Add the following:

▲⟨1503⟩ QUALITY ATTRIBUTES OF SYNTHETIC PEPTIDE DRUG SUBSTANCES

INTRODUCTION

The pharmaceutical industry's interest in peptides as drug candidates—based on their potency, enormous functional diversity, high degree of specificity, and general lack of toxicity—has existed since their general structure was first elucidated more than 100 years ago. The early development of synthetic peptides for therapeutic use was, however, challenged by the high costs of production, their inherently short in-vivo half-lives, the lack of sensitive and discriminating analytical methods for intermediates, and the relatively complex manufacturing methods, which are now referred to as "solution peptide synthesis" or "liquid-phase peptide synthesis" (LPPS).

The introduction of solid-phase peptide synthesis (SPPS) was a key factor in facilitating the expansion of peptide research and made the production of peptides with sequences longer than 20 amino acids feasible. Also, in recent years, new formulations and conjugation strategies have resulted in alternative routes of administration and longer half-lives for peptide therapeutics, which have facilitated patient compliance. As a result, more peptide drugs have been approved and are in clinical development. In spite of these successes—or perhaps because of them—some challenges in peptide manufacturing remain, especially with the increasingly complex molecules entering development.

Because synthetic peptides are not easily classified as small molecules or biological products, they are specifically excluded from many guidance documents. Hence, this chapter provides an overview of the current status and the quality attributes of synthetic therapeutic peptides, with particular emphasis on drug substance impurities and related contributing factors, including 1) manufacturing methods, 2) raw materials, 3) peptide general characteristics and specifications, 4) assay and peptide content, 5) impurities, 6) microbiological contamination, 7) bacterial endotoxins, and 8) specific tests.

Drug product quality attributes and recombinant DNA technologies, which can also be employed for manufacturing peptides for pharmaceutical use, are not included in this chapter.

PEPTIDE DEFINITION

Peptides are short chains of two or more amino acids covalently linked by amide bonds. They can be derived from rDNA or from a biological source or made by chemical synthesis. In the absence of clear scientific consensus on the criteria that distinguish proteins from peptides, including the exact size at which a chain or chains of amino acids become a protein, the FDA, in its final rule on the *Definition of the Term "Biological Product"* (*Federal Register* 85: 10057; February 21, 2020), distinguishes proteins from peptides based on size (i.e., number of amino acids).

FDA regulations state that a "protein is any alpha amino acid polymer with a specific defined sequence that is greater than 40 amino acids in size. When two or more amino acid chains in an amino acid polymer are associated with each other in a manner that occurs in nature, the size of the amino acid polymer for purposes of this interpretation will be based on the total number of amino acids in those chains, and will not be limited to the number of amino acids in a contiguous sequence" (21 CFR §600.3(h)(6)). FDA considers any polymer composed of 40 or fewer amino acids to be a peptide. Unless a peptide meets the statutory definition of a "biological product" (e.g., a peptide vaccine), it would not be considered a "biological product", and it would be regulated as a drug product under the Federal Food, Drug, and Cosmetic Act (FD&C Act). The concepts and approaches described in this chapter generally are applicable to chemically synthesized peptides.

MANUFACTURING METHODS

A number of chemistries and methods have been developed for building peptide chains. The strategies selected are largely determined by the size and characteristics of the peptide needed. The classical approach to peptide synthesis, LPPS, is often

chosen for manufacturing small peptides; however, SPPS has become the preferred option for manufacturing peptides of larger sizes. A hybrid SPPS/LPPS technique can also be used for manufacturing large peptides and peptide conjugates.

Differences in the LPPS and SPPS processes are particularly important, as they lead to differences in the impurity profiles of the final drug substance. In LPPS, potential impurities may be predicted based on the reaction scheme and usually can be identified and controlled individually for each reaction step. Typically, peptide-related impurities are easier to control in LPPS because they are less similar to the target peptide and their accumulation can be minimized by intermediate purification.

In SPPS processes, the peptide chain is assembled on a solid support, enabling excess reagents and amino acid derivatives to be washed off the resin-bound peptide on a filter screen at each step. Hence, non-peptide process-related impurities usually are not a major concern. However, all peptide-related impurities that are incorporated into the resin-bound peptide accumulate throughout the coupling and deprotection steps, leading to a complex profile of impurities very similar to that of the target peptide.

Regardless of the synthetic strategy, manufacturing highly purified peptides under good manufacturing practices (GMP) is a complex process and requires robust control strategies. Quality should be built into every step of the process, and a quality-by-design (QbD) strategy is often beneficial for a successful outcome.

RAW MATERIALS

Starting material (SM) selection, impurity characterization, control strategies, supplier qualifications, and regulatory acceptance are integral to the strategy for the development of any synthetic peptide manufacturing process. The term "raw materials" is used to describe the SMs, solvents, and reagents that are used in the production of the peptide drug substance.

A "starting material" is a raw material, intermediate, or an active pharmaceutical ingredient that is used in the production of—and incorporated as a significant structural fragment into—the drug substance, as described in the following International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use guidelines: ICH Q7—*Good Manufacturing Practice Guide for Active Pharmaceutical Ingredients*; ICH Q11—*Development and Manufacture of Drug Substances (Chemical Entities and Biotechnological/Biological Entities)*; and the ICH Implementation Working Group's Q11 *Questions and Answers* document.

