

〈1228〉 DEPYROGENATION

INTRODUCTION

The production of parenteral products requires not only that products be sterile, but that they are also free from harmful levels of pyrogens. Depyrogenation is defined as the direct and validated destruction or removal of pyrogens. For the purposes of this and subsequent chapters 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. The chapters in this series discuss depyrogenation procedures that are applicable to product streams, equipment, and drug product containers and closures.

The 〈1228〉 series builds on the tenets described in *Sterility Assurance* 〈1211〉, first published in *USP 20–NF 15*. In the time since that publication, the science of depyrogenation has advanced, as has control over manufacturing processes. Manufacturers are very aware of the impact that facility, equipment, and process design has on endotoxin levels and depyrogenation, and they are mindful of the effects of elevated endotoxin levels in raw materials and water systems. Risk management tools, including process evaluation tools such as Hazard Analysis Critical Control Point (HACCP), are helping companies to identify critical control points (CCP) for the control of endotoxin and other impurities or contaminants in the manufacturing process. As a result of these advances in science, technology, and philosophy, the 〈1228〉 series will also offer alternatives to historical or traditional thinking.

BACTERIAL ENDOTOXIN AND LIPOPOLYSACCHARIDE

Bacterial endotoxin is a component of the outer cell membrane of Gram-negative bacteria. The natural endotoxin complex contains many cell wall components including phospholipids, lipoproteins, and lipopolysaccharide (LPS), which is the biologically active component of endotoxin. Purified endotoxin is chemically defined as a LPS. LPS consists of three distinct regions:

1. The hydrophobic lipid A portion of the molecule is highly conserved among Gram-negative bacteria, and is largely responsible for most, if not all, of the biological activity of endotoxin.
2. A core oligosaccharide links the lipid A to the hydrophilic O-specific side chain or O-antigen.
3. The hydrophilic O-antigen is a highly variable region that confers serological specificity to the organism and is often used to distinguish strains of Gram-negative bacteria.

Due to the amphipathic nature of the LPS molecule [i.e., having both a polar (hydrophilic) end and a nonpolar (hydrophobic) end], purified LPS preparations, such as reference standard endotoxin (RSE) and control standard endotoxin (CSE) tend to form bilayers, micelles, ribbons, and other conformations when in solution, and they may adsorb, or “stick”, to surfaces, making them difficult to extract and detect. The degree of adsorption of LPS to solid surfaces is affected by the composition and finish of the material to be depyrogenated. The extent of aggregation of LPS in solution is affected by a host of formulation matrix attributes to which it is exposed, such as temperature, pH, salt concentration, divalent cation concentration, detergents or emulsifiers, and chelating agents.

When parenteral products are contaminated with endotoxin, the contaminant is not purified LPS, but rather whole cells or cell wall fragments generated during the normal growth cycle of the bacteria or disruption of bacteria, where the LPS remains embedded in or associated with other cell wall components. Purified LPS and native endotoxin are dissimilar in many respects, and the two terms should not be used interchangeably. Depending on the materials of construction or the formulation of the article to be depyrogenated, the use of native endotoxin as a challenge material in depyrogenation studies may be a consideration because a native endotoxin preparation better reflects operational reality, particularly for the depyrogenation of product streams, and because LPS molecules in natural endotoxin are embedded in cell wall complexes, they may be much less prone to the aggregation and adsorption issues seen with purified LPS.

For the purposes of the 〈1228〉 series, the term “challenge material” will be used to generically describe material (endotoxin or LPS) used as a spiking analyte for depyrogenation studies. “Endotoxin” will refer to the moiety in its natural state, meaning pieces of Gram-negative cell wall from a well-characterized source. LPS will refer to the purified material.

MEASURING ENDOTOXIN PRE- AND POST-PROCESSING

The primary procedure used for the measurement of bacterial endotoxin is the *Bacterial Endotoxins Test* 〈85〉 (BET). A well-controlled BET assay can provide assurance of accurate readings for the calculation of the reduction in challenge material activity pre- and post-processing, as well as provide consistent quantitation of levels of native endotoxin in raw materials, at CCP in the manufacturing process, and in finished products.

