorption is studied by beginning with a system already containing sorbed water and reducing the relative humidity. Ordinarily, if we are at equilibrium, moisture content at a particular relative humidity should be the same, whether determined from sorption or desorption measurements. However, it is common to see sorption-desorption hysteresis for certain types of systems, particularly those with microporous solids and amorphous solids, both capable of sorbing large amounts of water vapor. Here, the amount of water associated with the solid as relative humidity is decreased is greater than the amount that originally sorbed as the relative humidity is increased.

For microporous solids, vapor adsorption-desorption hysteresis is an equilibrium phenomenon associated with the process of capillary condensation. This takes place because of the high degree of irregular curvature of the micropores and the fact that they "fill" (adsorption) and "empty" (desorption) under different equilibrium conditions. For nonporous solids capable of absorbing water, hysteresis occurs because of a change in the degree of vapor-solid interaction due to a change in the equilibrium state of the solid, e.g., conformation of polymer chains, or because the time scale for structural equilibrium is longer than the time scale for water desorption.

In measuring sorption-desorption isotherms, it is important to establish that indeed something close to an equilibrium state has been reached. Particularly with hydrophilic polymers at high relative humidities, the establishment of water sorption or desorption values independent of time is quite difficult, since one is usually dealing with a polymer plasticized into its "fluid" state, in which the solid is undergoing significant change. Storing samples in chambers at various relative humidities and removing them to measure weight gained or lost is most commonly carried out. The major advantage of this method is convenience; the major disadvantages are the slow rate of reaching constant weight, particularly at high relative humidities, and the error introduced in opening and closing the chamber for weighing. Studies under vacuum in a closed system, using an electrobalance to measure weight change, avoid these problems but reduce the number of samples that can be concurrently run. It is also possible to measure amounts of water uptake not detectable gravimetrically by using volumetric techniques. In adsorption, to improve sensitivity, one can increase the specific surface area of the sample by reducing particle size or by using larger samples to increase the total area. It is important, however, that such comminution of the solid not alter the surface structure of the solid or render it more amorphous or otherwise less ordered in crystallinity. For absorption, where water uptake is independent of specific surface area, only increasing sample size will help. Increasing sample size, however, will increase the time to establish some type of equilibrium. To establish accurate values, it is important to dry the sample as thoroughly as possible. Higher temperatures and lower pressures (vacuum) facilitate this process; however, one must be aware of any adverse effects this might have on the solid, such as chemical degradation or sublimation. Using higher temperatures to induce desorption, as in a thermogravimetric apparatus, likewise must be carefully carried out with these possible pitfalls in mind. In some cases, direct analysis of water content by methods such as Karl Fischer titration or inverse gas chromatography may be advantageous. Sorption is usually expressed as weight of water taken up per unit weight of solid and plotted versus relative humidity. In most cases, the shape of the curve obtained resembles that normally seen for gas adsorption fitted to the Langmuir or Brunauer, Emmett, and Teller equations. Since crystal hydrate formation involving a phase change is usually a distinct first-order phase transition, the plot of water uptake versus pressure or relative humidity will in these cases exhibit a sharp increase in uptake at a particular pressure, and the amount of water taken up will usually exhibit a stoichiometric mole: mole ratio of water to solid. In some cases, however, crystal hydrates will not appear to undergo a phase change or the anhydrous form will appear amorphous. Consequently, water sorption or desorption may appear more like that seen with adsorption processes. X-ray crystallographic analysis and thermal analysis are particularly useful for the study of such systems. For situations where water vapor adsorption occurs predominantly, it is helpful to measure the specific surface area of the solid by an independent method and to express adsorption as weight of water sorbed per unit area of solid surface. This can be useful in assessing the possible importance of water sorption in affecting solid properties. For example, 0.5% w/w uptake of water could hardly cover the bare surface of 100 m<sup>2</sup>/g, while for 1.0 m<sup>2</sup>/g this amounts to 100 times more surface coverage. Since we generally find that pharmaceutical solids are in the range of 0.01 to 10 m<sup>2</sup>/g in specific surface area, what appears to be a low water content could represent a significant amount of water for the surface available.