Typically, the most relevant quality attributes to be controlled for SMs are identity and purity, including related impurities and stereoisomeric impurities content, due to their impact on the quality of the drug substance. The determination of adequate acceptance criteria for the SM quality attributes is based on development data, validation of the manufacturing process, and the corresponding risk assessment. Historical batch data may also be used to set SM specifications. Guidance is provided for the management of SMs and their suppliers in ICH Q7 Sections 7 and 13; ICH Q10—*Pharmaceutical Quality System*, Section 2.7; ICH Q11; and ICH Q11—*Questions and Answers*. It is recommended that SMs be manufactured under a quality system that assures the consistency of batches and the ability to trace the operations carried out to reach the desired material. SMs coming from different qualified suppliers should comply with the overall control strategy for the drug substance. Thus, the selection of SMs should be based on knowledge of the intended commercial process and should take into account the relationship of the SM to the impurity profile of the drug substance.

The ICH Q11 guideline emphasizes that appropriate controls should be in place for the impurities present in the SMs, and that the impact of the impurities on the drug substance quality should be assessed. ICH Q11 also makes an important distinction between commercially available chemicals and custom synthesized chemicals. ICH Q11 states that while the use of a commercially available chemical as an SM generally does not need to be justified, chemicals produced by custom synthesis are not considered to be commercially available. Thus, their proposed use as an SM should be justified.

In some cases, amino acids, protected amino acid derivatives, or any other compound such as dipeptide derivatives may not have a pre-existing, non-pharmaceutical use to meet the ICH Q11 definition of commercially available chemicals; but provided they are simple enough in structure, they may be acceptable as SMs with the appropriate justification. Per ICH Q11—*Questions and Answers*, Section 5.6, a rationale should be provided explaining why the SM is considered appropriate and why the proposed strategy is suitable for controlling impurities in the drug substance. This usually includes, but is not limited to, justification of the proposed SM specifications, including the acceptance criteria for amino acid impurities and their enantiomers [and diastereomers for isoleucine (Ile) and threonine (Thr)], as well as data to support that the proposed control strategy is adequate for manufacturing a peptide drug substance of acceptable purity. Likewise, fragments proposed as SMs in the synthesis of peptides are considered custom synthesized chemicals. Therefore, manufacturers should consider all of ICH Q11 Section 5.1, *General Principles*, and ICH Q11—*Questions and Answers*, clarifications for the selection and justification of such fragments as SMs.

The origin of the SMs should be known in order to evaluate their potential risk of contamination with adventitious agents, including agents that cause transmissible spongiform encephalopathies (TSE). When possible, use of materials from "non-TSE-relevant animal species" or non-animal origin is preferred. If animal sources are necessary, efforts should be made to minimize the risk of transmission of TSE, including not obtaining materials from specific geographic regions.

The most common impurities found in the SMs used for the synthesis of peptides are listed in *Table 1*.

Table 1. Potential Impurities in Starting Materials

Impurities	Origin
Incorrect enantiomers (e.g., D-amino acid instead of L-amino acid or L-amino acid present in D-amino acid)	Poor chiral purity of the SM
Dipeptides in fluorenylmethyloxycarbonyl protecting group (Fmoc) amino acid derivative (e.g., Fmoc-Leu-Leu-OH [Leu=leucine] instead of Fmoc-Leu-OH)	Synthetic impurities
β-Alanine-containing impurities (e.g., Fmoc-β-Ala-OH [Ala=alanine] and Fmoc-β-Ala-AA-OH [AA=amino acid or amino acid derivative])	Generated during Fmoc-protection of the amino acid when using 9-fluorenylmethyl *N*-succinimidyl carbonate
Free amino acids (not amine protected); partially protected amino acids	Loss of N-terminal α-amino-protecting group or incomplete protection

Table 1. Potential Impurities in Starting Materials *(continued)*

Impurities	Origin
Isomeric contaminants (e.g., residual isoleucine [Ile] in Leu derivatives)	Poor SM purity
Residual acetic acid (e.g., ethyl acetate)	From residual solvent used during synthesis of amino acid derivatives

Solvents, reagents, and resins (with the exception of resins preloaded with the first amino acid or amino acid derivative) are not considered SMs, but the impact of their quality on the final drug substance quality should be evaluated through a formal risk assessment. For solvents and reagents, it is recommended to use the highest quality material available. The use of lower grade raw materials is appropriate if their use has been justified. High quality grade water (i.e., at least purified water) should also be used for the purification of peptides.

PEPTIDE GENERAL CHARACTERISTICS AND SPECIFICATIONS

The specification for a drug substance needs to reflect the critical quality attributes proposed and justified by the manufacturer. The drug substance specification also needs to be approved by the regulatory authorities, as recommended by ICH Q6A—*Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances* and by the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) guideline GL-39—*Specifications: Test Procedures and Acceptance Criteria for New Veterinary Drug Substances and New Medicinal Products: Chemical Substances*.