There are many variables in study structure and test method that can affect the outcome of a depyrogenation study. Development of a test method depends on the material under test, the identification of an appropriate challenge preparation, and the method used to extract recoverable activity prior to processing and residual activity after processing. Once a test system is developed that includes the identification of a source for challenge material preparations, inoculation of the articles to be depyrogenated (including drying procedures), extraction or recovery methods, and appropriate BET test methodology and sensitivity, it is recommended that subsequent tests should use the same conditions to ensure the comparability of test results. Points to consider when constructing a depyrogenation study include the following:

1. **The challenge material:** Consider the source of the endotoxin (purified or natural). When using purified LPS, choose a preparation with no fillers, because the presence of these fillers can add to the variability and therefore decrease the

accuracy of the assay. Once challenge material preparation is chosen, it is recommended to use material from the same source in subsequent studies to reduce variability.¹

2. **The characteristics of the material being depyrogenated:** It is important to understand the characteristics of the material being tested. For example, LPS may adsorb to plastics, and although two objects may be made of the same plastic, surface finish, surface area, and conformation differences may affect extraction efficiency and LPS recovery. For solutions, formulation matrices may affect aggregation of purified LPS in that pH, salt concentration, chelating agents, surfactants, and the presence of divalent cations may all have an impact on the recovery of the challenge material. The use of natural endotoxin may mitigate some of these recovery issues. For materials that are received with low or undetectable levels of endotoxin, depyrogenation studies using endotoxin or LPS challenge materials may be unnecessary if control is demonstrated and decisions are scientifically justified.
3. **The level of activity needed for the study:** How much pre-processing activity do you need to execute the study? The current industry standard is to add enough endotoxin to the system so that at least 1000 EU can be recovered prior to depyrogenation. However, depending on the test system, 1000 EU may be either excessive or insufficient. For example, when designing a study for the depyrogenation of a product stream that normally contains <1 EU in a certain volume, a spike of 1000 EU of a CSE in that same volume may be excessive. For solid-surface materials, the level of activity in the challenge material should be established taking into account the materials of construction and finish as they may contribute to LPS adsorption. Knowledge of historical levels of endotoxin in or on the surface, the efficiency of the depyrogenation processes, the efficiency of the challenge material extraction or recovery method, and the log reduction or safety level target acceptance criterion are all important to the setting of a pre-processing activity requirement. If a reduction study for a product stream is required, it may be more appropriate to add an amount of naturally occurring endotoxin to the product consistent with the maximum expected endotoxin load ("worst case"), based on known endotoxin contributors (e.g., raw materials and water) and process capability to demonstrate reduction to safe levels. Whatever the procedure, the logic and methodology for endotoxin reduction studies should be justified and documented. For those materials that routinely contain a level of endotoxin, such as fermentation broths where there are high levels of activity to begin with, it may not be necessary to add additional challenge material.
4. **Preparation of test samples:** The method used to affix challenge materials to the surface of materials to be depyrogenated may affect its removal or recovery. Air-drying is the most convenient method of affixing challenge material to hard surfaces, but freeze drying and vacuum drying also have been used. To improve drying efficiency, it is suggested that a small volume of a highly concentrated activity of challenge material be used. This volume may be added to the surface of the item in an area that has been defined as "hardest to depyrogenate" or may be dispersed to represent the more likely natural occurrence. Inoculation methodology must be well defined for comparability across studies. Decisions regarding the design of studies must be documented and justified.
5. **Recovery methods:** Although there are standard methods for the recovery of endotoxin from medical devices, there is no standard method for the recovery of endotoxin dried onto solid surfaces that are used as indicators in depyrogenation studies. Although it is most convenient to adopt the standard extraction methods described in *Medical Devices—Bacterial Endotoxin and Pyrogen Tests* (161) and ANSI/AAMI ST72:2011,² a laboratory may choose to develop and validate a method that better suits the material under test. The efficiency of the recovery of endotoxin will depend on the attributes of the material under test, the composition of the challenge material, the concentration of the challenge material spike, and the method of drying. Recoveries of less than 100% of the challenge material nominal spike in positive controls are not uncommon. Perhaps more important than the percent recovery in positive controls is consistent recovery across lots of the same material and across depyrogenation studies.^{3, 4}
6. **Test method:** Lysate formulations differ, and they may be subject to different interferences such as leachables, chelators, and salts. If interferences are encountered during method development that are difficult to overcome, consider trying another test method (e.g., gel clot, kinetic chromogenic, kinetic turbidimetric, endpoint chromogenic) or a different source of reagent.
7. **Depyrogenation method:** Variability in the depyrogenation treatment may impact recovery. Treatments vary in efficiency, both within and between treatment types. For example, a large difference in efficiency will be seen when comparing the depyrogenation of glass vials using dry heat versus rinsing in water for injection (WFI), and differences may be seen in the reduction of challenge material by the filtration of a solution depending on the type of filter chosen. Likewise, variability can be seen when using a single depyrogenation methodology with two different materials, such as the filtration of two different solutions using the same type of filter medium.