Since the "dry surface area" is not a factor in absorption, sorption of water with amorphous or partially amorphous solids is best expressed on the basis of unit mass corrected for crystallinity when the crystal form does not sorb significant amounts of water relative to the amorphous regions.

**Rates of Water Uptake**— The rate at which solids exposed to the atmosphere might either sorb or desorb water vapor can be a critical factor in the handling of solids. Even the simple act of weighing out samples of solid on an analytical balance and the exposure, therefore, of a thin layer of powder to the atmosphere for a few minutes can lead to significant error in, for example, the estimation of loss on drying values. It is well established that water-soluble solids exposed to relative humidities above that exhibited by a saturated solution of that solid will spontaneously dissolve via deliquescence and continue to dissolve over a long time period. The rate of water uptake in general depends on a number of parameters not found to be critical in equilibrium measurements because rates of sorption are primarily mass-transfer controlled, with some contributions from heat-transfer mechanisms. Thus, factors such as vapor diffusion coefficients in air and in the solid, convective airflow, and the surface area and geometry of the solid bed and surrounding environment, can play an important role.

Indeed, the method used to take such measurements can often be the rate-determining factor because of these environmental and geometric factors.

**Physical States of Sorbed Water**— The key to understanding the effects water can have on the properties of solids, and vice versa, rests with an understanding of the location of the water molecule and its physical state. More specifically, water associated with solids can exist in a highly immobile state, as well as in a state of mobility approaching that of bulk water. This difference in mobility has been observed through such measurements as heats of sorption, freezing point, nuclear magnetic resonance, dielectric properties, and diffusion. Such



Changes in mobility have been interpreted as arising because of changes in the thermodynamic state of water as more and more water is sorbed. Thus, water bound directly to a solid is often thought of as "tightly" bound and unavailable to affect the properties of the solid, whereas larger amounts of sorbed water tend to become more clustered and form water more like that exhibiting solvent properties. In the case of crystal hydrates, the combination of intermolecular forces (hydrogen bonding) and crystal packing can produce very strong water-solid interactions. However, there are reported situations where hydration and dehydration of crystals occur quite easily at low temperatures. More recently, the concept of "tightly" bound water in amorphous systems has been questioned. Recognizing that the presence of water in an amorphous solid can affect the glass transition temperature and hence the physical state of the solid, it is argued that at low levels of water, most polar amorphous solids are in a highly viscous glassy state because of their high values of  $T_g$ . Hence, water is "frozen" into the solid structure and is rendered immobile by the high viscosity, e.g., 1014 poise. As the amount of water sorbed increases and  $T_g$  decreases and approaches ambient temperatures, the glassy state approaches that of a "fluid" state, and water mobility along with the solid itself increases significantly. At high RH, the degree of water plasticization of the solid can be sufficiently high that water and the solid now can assume significant amounts of mobility. In general, therefore, this picture of the nature of sorbed water helps to explain the rather significant effect moisture can have on a number of bulk properties of solids such as chemical reactivity and mechanical deformation. It suggests strongly that methods of evaluating chemical and physical stability of solids and solid dosage forms should take into account the effects water can have on the solid when it is sorbed, particularly when it enters the solid structure and acts as a plasticizer. Much research still remains to be done in assessing the underlying mechanisms involved in water-solid interactions of pharmaceutical importance.

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Gary E. Ritchie, M.Sc.</a> Scientific Fellow 1-301-816-8353	(PW05) Pharmaceutical Waters 05

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### 1251 WEIGHING ON AN ANALYTICAL BALANCE

Weighing is a frequent step in analytical procedures, and the balance is an essential piece of laboratory equipment in most analyses. In spite of this, weighing is a common source of error that can be difficult to detect in the final analytical results. The procedure described here applies directly to electronic balances; therefore, certain portions of the procedure are not applicable to other types of balance. The weighing procedure can be separated into three basic steps: planning, checking the balance, and weighing the material.

