⟨1228.4⟩ DEPYROGENATION BY RINSING

INTRODUCTION

The production of parenteral products requires that products be sterile, but also that they are free from harmful levels of pyrogens, or fever causing agents. For the purposes of the ⟨1228⟩ series, the term "depyrogenation" refers to the destruction or removal of bacterial endotoxins, the most prevalent and quantifiable pyrogen in parenteral preparations. Effective depyrogenation depends on the product and the method of removal and/or destruction. Although depyrogenation of heat-stable articles may be best accomplished by dry heat, heat-labile equipment, components, ingredients, or materials such as drug product containers and closures, and some medical devices may be depyrogenated through physical means such as rinsing. This chapter provides an overview of the depyrogenation process, its validation, and routine process control.

RINSING PROCESS

Rinsing is the most common means of reduction or removal of bacterial endotoxins on closures (such as elastomeric stoppers), medical devices, and other materials that are not compatible with the temperatures used in dry heat depyrogenation. The mechanism for this method of depyrogenation is removal of the endotoxin, followed by dilution (*1*). The process of general rinsing to remove pyrogens is accomplished by using high-purity water such as *Water for Injection* preferably above 60°. Multiple rinses may be necessary with proper controls to ensure that the *Water for Injection* does not become contaminated with Gram-negative bacteria and bacterial endotoxins during processing.

The use of high-purity rinse water is often the key to successful reduction of bacterial endotoxin activity on the surfaces of the materials being processed. At a minimum, the rinse water quality should meet the bacterial endotoxin limit of *Water for Injection*, which is <0.25 USP Endotoxin Units (EU)/mL. Using water that risks having bacteria grow in it may result in the deposition of bacterial endotoxins onto the items, or in the case of depyrogenated articles, the re-deposition (re-pyrogenation) of endotoxins from the rinse water itself. The advent of *Water for Injection* for use in the healthcare manufacturing industry has ensured that the most prevalent ingredient in most of our products is no longer a source of endotoxin. Additionally, re-pyrogenation of articles due to subsequent microbial proliferation on the rinsed items should be minimized either by a prompt subsequent sterilization or by drying the items after rinsing.

It is important to note that starting with *Water for Injection*, while essential, does not ensure that such water remains suitable for use through the duration of the depyrogenation process. When cooled from the elevated circulation temperatures used for its distribution, *Water for Injection* is vulnerable to microbial proliferation. *Water for Injection* held at temperatures below 55° and above 8° should be considered at risk for microbial contamination unless sterilized and held in a sterile vessel. Therefore, holding water for more than 3–4 h within this temperature danger zone is an unsuitable practice. Systems that recirculate *Water for Injection* can be effective but process conditions and water storage times and temperatures should be carefully controlled and validated.

Solvents other than *Water for Injection*, such as caustic alkali or detergents, have been used for depyrogenation by rinsing. The concern with these solvents is the removal of residuals that may ultimately be harmful to patients or the product, so care must be taken to test for residuals during validation of the method.

For any process that purports to depyrogenate by rinsing, there are a number of critical factors that must be defined and controlled during the validation study and beyond:

1. Solvent description, including normality or concentration if caustic alkali or detergents are used; if high-purity water is used, the source should be clearly described
2. Solvent temperature
3. Solvent pressure, particularly where rinsing is used to depyrogenate glass or other articles that remain stationary during the process
4. Solvent flow rate through the system
5. A justification for the recirculation of solvents

VALIDATION

Validation of depyrogenation by physical means is not different, in principle, from the validation of other depyrogenation methods. Endotoxin indicators, which are articles representative of the material to be depyrogenated spiked with a known amount of endotoxin, are prepared in a laboratory (see *Endotoxin Indicators for Depyrogenation* ⟨1228.5⟩). Quantitation of endotoxin activity prior to and subsequent to the rinsing process will demonstrate the effectiveness of the process in removing endotoxins. To prepare endotoxin indicators, inoculate each indicator with a known level of activity of Control Standard Endotoxin (CSE), USP Endotoxin RS (which, by convention, is abbreviated as RSE), or Naturally Occurring Endotoxin (NOE), which is calibrated against RSE before processing. Generally speaking, a small volume (e.g., <100 µL) of a highly concentrated analyte (purified LPS or NOE) is inoculated onto a section of the article that is the most difficult for the rinse solvent to reach. For example, stoppers are generally inoculated onto the product contact portion of the stopper. A low volume of inoculum is used to ensure rapid drying. The inoculated indicators may be dried in a unidirectional air flow cabinet or other validated means to hasten drying and limit the possibility of extrinsic microbial contamination that can arise in uncontrolled environmental conditions, particularly with moisture present. Historically, 1000 EU/article has been the target spike value, but depending on 1) the level of endotoxins historically found on the material, 2) the amount of recoverable endotoxins observed after the drying

process, and 3) the "safe" level of endotoxin activity that must be attained post-processing, an inoculum at a level other than 1000 EU may be justified.

Because the prepared endotoxin indicators may be mixed with many uninoculated articles (e.g., stoppers), it is essential to provide a means to identify the indicators. For example, if the stoppers used by the company are gray, an identical stopper that is a different color but has the same elastomeric material, or a stopper of the same composition but a different shape, could be used for the indicators. If a stopper of different color or other identifying mark is not available, the inoculated indicator stoppers might be placed in individual small mesh bags. For glass containers, the indicator containers might be amber rather than clear, or they may be marked with indelible or heat resistant ink. For metal articles, a heat-resistant marker might be used to identify the indicators. In any event, the endotoxin indicators must be identical in materials of composition to the other articles in the study, but must be prepared so that they can be easily retrieved for analysis.