The process of setting a specification ensures that the quality of a drug substance is controlled through general characteristics, identity, assay, impurities determination, and specific tests. For reference, the *USP* general chapters that describe the test methods most frequently used for characterization and quality control of peptide drug substances are listed in *Table 2*.

Table 2. Summary of Tests for Characterization and Quality Control of the Drug Substance

Test	Method	Comments
Characteristic		
Appearance[a]	Visual inspection	—
Identification		
High-performance liquid chromatography (HPLC) coelution with reference standard[a]	⟨621⟩[b]	The method used for the identification of the active pharmaceutical ingredient (API) may be the same as the one used for the detection of related substances or for determination of the assay based on comparison with a reference standard
Mass spectrometry (MS)[a]	⟨736⟩[c]	Monoisotopic mass
Amino acid analysis (AAA)	⟨1052⟩[d] and ⟨507⟩[e]	Hydrolysis and derivatization protocols should be specified
Tandem mass spectrometry (MS–MS) sequencing	⟨736⟩[c]	May be complicated for longer sequences
Nuclear magnetic resonance (NMR) spectroscopy (i.e., ^{1}H, ^{13}C and ^{15}N)	⟨761⟩[f]	Complex interpretation for longer sequences
Peptide mapping by chemical or enzymatic cleavage methods	⟨1055⟩[g]	Used for longer sequences (e.g., >20 amino acids); equivalent to MS–MS
Enantiomeric purity	⟨621⟩[b] and ⟨736⟩[c]	AAA in combination with chiral chromatography and MS detection
N-Terminal sequence analysis	Edman degradation	Complicated analysis, especially for sequences that are N-terminally blocked; largely superseded by LC with MS–MS (LC–MS/MS)
Infrared spectroscopy	⟨197⟩[h] and ⟨854⟩[i]	Limited use for peptides
Higher order structures	Circular dichroism, NMR, and Fourier-transform infrared spectroscopy (FTIR)	Used for investigation of secondary and tertiary structures in aqueous solutions
Bioidentity	⟨1032⟩[j], ⟨1033⟩,[k] and ⟨1034⟩[l]	No longer a routine test for most peptides; may be required for large peptides or those with complex sequences
Assay		
Assay by HPLC[a]	⟨621⟩[b]	Method is based on a comparison with a reference standard and may be the same method used to measure related substances and for identification
Peptide Content		
Peptide content by AAA	⟨1052⟩[d] and ⟨507⟩[e]	Hydrolysis protocols must be validated; only well-recovered amino acids should be included in the calculation of mean peptide content

Table 2. Summary of Tests for Characterization and Quality Control of the Drug Substance *(continued)*

Test	Method	Comments
Peptide content by nitrogen analysis	⟨461⟩[m]	Direct analysis by elemental analysis, or Kjeldahl, or using HPLC with chemiluminescence nitrogen detector (CLND)
UV Spectroscopy	⟨197⟩,[h] ⟨857⟩,[n] and ⟨507⟩[e]	Only useful for drug substances containing amino acids with suitable chromophores
Quantitative NMR (qNMR)	⟨761⟩[f] and ⟨1761⟩[o]	A suitable internal standard is required
Impurities		
Peptide-related substances[a]	⟨621⟩[b], LC–MS	Method specific for drug substances; must be validated for both process-related impurities and degradation products; limits for total and individual impurities should be specified, LC–MS is a commonly used method for characterization
Residual solvents[a]	⟨467⟩[p]	If justified, may be limited to solvent used in the final steps of the manufacturing process
Elemental impurities[a]	⟨232⟩,[q] ⟨233⟩,[r] and ⟨1065⟩[s]	Required if metal catalysts are used in the manufacturing process; elemental impurities may be required based on the risk assessment
Residual trifluoroacetic acid[a]	⟨503.1⟩[t]	Required if TFA is used during the manufacturing process
Residual fluoride[a]	⟨1065⟩[s] or ion-selective electrodes	Required if hydrofluoric acid (HF) is used during the manufacturing process (e.g., due to *tert*-butyloxycarbonyl protecting group or *tert*-butoxycarbonyl protecting group [Boc]-chemistry)
Other small-molecule impurities	Impurity dependent	Non-peptide impurity limits are recommended to follow ICH Q3A(R2), *Impurities in New Drug Substances*; potentially mutagenic impurities require additional evaluation
Specific Tests		
Counter-ion content[a]	For acetate, ⟨503⟩[u], for others, ⟨1065⟩[s]	Titration with silver nitrate may be used to determine chloride
Water content[a]	⟨921⟩[v]	⟨921⟩, *Method I, Method Ic (Coulometric Titration)*[v] preferred
Optical rotation	⟨781⟩[w]	For characterization only
Thiol groups	Ellman's test	Only required if the reduced form(s) of a peptide containing disulfide bonds cannot be determined using the HPLC methods for related substances
Bioburden[a]	⟨61⟩[x] and ⟨62⟩[y]	Often required for the drug substances used in the manufacture of parenteral drug products
Bacterial endotoxins[a]	⟨85⟩[z]	Required for the drug substances used in the manufacture of parenteral drug products