#### PLANNING

The initial step is to assemble the proper equipment, such as containers for weighing, receiving vessels, forceps, pipets, spatulas of proper size, and so forth. Use containers of size such that the loading capacity of the balance is not exceeded. Make sure that the containers selected to receive the weighed material are clean and dry. Assemble the necessary chemicals if solutions or reagents are required.

Preparation of the material to be weighed is often necessary. The material may require grinding or drying. Some materials may have been heated or stored in a refrigerator. Materials must be brought to the temperature of the balance before they are weighed. To avoid condensation of moisture, refrigerated materials must be allowed to come to room temperature before the container is opened.

#### CHECKING THE BALANCE

In the next step it is important to remember that, unless the balance is checked before each weighing operation is performed, errors can easily occur, resulting in faulty analytical data. The balance user should check the Balance Environment, Calibration, and Balance Uncertainties. Do not assume that the balance has been left in the proper operating condition by the previous user.

##### Balance Environment

The balance is placed in a suitable location with sufficiently low levels of vibration and air current. It must have a constant electrical supply. The balance and the surrounding work area have to be kept neat and tidy. It is good practice to use a camel's hair brush or its equivalent to dust the balance pan before any weighing so as to remove any materials that may have been left by the previous operator. [note—Individuals must clean up debris, dispose of any spilled materials or paper, and remove the vessels and apparatus used in making the measurements.] When a balance is moved, it must be allowed to adjust to the temperature of its new environment and be recalibrated.

##### Calibration

If necessary, turn on the power, and allow the balance to equilibrate for at least 1 hour before proceeding with the calibration. (Microbalances may require up to 24 hours to reach equilibrium.) If the balance power has gone off and then has come back on, as in a power outage, certain types of balance may display a message indicating that the balance must be calibrated before a weighing is made. If the operator touches the balance bar, the message may be cleared and the balance may display zeros; however, the balance will not give the correct weighing until it has been calibrated. Electronic analytical balances have an internal calibration system based on an applied load. The calibration applies for the current ambient temperature.

##### Balance Uncertainties

###### drift reduction

Drift is one of the most common errors, and it is also one of the easiest to reduce or eliminate. Balance drift can be present without the operators being aware of the problem. Check the sample, the balance, and the laboratory environment for the following causes of errors, and eliminate them:

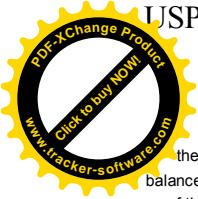
1. A balance door is open.
2. Temperatures of the balance and the material to be weighed are not the same.
3. The sample is losing or gaining weight.
4. The balance has been recently moved but has not been allowed to equilibrate to its surroundings or has not been recalibrated.
5. Air currents are present in the laboratory.
6. Temperatures in the laboratory vary.
7. The balance is not properly leveled.
8. Laboratory operations are causing vibration.
9. Hysteresis of the mechanical parts occurs during weighing.

##### mechanical hysteresis

Hysteresis in the balance is caused by excessive stretching of the springs, and it is primarily due to overloading or to the accidental dropping of an object onto the pan. Microbalances are very sensitive to overload and shock. When using a microbalance, set the lever to the rest position when adding or removing material; turn the lever to the weigh position to register the weight. In some cases, drift due to hysteresis can be eliminated by allowing the balance to stand without weighing long enough for it to recover. If stretching of the springs is excessive, an expensive balance overhaul may be needed. In the case of electronic force restoration balances, springs are replaced by flexures, and the term creep is more appropriate than hysteresis.

##### quality assurance procedure for measurement of balance drift

Over an extended period of time, balance drift and other day-to-day variations are monitored by weighing a fixed check-weight on a regular basis; this check should be performed after



the balance has been calibrated at the ambient laboratory temperature. The check should be made before the first weighing of the day or after any event that might disturb the balance's calibration (power failure, moving the balance to a new location, etc.). The check-weight may be any object whose mass remains constant and does not exceed the load limit of the balance. A balance weight makes a reliable check-weight. Each balance should be provided with a check-weight, which should be stored in a protective container near the balance.