The efficiency of a depyrogenation process has been historically measured in terms of a logarithmic (log) reduction of a large "spike" or bolus of purified endotoxin that is added to or dried onto a material prior to treatment. Although log reduction is a convenient measurement benchmark, the more relevant and pragmatic indicator of depyrogenation efficiency is one based on process capability and patient safety, which is the reduction of the measured or anticipated worst case natural levels of contaminating endotoxin to safe levels as defined by reference to calculated endotoxin limits for the material.

With the implementation of the principles of quality by design (QbD) and risk management, the 3-log reduction that was first introduced in 1984 may be inappropriate as a universal benchmark for modern depyrogenation processes. For example, materials with a high level of native endotoxin, such as fermentation broths, could require more than a 3-log reduction to reach a safe level, whereas dry heat depyrogenation of materials with normally low or undetected levels of native endotoxin, such as washed glass vials, will require substantially less than a 3-log reduction. The appropriate endotoxin log reduction for the process should be determined by the user based on a full understanding of the product and process capability including

¹ Ludwig JD, Avis KE. Recovery of endotoxin preparations from the surface of glass capillary tubes. *J Parenter Sci Technol.* 1989;43(6):276–278.

² ANSI/AAMI ST72:2011. Bacterial endotoxins—Test methods, routine monitoring, and alternatives to batch testing, AAMI, Arlington, VA.

³ LAL Users Group. Preparation and use of endotoxin indicators for depyrogenation process studies. *J Parenter Sci Technol.* 1989;43(3):109–112.

⁴ PDA Technical Report 3 (Revised 2013). Validation of dry heat processes used for depyrogenation and sterilization. Bethesda, MD: Parenteral Drug Association; 2013.

input sources, levels of endotoxin, efficiency of depyrogenation methods, and output (product- or process-specific) endotoxin requirements. Effective process control requires knowledge of input, in-process (where appropriate), and output endotoxin levels. Under these circumstances, with appropriate process development, justification for reduced endotoxin challenges or the elimination of endotoxin challenges may be made based on historical data and demonstration of continued control.

CONTROL OF ENDOTOXIN IN PARENTERAL PRODUCTS

The best control of endotoxin levels in parenteral products is the control of Gram-negative bioburden in raw materials, equipment, process streams, and manufacturing environment and operators. Parenteral manufacturers may exercise three categories of control to keep endotoxin content in drug products at safe levels.

The first category is "indirect control", which is comprised of a series of preventive measures that control bioburden, the potential endotoxin contribution by formulation components (e.g., raw materials, APIs, excipients), water, primary packaging components, equipment, and the manufacturing environment, including personnel.

The second category is "process control", in which endotoxin is monitored at CCP during processing to ensure that there is no increase in endotoxin. These process control elements are subject to validation or qualification.

The third category is "direct control", or the direct destruction or removal of endotoxins from product streams, equipment, and primary packaging materials. As with controls on processing, direct measures of endotoxin destruction or removal must be validated.