[a] Recommended test to be performed as part of the release.
[b] *Chromatography* ⟨621⟩.
[c] *Mass Spectrometry* ⟨736⟩.
[d] *Biotechnology-Derived Articles—Amino Acid Analysis* ⟨1052⟩.
[e] *Protein Determination Procedures* ⟨507⟩.
[f] *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩.
[g] *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩.
[h] *Spectroscopic Identification Tests* ⟨197⟩.
[i] *Mid-Infrared Spectroscopy* ⟨854⟩.
[j] *Design and Development of Biological Assays* ⟨1032⟩.
[k] *Biological Assay Validation* ⟨1033⟩.
[l] *Analysis of Biological Assays* ⟨1034⟩.
[m] *Nitrogen Determination* ⟨461⟩.
[n] *Ultraviolet-Visible Spectroscopy* ⟨857⟩.
[o] *Applications of Nuclear Magnetic Resonance Spectroscopy* ⟨1761⟩.
[p] *Residual Solvents* ⟨467⟩.
[q] *Elemental Impurities—Limits* ⟨232⟩.
[r] *Elemental Impurities—Procedures* ⟨233⟩.
[s] *Ion Chromatography* ⟨1065⟩.
[t] *Trifluoroacetic Acid (TFA) in Peptides* ⟨503.1⟩.
[u] *Acetic Acid in Peptides* ⟨503⟩.
[v] *Water Determination* ⟨921⟩.
[w] *Optical Rotation* ⟨781⟩.
[x] *Microbial Enumeration Tests* ⟨61⟩.

[y] *Tests for Specified Microorganisms* ⟨62⟩.
[z] *Bacterial Endotoxins Test* ⟨85⟩.

Color and Appearance

The color and appearance test by visual inspection is included in the specification of a drug substance because it gives a qualitative description of the color and physical state. The test also ensures a consistent appearance from batch to batch. However, because the processes adopted by different manufacturers can result in subtle variations in the color and appearance, tests by visual inspection are not included in *USP* monographs. In some cases, the description is available in *Reagents and Reference Tables, Reference Tables, Description and Relative Solubility*.

Identification

Identification tests are specific for a peptide drug substance and, as stated by ICH Q6A, "should optimally be able to discriminate between compounds of closely related structure which are likely to be present". Identity is normally verified by multiple orthogonal techniques, generally including at least one specific test. The most commonly used identification tests for synthetic peptides are reverse-phase high-performance liquid chromatography (RP-HPLC) (see *Chromatography* ⟨621⟩) and mass spectrometry (MS) (see *Mass Spectrometry* ⟨736⟩). Other identification tests such as amino acid analysis (AAA), peptide mapping, or nuclear magnetic resonance (NMR) spectroscopy may be used.

HPLC identification tests rely on the comparison of the retention times of the peptide drug substance's main peak from a reference standard and the test sample, when injected separately, or by coelution of a reference standard and the test sample when co-injected as an equal mixture. Co-injection of an equal mixture of a reference standard and the sample is the recommended best practice. If the HPLC method is capable of distinguishing between the peptide and its closely related impurities, the same method can be used for the identification and impurity measurements.

The monoisotopic mass can be determined by MS, an orthogonal identification test. For peptides larger than 2 kDa, the use of high-resolution instruments may be necessary. In addition, tandem mass spectrometry (MS–MS) can be used for peptide mapping and sequencing to further characterize the synthetic peptide drug substance and to confirm the primary sequence of the peptide. However, peptide mapping and sequencing by MS–MS are normally not part of routine release testing of peptides.

Although AAA was previously a commonly used technique for identification, it has been gradually replaced by techniques such as HPLC and MS. Despite providing the amino acid composition of a peptide, AAA is unable to differentiate aspartic acid (Asp) from asparagine (Asn) and glutamic acid (Glu) from glutamine (Gln) or pyroglutamic acid (Pyr). However, AAA is still a useful tool when discrimination between leucine (Leu) and isoleucine (Ile) is required.

Infrared spectroscopy (IR) is of limited use for identification of a peptide drug substance due to the complexity of peptide molecules, but it is sometimes still used as an orthogonal identification test.

Bioassays

Bioassays are laboratory tests that mimic the drug substance's mechanism of action towards a therapeutic target. In the past, many of these methods used animal models. Today, depending on the complexity of the biologic response, cell-based, or ligand- or receptor-based binding methods are used instead. During drug discovery and development, bioassays are commonly used to discover new chemical or biologic entities that may be agonists or antagonists of a specific therapeutic target. In later stages, for small molecules and many simple, small peptides, bioassay methods are not used for release once the more precise physicochemical methods are validated and justified. ICH Q6A does not mention bioassay use.

For more complex or long peptides, regulatory authorities may require that a bioassay remain as a release test to measure potency or to confirm that the functional activity of the peptide drug is maintained throughout the manufacturing process. Bioassays may be needed because a very small chemical change may cause a loss of functional activity. A "bioidentity" test, sometimes found in *USP* monographs, is a subset of the bioassay class and is used as an orthogonal identity test and to confirm the functionality of a drug substance or drug product. In the case of peptide monographs that include an HPLC-based assay and a bioidentity test, the number of replicates may be reduced because the acceptance criterion is often a limit (e.g., "activity not less than").