Perform the following procedures to reduce balance errors and the possibility of an incorrect reading because of drift:

1. Make certain that the electrical power to the balance is on and that the level bubble is in the center of the indicator.
2. Calibrate the analytical balance or the microbalance. [note—Some balances have a calibration lever, which must be returned fully to its original weighing position. Do not depend upon any prior calibration.]
3. The first person to use the balance each day should weigh the check-weight and record the weight in the log book for comparison with previous readings. If a deviation greater than those indicated below for Analytical Balances and Microbalances is observed, the balance should be reported for service. [note—Check-weights tend to gain weight upon standing because of mishandling and exposure to contaminants in the atmosphere. These weights can be cleaned by wiping with a lint-free cloth moistened with a small amount of an appropriate solvent such as diethyl ether.]

**Analytical Balances**— Select a check-weight of an appropriate mass to examine an analytical balance. If possible, set the balance to read to 5 decimal places. Follow the manufacturer's operating instructions. Pick up the check-weight with a forceps, place it carefully on the balance pan, and weigh it. [note—Do not drop the weight on the balance pan, because damage to the balance could result.] Place the weight in the center of the pan to eliminate corner-weighing differences. The accuracy of the weight is not important: the only factor of interest is whether any drift has occurred. If no drift has taken place, the value should remain constant. Periodic weighing of a fixed weight will determine whether the boards (or knife edges in mechanical balances) in the instrument are defective. The check for drift at the most sensitive position will show whether a problem exists; the variation in the observed weight does not exceed  $\pm 0.2$  mg. For example, with a 20-g weight, if the mean value of the readings were 19.9984, the tolerance would be from 19.9982 to 19.9986 g. Thus, several readings must be taken before one can establish a tolerance. [note—The check-weight need not be of high accuracy, but it is essential that its mass remain constant. In addition, the tolerance does not correspond to the value of 0.1%, specified under [Weights and Balances](#), for weighing material accurately. Rather, the tolerance is purposefully tight to reveal possible drift or calibration errors; this tolerance is readily achievable with modern electronic balances.]

**Microbalances**— Proceed as directed for Analytical Balances, but use a check-weight appropriate for the particular balance. For example, a 100-mg check-weight might be selected for a balance that has a load limit of 150 mg; or a 10-mg check-weight might be used for an ultramicrobalance with a load limit of 15 mg. (The operator must know the maximum capacity of the balance to select the correct check-weight.) The balance indicates the weight in milligrams. Record the weight as soon as the reading is stable for a few seconds. The variation in weighings ought to be within a range commensurate with the specifications given by the balance manufacturer, but not greater than 0.1% of the amount of material typically weighed on the particular balance. For example, if 10-mg samples are routinely weighed, the variation in the weighings of the check-weight cannot exceed 0.01 mg.

#### WEIGHING THE MATERIAL

In this final step, select the number of decimal places required for the analytical procedure. In most pharmaceutical analyses small quantities of material are used, requiring the balance reading to be set to the fifth decimal place to achieve the necessary accuracy. Weighing read with four decimal places is preferred for weighing near-gram quantities. Do not allow the material to remain on the balance for an extended period of time because changes, caused by interaction with atmospheric water or carbon dioxide, may take place.

##### Load Limit

Select the appropriate balance for the quantity and accuracy needed. Each balance has a load limit, which should not be exceeded. Each balance manufacturer supplies the maximum loading condition, and this limit varies with the type of balance. The operator should know this limit so that the balance will not be damaged. [note—Electronic balances operate on a "load cell" principle that produces an electrical output proportional to the movement of the strain gauge and is linear over the range.]

##### Receivers

The proper receiver for the material must be selected. The receiver's weight plus the weight to be measured must not exceed the maximum load for the balance; the size and shape of the receiver should permit it to fit into the space and on the balance pan without interfering with any operation. It is important that the receiver be clean and dry. Common receivers are weighing bottles, weighing funnels, flasks, and weighing paper. The correct receiver depends upon the quantity and type of material (liquid, solid, or powder) to be weighed. All other things being equal, a vessel of low mass should be chosen when small amounts of material are to be weighed. It is recommended that gloves, forceps, or another type of gripping device be used when handling receivers, because oils from the hands will add weight.