Once dry, the endotoxin indicators are analyzed for the recoverable level of endotoxins activity using the *Limulus* amoebocyte lysate (LAL) assay. To determine the recoverable activity, follow the process for extracting endotoxin from medical devices (see *Medical Devices—Bacterial Endotoxin and Pyrogen Tests* ⟨161⟩). Submerge the article or a number of articles in Water for Bacterial Endotoxins Test (BET; see *Bacterial Endotoxins Test* ⟨85⟩*, Reagents and Test Solutions, Water for Bacterial Endotoxins Test*) prewarmed to 37°. Allow the article(s) to remain in contact with the Water for BET for an hour. Agitation such as intermittent vortexing or sonication may be added to enhance recovery. If methods other than immersion in Water for BET are used, they must be validated to demonstrate that they do not result in a loss of endotoxin activity and identified in the protocol so that subsequent studies use the same process. Regardless of the method used, a statistically significant number of samples representing the load under study should be evaluated to ensure adequate reproducibility.

Controls

Two sets of controls are recommended for validation studies:

1. Testing of uninoculated indicators—provide data on the resident level of endotoxin activity on the lot of stoppers to be tested.
2. An appropriate number of endotoxin indicators should be retained by the laboratory and used as positive controls. After the depyrogenation process is completed, extract both the processed units and positive control indicators as described above and test using a qualified BET method.

Any activity detected on the retained positive controls is called “recoverable” activity and activity detected on the processed indicators is called “residual” activity (*2*). The following factors should be considered during the extraction of endotoxin activity:

1. Inoculated units should not be pooled for analysis. The conceptual basis for this study design is to determine the depyrogenation capability of the process, and pooling extracts from a number of units would not allow evaluation of reproducibility and also bias the results. However, there may be a unique circumstance in which accurate or valid results may not be possible because of a small sample size or other logistical or practical considerations. If articles are pooled, the reason for pooling should be documented in the body of the protocol.
2. The test result must account for all sources of dilution. For example, the units for an LAL test are EU/mL. If the extraction volume is 1 mL/stopper, then the result of the test is really EU/stopper. But, for example, if the extraction volume is 5 mL/stopper, then each milliliter of the extract is a 1:5 dilution of any endotoxin activity that has been extracted from the indicator.

The log reduction is calculated using the following formula:

$$\text{Log}_{10}\text{ reduction} = (\log_{10}\text{ recoverable activity}) - (\log_{10}\text{ residual activity})$$

For example, if a laboratory detects 4500 recoverable EU/stopper in the controls and 0.3 residual EU/stopper, the log reduction is calculated as:

$$\text{Log}_{10}\text{ reduction} = (\log_{10} 4500\text{ EU}) - (\log_{10} 0.3\text{ EU})$$

$$\text{Log}_{10}\text{ reduction} = (3.65) - (-0.52) = 4.17$$

Historically, laboratories added sufficient inoculum to each endotoxin indicator unit so that at least 1000 EU could be recovered in the retained controls. However, there are factors that the analyst and the validation team should be aware of in conducting these studies. Foremost among these factors is an understanding that the lipopolysaccharides extracted via the Westphal procedure, of which CSE and RSE consist, have physicochemical properties that can make achieving a uniform dispersion of a highly concentrated quantity of this material difficult or impossible. Also, CSE tends to adsorb to surfaces and therefore may be difficult to remove from some materials resulting in control recoveries of less than 50%. In some cases, the recovery may be substantially less than 50%. There may also be considerable variability in recovery from test to test or even within a single test run. This is to be expected and history has taught us that this does not impact the usefulness or effectiveness of a depyrogenation study.

A 3-log reduction of the recoverable endotoxin is a common target for acceptable depyrogenation effectiveness (*3*). However, this may not be attainable in all test systems or under all test conditions nor may it be necessary. Where a 3-log reduction is not attainable it is important to carefully evaluate endotoxin risk in the process based extensively on inherent endotoxin burden. Rather than strict reference to a 3-log reduction, the depyrogenation program must prove that endotoxin activity at levels below a scientifically established target can be consistently attained. The target should consider the use of the article and the potential contribution to the final endotoxin limit established for the drug product. Although the endotoxin limit is established based on calculations of active pharmaceutical ingredient (API) dosage contained in the drug product, any contributions by the excipients and container/closure must be considered in any validation study, and the sum of all these contributions must meet this limit.

Elastomeric closures are generally received with little or no detectable endotoxin activity. It is possible that a 2-log reduction by rinsing could be sufficient for some packaging components, excipients, or other materials. An important consideration in making this determination is the incoming pyroburden and bioburden presented by any material to the depyrogenation process.

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ROUTINE PROCESS CONTROL

Proactive control of Gram-negative bacterial contamination is essential to the production of safe parenteral products. The use of properly generated, stored, and distributed ▲*Water for Injection*▲ (ERR 1-Nov-2019) ensures that the most prevalent ingredient in most products is not a significant source of endotoxin.

Once a depyrogenation process has been effectively developed and validated, it must be maintained in that state to ensure continued acceptability. Chapter ⟨1228⟩ details the general practices that are appropriate for all depyrogenation systems. This is accomplished by a number of related practices that are essential for the continued use of the process over an extended period of time. The essential practices to maintain validated status include calibration, physical measurements, periodic endotoxin assessment on incoming materials, ongoing process control, change control, preventive maintenance, and periodic reassessment and training.

REFERENCES

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