ICH Q6B—*Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products*, which applies to proteins, allows bioassays to be replaced by physicochemical tests when a well-established manufacturing history exists and when "sufficient physicochemical information about the drug, including higher-order structure, can be thoroughly established by such physicochemical methods, and relevant correlation to biologic activity demonstrated".

Manufacturers are encouraged to contact regulators early in development for control strategy related to bioassays, e.g., the need for a bioassay, its purpose, etc. Additional guidance for development, validation, and analysis of bioassays can be found in *Biological Assay Chapters—Overview and Glossary* ⟨1030⟩, *Design and Development of Biological Assays* ⟨1032⟩, *Biological Assay Validation* ⟨1033⟩, and *Analysis of Biological Assays* ⟨1034⟩.

Assay and Peptide Content

The peptide drug substance assay is normally defined on an anhydrous, counter-ion free basis. Routine assay testing is normally performed by a specific chromatographic method. Assay by HPLC, based on the use of an established, quantitative standard, e.g., a pharmacopeial reference standard or an in-house developed peptide standard, is a relative method that is often the method of choice for peptides. If the HPLC method separates all peptide-related impurities, no correction for impurities is generally required. However, a correction for levels of additional impurities determined from a separate method may be applied. The specific method(s) used and the basis for the calculation should be defined and justified for each peptide drug substance. It is important to note that significant variability of assay results must be considered when establishing acceptable

ranges. The main causes for assay variability are difficulty of sample preparation due to the hygroscopicity of peptides and batch-to-batch variability of reference standards. Peptide content (sometimes referred to as net peptide content) is the percentage of total peptides (i.e., drug substance plus peptide-related impurities) relative to everything else present in the sample, such as water, counter ions, residual solvents, and salts. Peptide content is usually determined by an absolute method such as quantitative AAA or nitrogen content determination. Peptide content may be helpful in the following cases:

- Drug substance testing, if no quantitative reference standard is available
- Qualification of a quantitative reference standard
- Investigations of an assay out-of-specification result, especially of the reference standard used

Peptides normally contain residual water, a counter ion, peptide-related impurities, and residual solvents. While all of these components can be determined individually, there are tests that will determine combinations of them. While primary or absolute methods do not require a reference standard, relative methods are based on a comparison to a reference standard. The quantitative qualification of a primary reference standard requires absolute methods. There are several absolute methods available to determine the peptide content.

It is important to consider that most absolute methods are non-specific and will only determine the peptide content, not discriminating between the drug substance and peptide-related impurities. If a specific result for the peptide drug substance assay is needed to substitute for an HPLC assay result, the peptide content may be corrected for the total related impurities, which are usually determined by HPLC. Where relevant, other impurities, such as oligomers measured by size-exclusion chromatography (SEC) or peptide impurities measured by ion-exchange chromatography, should also be included in the correction.

The easiest approach to determine the net peptide content is a simple mass balance calculation that consists of deducting the percentage of water, of counter ion, and, if relevant, of total other non-peptide impurities from 100%.

Peptide content can also be determined by elemental nitrogen analysis, Kjeldahl analysis, or chromatographic methods with nitrogen chemiluminescence detection (NCD) (see *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩ and *Nitrogen Determination* ⟨461⟩).

Quantitative AAA (see *Biotechnology-Derived Articles—Amino Acid Analysis* ⟨1052⟩ or *Protein Determination Procedures* ⟨507⟩, *Procedure, Method V. Amino Acid Analysis*) has traditionally been used for the determination of peptide content; however, because the method involves a relatively complex sequence of operations, results may vary. Peptide content by quantitative AAA compares the peak areas of the amino acids in a hydrolyzed sample with the peak areas of the same amino acids in an external standard. The peptide hydrolysis procedure should be validated to ensure good recovery, and only well-recovered amino acids should be used in the calculation of mean peptide content. Some peptide sequences containing multiple, adjacent hydrophobic residues, such as isoleucine (Ile), phenylalanine (Phe), leucine (Leu), and valine (Val), or sterically hindered amino acids, such as valine (Val) and isoleucine (Ile), may give a lower recovery than expected.

Quantitative NMR (qNMR) is also a useful tool for peptide content determination, although it requires a suitable internal standard with signals that fully resolve from the signals associated with the peptide. The versatility of qNMR for quantitative analysis of peptides that do not contain an appropriate UV chromophore makes it well-suited for the determination of peptide concentration in aggregation studies.

UV at 280 nm is used during characterization to determine the content of peptides containing tryptophan (Trp) or tyrosine (Tyr). The molar absorption coefficient required for the measurements at UV 280 nm can be calculated theoretically based on knowledge of the sequence; however, the method should be independently validated using orthogonal techniques. Peptide content by UV absorption may be applied as a fast and simple method for frequent or on-line, in-process determination of peptide concentrations.

Impurities

A peptide impurity profile may differ significantly depending on the technology used for manufacturing. Hence, it is difficult to generalize the impurities and their control strategies. In addition, because the manufacturing technologies for the production of peptides are diverse and complex compared to small molecules, the impurity guidelines applicable to small molecules usually cannot be applied to peptides.