The weighing funnel is often the most satisfactory receiver, because it can function as both a weighing dish and a transfer funnel, allowing easy transfer to volumetric flasks. Weighing funnels come in various sizes; the size suitable for the operation should be selected.

Weighing paper may be used for solids. Paper receivers must be handled by hand, and great care must be used to prevent spills.

##### Weighing by Difference

Weighing is usually done by difference. The following methods are acceptable for good analytical results.

###### method 1

Tare the empty receiver as follows. Place the receiver on the balance in the center of the pan, and press the appropriate tare key on the balance. This operation electrically sets the signal from the strain gauge to zero so that the weight of the receiver is no longer indicated. Add the material to the receiver, and record the weight. Transfer the weighed material to the final flask or receiver; then reweigh the original weighing receiver by placing it in the same position on the pan. [note—Do not change the set tare of the balance between these two weighings.] The second weight represents the untransferred material and is subtracted from the total material weight to determine the weight of the transferred material.

###### method 2

If the empty receiver is not going to be tared, add the material to the receiver, and place the receiver on the balance in the center of the pan. Record the weight, and transfer the weighed material to the final flask or receiver; then reweigh the original weighing receiver by returning it to the same position on the pan. The second weight represents the sum of the weights of the receiver and the untransferred material; subtract this sum from the sum of the total material weight and the receiver weight to determine the weight of the transferred material.

###### method 3

This method may be described as quantitative transfer. The material is added to the tared receiver, the amount is determined by difference, and then the whole amount is transferred quantitatively (e.g., by using a solvent) to the final receiver.

##### Materials-Handling Safety Procedures

The operator must be familiar with precautions described in the Material Safety Data Sheet for the substance before weighing it. Hazardous materials must be handled in an enclosure having appropriate air filtration. Many substances are extremely toxic, are possibly allergenic, and may be liquids or finely divided particles. A mask that covers the nose and mouth should be used to prevent any inhalation of chemical dust. Gloves should be used to prevent any contact with the skin. [note—The use of gloves is good practice for handling any chemical. If it is necessary to handle the container being weighed, the operator should put on gloves, not only for self-protection but also to prevent moisture and oils from being deposited on the weighed container.] During a weighing, the operator may be exposed to high concentrations of the pure substance; therefore, the operator must carefully consider these possibilities at all times.

Weighings are made on many different types of materials, such as large solids, finely divided powders, and liquids (viscous or nonviscous, volatile or nonvolatile). Each type of material requires its own special handling.



### Weighing Solids

Solids come in two forms: large chunks, with or without powdery surface, and finely divided powders or small crystals. If large chunks with a powdery surface are to be weighed, at least a piece of weighing paper must be placed on the balance pan to protect it from damage. Large nonreactive chunks that have no powdery surface may be placed directly on the pan (for example, a coated tablet). [note—Solid pieces must be handled with forceps, never by hand.]

#### static charge

Fine powders have a tendency to pick up static charge, which will cause the particles to fly around. This static charge must be eliminated before a suitable weighing can be made. An antistatic device may be used to minimize this problem. [note—Such devices may use piezoelectric components or a very small amount of a radioactive element (typically polonium) to generate a stream of ions that dissipate the static charge when passed over the powder to be weighed.] The static charge depends upon the relative humidity of the laboratory, which in turn depends upon the atmospheric conditions. In certain conditions, static charge is caused by the type of clothing worn by the operator; this charge causes large errors in the weighing when discharged.

#### weighing procedure

Place the receiver on the balance pan, close the balance door, and weigh as indicated for Weighing by Difference, with the following additions. Carefully add the powdered material from a spatula until the desired amount is added. Use care to avoid spilling. Close the balance door, and record the weight as soon as the balance shows a stable reading.