CLASSIFICATION

Impurities in Drug Substances and Drug Products ⟨1086⟩ classifies impurities into three categories: organic impurities, inorganic impurities, and residual solvents. However, from a practical perspective, impurities can be classified as either peptide related or non-peptide.

Peptide-related impurities: Peptide-related impurities, also referred to as "related substances" in this chapter, comprise all impurities that are related to the structure of the target molecule. These are by-products derived from SM impurities, side-reactions during synthesis, or degradations during storage. Thus, peptide-related impurities are either process related or drug related. Stereoisomers are also considered process-related impurities or degradation products. *Table 3* summarizes the categories and types of peptide-related impurities, their origin and/or relevance, and the recommended analytical techniques for their identification. The information provided focuses on the most-common examples and should not be considered exhaustive.

Non-peptide impurities: Non-peptide impurities are impurities that are introduced by the manufacturing process and are not related to the structure of the target molecule. These comprise residues of raw materials, reagents, and auxiliary materials such as catalysts and solvents. Such residues also include reaction by-products and contaminants carried over by these materials, as shown in *Table 4*. Peptide drug substances are often defined with counter ions such as acetate or chloride. These counter ions are not considered impurities but concomitant components according to *Ordinary Impurities* ⟨466⟩. Nevertheless, excess bases or acids should be limited.

IMPURITIES IN SYNTHETIC PEPTIDES

The purity of a peptide is usually determined by HPLC. The method should be capable of separating SM, process-related, and degradation impurities. When using multiple methods, orthogonal separation techniques are recommended. One or more analytical procedures may be required for comprehensive quantitation of all relevant peptide-related impurities. For peptides containing hydrophobic residues, RP-HPLC methods may be appropriate, although alternatives, such as ion-exchange or mixed-mode methods could be considered. For hydrophilic peptides, hydrophilic interaction chromatography stationary phases may achieve the necessary resolution. Anion- and cation-exchange chromatography may be also useful. Liquid chromatography–mass spectroscopy (LC–MS) compatible methods are also advantageous for the identification of impurities. At least one of the methods should be a stability-indicating method.

In order to set acceptance criteria for peptide-related impurities, it is helpful to further classify impurities as specified or unspecified. Specified impurities can be identified or nonidentified. Impurities are classified as identified when their structure has been elucidated. Impurities are classified as qualified when their limits have been justified by toxicological studies, clinical trials, or by a structure-based scientific rationale. If present in the drug substance, qualified identified and unidentified impurities are usually included in the final release specification as specified impurities. Non-qualified impurities should be limited by a general acceptance criterion for "any unspecified impurity". While ICH Q3A(R2), *Impurities in New Drug Substances*, limits each unspecified impurity to 0.10% or 0.05%, peptides are excluded from this guideline. The higher threshold (0.5% for identification) adopted by the *European Pharmacopoeia* (*Ph. Eur.*) is recognized by authorities of *Ph. Eur.* member states. Other authorities, e.g., the FDA may consider the limits for unspecified impurities for new peptides on a case-by-case basis.

In order to define appropriate targets for process and analytical development, regulators encourage case-specific meetings [e.g., pre-investigational new drug (pre-IND)]. As appropriate, the specification parameters should include each specified identified impurity, each specified unidentified impurity, any unspecified impurity, and the total impurities as the sum of all impurities.

Peptide-related impurities: A control strategy for peptide-related impurities, built on process characterization, should be implemented. The control strategy should account for the impurities introduced with the SMs and the impurities generated during the peptide synthesis and purification processes. Appropriately validated in-process control analytical procedures may be used to ensure the quality of the final drug substance, especially when an impurity cannot sufficiently be controlled by the release method.

The purity of a very complex peptide (i.e., very large or conjugated peptides) may be difficult to monitor due to broad signals and poor resolution in HPLC. The control strategy should then comprise the control of related substances for appropriate key intermediates, e.g., the peptide prior to conjugation. To determine possible peptide polymerization, a test for the presence of multimers is needed. A specific methodology such as SEC can be used to quantitate multimers.

HPLC with UV detection is often chosen for the determination of impurities. Because of the frequent similarities between the peptide-related impurities and the drug substance, and because of their potential coelution, smaller particle sizes allow for superior resolution of related compounds on C18, C8, and biphenyl columns. Incomplete separation may be observed with conjugated peptides or as the peptide chain length increases. UV detection is not specific for peptide-related impurities and cannot unequivocally identify these impurities by retention time and UV absorbance, or quantify impurities that coelute. Understanding the impurity profile is important to ensure drug quality and safety, such as any adverse events associated with immunogenicity. Hence, more sensitive and specific LC-HRMS method is recommended for characterizing peptide-related impurities.