#### spills

If solids are spilled, remove the receiver, and sweep out all of the spilled material from the balance. The spilled material must be properly disposed of and must not be swept out onto the balance table where other operators may come in contact with the chemical. Then either start the process over or reweigh the remaining material. [note—Never return any excess material to the original container. Any excess material must be disposed of in a proper manner.]

### Weighing Liquids

Liquids may be volatile or nonvolatile and viscous or nonviscous. Each type requires special attention.

#### weighing procedure

Weigh as directed for Weighing by Difference, with the following additions. Liquids should always be weighed into a container that can be closed so that none of the material is lost. It is best if the liquid can be added to its receiving container outside the balance because of the possibility of a spill. [note—Liquids spilled within the balance housing can cause serious damage to the balance, and they may be difficult to remove.]

Nonviscous liquids can be handled with a Pasteur capillary pipet equipped with a small rubber bulb such as a medicine dropper bulb. The liquid is discharged into its receiver, the top is closed or stoppered, and the receiver and contents are weighed. Small quantities of viscous liquids can be handled by touching a glass stirring rod to the surface of the liquid and then carefully touching the rod to the side of the receiving vessel, which allows some of the material to be transferred.

#### Weighing Corrosive Materials

Many chemicals, such as salts, are corrosive, and materials of this nature should not be spilled on the balance pan or inside the balance housing. Extreme care is essential when materials of this nature are being weighed.

### CONCLUSION

By carefully following the procedures outlined above, laboratory personnel will eliminate many errors that might be introduced into weighing procedures. However, it is important for each balance to be serviced and calibrated regularly by a specially trained internal or external service person. The balance should be tested using weights traceable to standardization by the National Institute of Standards and Technology. No repairs should be made to any balance by anyone other than a qualified maintenance person.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Horacio N. Pappa, Ph.D.</a> Senior Scientist and Latin American Liaison 1-301-816-8319	(GC05) General Chapters 05

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Pharmacopeial Forum: Volume No. 34(3) Page 798

### 1265 WRITTEN PRESCRIPTION DRUG INFORMATION—GUIDELINES

The purpose of these guidelines—comprising format, content, and accessibility of prescription drug leaflets—is to help ensure that leaflets are useful. In this context, “useful” means that recipients receive, understand, and are motivated to apply written information about their medicines to achieve maximum benefit and minimize harm. Dispensers, prescribers, health care providers who counsel patients about their medicines, and the patients themselves are intended to be the primary beneficiaries for these guidelines.

#### CRITERIA (from the Keystone Action Plan<sup>1</sup>)

Written prescription medicine information should be based on the following criteria:

1. Scientifically accurate,
2. Unbiased in content and tone,
3. Sufficiently specific and comprehensive,
4. Presented in an understandable and legible format that is readily comprehensible to consumers,
5. Timely and up-to-date, and
6. Useful.

### FORMAT GUIDELINES

1. Group all information from the same category, using brief, clear titles and bullets or subheadings as needed. Avoid symbols and subheadings not directly connected to the information they mark.
2. Be consistent in the placement and labeling of categories of information in all leaflets.
3. Provide information at the sixth-grade reading level or below, if possible (never above eighth-grade level). Do not exclude information to achieve a lower reading level.
4. Use simple, common, accurate terms (for example, use “noise in the ears”, not “tinnitus”).
5. Use direct language that avoids words with opposite meanings (for example, use “decrease blood pressure”, not “increase low blood pressure effect”).
6. Provide reasons for instructions (for example, “take with food to avoid upset stomach”).
7. Emphasize the most important information. Clearly distinguish warnings from instructions or from other text that may be misinterpreted as warnings.
8. Accompany each pictogram, if used, with corresponding text placed close to the pictogram. Use the simplest pictograms possible. For pictograms intended to prompt patients to ask questions or inform health care providers, add text such as “Tell Doctor” or “Ask Pharmacist”.



9. Make text readable by using 12-point or larger type, both uppercase and lowercase letters, an easy-to-read font (for example, a serif font), and adequate space between lines and paragraphs. To call attention to important information, use a larger, boldface type.
10. Evaluate format by performing tests of readability, comprehension, memory, problem solving, and behavioral efficacy and intention, using representative samples of the target population.