Table 3. Peptide-Related Impurities: Origin and Commonly Used Analytical Techniques

Type	Description	Origin	Analytical Techniques
Deletion (clipped forms, fragments, truncations)	Loss of one or more amino acids	Synthesis (incomplete coupling or deprotection) or storage (hydrolysis of N- or C-terminal amino acids or fragments)	LC–MS or LC–MS/MS
Insertion	Presence of one or more additional amino acids	Raw materials (SMs containing the respective protected dipeptide) or synthesis (loss of the N-protecting group during coupling, presence of unprotected amino acids in the starting material)	LC–MS or LC–MS/MS
Substitution	Presence of one or more different amino acids	SM contaminants or insufficient washes during peptide-resin synthesis	HPLC/UHPLC spiking with synthesized analogs or isolation/AAA/LC–MS or LC–MS/MS
Truncation	Sequences containing N-terminal deletions	Raw materials (materials containing residual acetic acid), synthesis (steric hindrance, deliberate acetylation of incomplete coupling reactions), or storage	LC–MS or LC–MS/MS
Stereoisomers (diastereomers)	Sequences containing epimerized amino acids	SMs, synthesis, or degradation	HPLC/UHPLC spiking with synthesized diastereomeric analogs or chiral analysis

Table 3. Peptide-Related Impurities: Origin and Commonly Used Analytical Techniques *(continued)*

Type	Description	Origin	Analytical Techniques
Asp/Asn-related impurities	Aspartimide/succinimide-containing sequences	Cyclization to the backbone via loss of water or ammonia, respectively, from the sidechain of Asp or Asn	HPLC, LC–MS, LC–MS/MS
	β-Asp-containing sequences	Hydrolytic ring-opening of the aspartimide/succinimide intermediate during processing or storage	HPLC/UHPLC spiking with synthesized analogs or isolation
	Asp stereoisomers (diastereomers)	Epimerization of the aspartimide/succinimide intermediate followed by ring-opening during processing or storage	HPLC/UHPLC spiking with synthesized analogs or isolation
	Chain cleavage products	Total hydrolysis of the aspartimide/succinimide intermediate during processing or storage	HPLC/UHPLC spiking with synthesized analogs or isolation
β-Alanine insertion	Sequences containing β-alanine	SM contaminants	LC–MS or LC–MS/MS
Pyroglutamic acid	Sequence containing N-terminal glutamine or glutamic acid	Synthesis	HPLC or LC–MS
Oligomers	Polymers or aggregates	Conformational changes or peptide polymerization	SEC or SEC–multi-angle light scattering (SEC–MALS), sedimentation velocity analytical ultracentrifugation (SV–AUC), dynamic light scattering (DLS), field-flow fractionation (FFF), or ion mobility spectrometry (IMS)
Disulfide reduction	Sequences containing disulfides	Synthesis or storage	LC–MS or LC–MS/MS
Others	Deamidation of glutamine, asparagine, or C-terminus	Synthesis or storage	LC–MS or LC–MS/MS
	Acetylation of amino functional group	Synthesis or storage	LC–MS or LC–MS/MS
	Oxidation of certain residues, e.g., aromatic or sulfur-containing side-chains	Synthesis, storage, or analytical sample preparation	LC–MS or LC–MS/MS
	Alkylation, acylation of amino acids	Deprotection (cleavage)	LC–MS or LC–MS/MS

Stereoisomers: Non-routine determination of stereoisomeric purity is possible using chiral AAA. The method is based on acid hydrolysis of the peptide, suitable derivatization of the resulting amino acids, and determination of the optical isomers of the constituent amino acids, e.g., by chiral gas chromatography with mass spectrometry (GC–MS) detection. HPLC-based methods of chiral AAA involve pre- or post-column derivatization of amino acid hydrolysates, e.g., using Marfey's reagent or *o*-phthalaldehyde (OPA), respectively. The method should be validated to account for potential epimerization during hydrolysis or subsequent derivatization. The use of deuterated reagents, e.g., deuterium chloride (DCl) and deuterium oxide (D_2O), results in substitution of the α-carbon hydrogen with deuterium during the epimerization process, permitting correction for the epimerization during hydrolysis and derivatization when using MS-based detection methods. It is recommended that stereoisomeric purity data are established for at least three representative batches to demonstrate that the manufacturing process does not lead to unacceptable levels of diastereomers. If there are indications of significant epimerization, the respective diastereomers should be identified and routinely controlled.

Non-peptide impurities: Reagents used in the manufacturing process should be included in the drug substance specification if there is a risk of residues of such reagents in the drug substance. One example is trifluoroacetic acid (TFA) used in the cleavage and deprotection steps when fluorenylmethyloxycarbonyl protecting group (Fmoc) chemistry is utilized. The drug substance specification should include tests for the determination of residual solvents "likely to be present" in accordance with ICH Q3C(R7)—*Impurities: Guideline for Residual Solvents*; acceptance criteria are to be set in accordance with the same guideline.

Other examples of potential process residues include elemental impurities. As described in ICH Q3D(R1)—*Guideline for Elemental Impurities*, *Elemental Impurities—Limits* ⟨232⟩, and *Elemental Impurities—Procedures* ⟨233⟩, elemental impurities may arise from several sources: they may be residual catalysts that were added intentionally during synthesis, they may be present as impurities (e.g., through interactions with processing equipment or container–closure systems), or they may be derived from the raw materials.