#### CONTENT GUIDELINES

1. Provide enough detail to facilitate correct use, achieve maximum benefit, and minimize harm, including a statement that identifies activities (such as driving or sunbathing) that the patient should avoid.
2. Write text that is unbiased in content and tone and scientifically accurate. The uses described should be consistent with FDA-approved labeling or otherwise permitted by FDA, or should appear in federally recognized drug compendia. Distinguish unlabeled from labeled use.
3. For drugs sold under a brand name, provide both brand and generic names, and include a pronunciation guide for each.
4. Describe the drug and its dosage form. Include indications and contraindications, specific directions for use, what to do if a dose is missed, and what to do in the event of an overdose or poisoning.
5. Do not use abbreviations.
6. Indicate the intended type of benefit (for example, "cure", "prevention", "to help relieve symptoms"). Indicate how—and how soon—the patient should recognize the benefit and what to do if none is observed.
7. Give a balanced evaluation of risks and benefits.
8. List side effects, in order of severity, such as "serious", "most common", and other similar type groupings. It may not be appropriate to provide sufficient detail for the patient to be able to monitor serious or common side effects. Provide guidance to consult the doctor or pharmacist, and indicate that not all the side effects are listed.
9. List sufficiently specific and comprehensive information that includes the provision of all important risk information. Patients should be advised to be sure to inform the provider about all the medicines they are taking.
10. Indicate the potential for therapeutic duplication if the drug is available under multiple names or over-the-counter, or if the active ingredient is contained in other products.
11. If known, include a statement concerning the safety of use in the presence of other conditions and during pregnancy or breast-feeding. Direct affected patients to discuss their condition with health care providers. If the safety of use during pregnancy or breast-feeding has not been established, say so.
12. State whether safety and efficacy have been established in pediatric, geriatric, and other special populations. Patients should be encouraged to discuss with their health care provider any recommendations for dosage adjustment.
13. Illustrate information with diagrams when appropriate. Label the diagram components (for example, device parts) if they are not obvious. The words on the label should be prominently placed thereon with such conspicuousness and in such terms as to render them likely to be read and understood by the ordinary individual under customary conditions of purchase and use.
14. Include the following:
  - a. A statement that the product is to be used only by the person for whom it was prescribed,
  - b. Storage information,
  - c. A completeness disclaimer advising the patient to discuss this issue with the health care provider,
  - d. The publisher of the leaflet and the date the leaflet was developed or revised,
  - e. Sources of in-depth information and answers to questions, and
  - f. Other relevant general statements.
15. The patient should be advised about risks of developing dependence on, or tolerance to, the medication.

#### ACCESSIBILITY GUIDELINES

1. Write text that is relevant to the intended use of the drug.
2. Design the leaflets to be easy to recognize, consistent in format, and easy to store and retrieve.
3. Supplement the leaflets with oral counseling of patients, including children, the elderly, and caregivers.
4. Include a statement asking the patient to reread the leaflet.
5. Distribute the leaflets with all prescription medicines to consumers (namely, persons independently responsible for any aspect of medicine use or for giving medicines to others).
6. Produce leaflets in Spanish, English, or other languages; and establish criteria for producing them in other languages and for special populations (for example, children, visually handicapped) [note—Ideally, prescription drug information leaflets would be customized for the patient's condition and for other relevant information (for example, gender, age, or physical limitations), and would be available in the patient's primary language. Currently, such customization is neither feasible nor practical, but it remains a goal.]

1 In December 1996, the "Action Plan for the Provision of Useful Prescription Medicine Information" was presented to the Secretary of Health and Human Services. The plan, commonly known as the "Keystone Plan," described certain criteria for written prescription medicine information. These criteria are described in detail in the action plan, which can be found at [www.fda.gov/cder/offices/ods/keystone.pdf](http://www.fda.gov/cder/offices/ods/keystone.pdf).

Auxiliary Information—Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Desmond G. Hunt, Ph.D.</a> Scientist 1-301-816-8341	(PS05) Packaging and Storage 05