The acceptance criteria for residual elemental impurities are established based on permitted daily exposure (PDE) for each element of toxicological concern. Guidance for elemental impurity limits is provided in ICH Q3D(R1). If the risk assessment fails to demonstrate that an elemental impurity level is consistently below the control threshold, additional control should be implemented to ensure that the elemental impurity level in the drug product does not exceed the PDE. In some cases, lower levels of elemental impurities may be warranted when levels below toxicity thresholds have been shown to have an impact on other quality attributes of the drug product (e.g., element-catalyzed degradation of drug substances).

For many process-related impurities, it is possible to rely on the manufacturing process to remove them. HPLC purification methods may also be able to remove residual process materials. For analysis of residual solvents, elemental impurities, and residual anions and cations, methods such as GC, inductively coupled plasma mass spectrometry (ICP–MS), and ion chromatography (IC) should be used, respectively. NMR is particularly useful during process characterization because diffusion experiments allow for rapid differentiation between small-molecule organic impurities and peptide-bound modifications.

Table 4. Non-Peptide Impurities: Origin and Commonly Used Analytical Techniques and Reference Guidance

Type	Origin	Analytical Techniques and Reference Guidance
SMs, intermediates, reagents, and their respective reaction or degradation products	SMs or reagents	⟨621⟩[a] (e.g., HPLC or GC)
Residual solvents	Solvents used in late stages of manufacturing (e.g., acetonitrile used as eluant for the preparative HPLC purification)	⟨467⟩[b] and ICH Q3C(R7)
Inorganic impurities, residual anions of organic acids, and residual cations of bases	Residual anions or cations present as counter ions or buffer components (e.g., TFA or triethylammonium phosphate (TEAP), or fluoride originating from hydrogen fluoride used with Boc-SPPS)	⟨503⟩,[c] ⟨503.1⟩,[d] and ICH Q3C(R7) (e.g., for acetic acid and triethylamine)
Elemental impurities	SMs, catalysts during synthesis; equipment	⟨232⟩,[e] ⟨233⟩,[f] and ICH Q3D(R1)
Bioburden	Aqueous solutions or buffers at neutral pH	⟨61⟩,[g] ⟨62⟩,[h] and ⟨1111⟩[i]
Bacterial endotoxins	Materials used in late stages of manufacturing	⟨85⟩[j]

[a] *Chromatography* ⟨621⟩.
[b] *Residual Solvents* ⟨467⟩.
[c] *Acetic Acid in Peptides* ⟨503⟩.
[d] *Trifluoroacetic Acid (TFA) in Peptides* ⟨503.1⟩.
[e] *Elemental Impurities—Limits* ⟨232⟩.
[f] *Elemental Impurities—Procedures* ⟨233⟩.
[g] *Microbial Enumeration Tests* ⟨61⟩.
[h] *Tests for Specified Microorganisms* ⟨62⟩.
[i] *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* ⟨1111⟩.
[j] *Bacterial Endotoxins Test* ⟨85⟩.

Microbiological Contamination

In contrast to biotechnological processes, the manufacturing of peptides by chemical synthesis normally involves organic solvents and conditions that do not support microbiological growth. Unless pure aqueous solutions or buffers at neutral pH are involved, microbiological growth can easily be prevented. Bioburden should be controlled according to *Microbial Enumeration Tests* ⟨61⟩ and *Tests for Specified Microorganisms* ⟨62⟩. Appropriate acceptance criteria are recommended in *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* ⟨1111⟩.

Bacterial Endotoxins

In most cases, when used in injection products, peptides should be controlled for bacterial endotoxins according to *Bacterial Endotoxins Test* ⟨85⟩. The final endotoxin content of a synthetic peptide is mainly dependent on the endotoxin content of materials introduced in late manufacturing stages, as synthesis conditions will normally not favor microbiological growth. Consequently, the endotoxin contribution of these materials needs to be controlled.

Specific Tests

WATER

Water is often a constituent of the peptide drug substance when it is isolated from aqueous solutions by, for example, lyophilization. As peptides are typically hygroscopic, the water content can significantly affect the peptide content and stability of the drug substance and should therefore be controlled. The acceptance criterion for water content is specific to each drug substance and is usually set based on batch history. This specification parameter may contribute to the peptide stability. It is usually included in the stability study of the peptide drug substance because it is required for the calculation of the assay by HPLC. As per ICH Q6A, the acceptance criterion may be justified with data on the effects of hydration or moisture absorption.

Typically, *Water Determination* ⟨921⟩ is used to monitor the water content. The more specific Karl Fischer titration in ⟨921⟩, *Method I, Method Ic* is preferred. If volatile components in addition to water are also expected, then *Loss on Drying* ⟨731⟩ is a suitable alternative. In addition, if residual solvents are expected, they should be quantitated independently (see *Residual Solvents* ⟨467⟩).

COUNTER ION

Most peptide drug substances contain a counter ion, commonly acetate or chloride. The counter ion acceptance criterion is specific to each drug substance and is usually set based on batch data history. Counter-ion determination usually is not included in stability studies of peptide drug substances. A common method for acetate determination is described in *Acetic Acid in Peptides* ⟨503⟩. Alternatively, *Ion Chromatography* ⟨1065⟩ provides additional information on common techniques used for this purpose.▲ (USP 1-Aug-2021)