INDIRECT CONTROL

Reducing opportunities for Gram-negative microbial proliferation at any stage of manufacturing will reduce the likelihood of endotoxin contamination in the following ways:

- Exercising control over the endotoxin content of incoming materials, particularly materials derived from natural sources or those with high water activity, will reduce the opportunity for Gram-negative microbial proliferation and therefore reduce or eliminate the need for endotoxin removal downstream. Because of their manufacturing processes, glass and plastic containers as well as elastomeric closures are often received with very low or undetectable levels of endotoxin. Qualification of primary packaging suppliers should include an audit that examines and confirms the supplier's consistent and documented control over applicable manufacturing processes.
- Bioburden and endotoxin control should be a component of a vendor audit and supplier qualification program for formulation materials that could potentially contribute endotoxin to parenteral products.
- Water is the most ubiquitous raw material in the manufacturing of parenteral products, but unless the generation and distribution of high-quality water is properly validated and controlled, the system will be prone to contamination by Gram-negative bacteria and the establishment of biofilms that can contribute significantly to the endotoxin load of the product (see *Water for Pharmaceutical Purposes* (1231) for a discussion of the types of waters used in pharmaceutical manufacturing and guidance for validation, maintenance, sampling, and testing of systems).

PROCESS CONTROL

Product-specific process control requires the identification of CCP for the introduction or removal of endotoxin. Process control requires good process and equipment design consistent with QbD using a risk management tool such as HACCP. Process control measures include but may not be limited to the following:

- Control of manufacturing practices is essential to endotoxin control. Endotoxin control should be a part of validated cleaning procedures. The use of product contact materials for which endotoxin control cannot be established should be avoided. Clean product-contact equipment should be stored dry to avoid bioburden and Gram-negative bacterial proliferation.
- Hold times during manufacturing, particularly for nonsterile bulk in-process materials and drug product, should be validated to ensure that the hold conditions do not support microbial proliferation and therefore potential endotoxin production by Gram-negative bacteria.
- Environmental control, including good manufacturing practices (aseptic, as appropriate), is essential to process management of endotoxin. Operators should be properly garbed and trained. Housekeeping and disinfecting practices should be established to reduce the possibility of microbial proliferation in critical areas. Cleaning regimens should emphasize that standing water be removed at the end of the cleaning process.
- Endotoxin control and monitoring will be covered in a forthcoming chapter.

DIRECT CONTROL

These processes require validation to ensure that endotoxins are removed or reduced to safe levels. Direct control may be accomplished by a variety of methods and processes that may be combined to ensure endotoxin reduction to a safe level. The most commonly used depyrogenation processes and the associated control measures are the subject of the (1228) series.

SELECTION OF AN APPROPRIATE DEPYROGENATION METHOD

The basic principles for the control and validation of a depyrogenation process using a life cycle approach include:

- Assessment of manufacturing processes to identify materials and process components that are essential to the control of endotoxin
- Focused depyrogenation process development that is consistent with the material to be depyrogenated, the resident endotoxin level of the material to be depyrogenated, and the limit of endotoxin for the finished article
- Adequate validation studies
- Ongoing monitoring of process controls to ensure continued efficacy of the depyrogenation process
- Identifying and documenting changes (a change control program) to the depyrogenation processes over time

Known or anticipated levels of endotoxin can be determined so that appropriate indirect or direct measures of control, consistent with the product or materials of construction, will ensure that endotoxin is eliminated or reduced to levels that ensure product and patient safety.

Should direct methods of depyrogenation be required for the control of endotoxin in or on the article, an important consideration during manufacturing process development is the selection of an appropriate method from the possible alternatives: dry heat, chemical, filtration, or physical removal. In some instances this selection is limited by the potential effects of the depyrogenation treatment may have on the materials themselves. The choice of the appropriate process for a given item requires knowledge of depyrogenation techniques and information concerning effects of the process on the material being processed. The selection of a particular treatment (and the details of its execution) often represents a compromise between those conditions required to destroy the endotoxin or remove it to the desired level and the effect of the process on the materials. Depyrogenation processes should be no more aggressive than required for effective process control to avoid adverse consequences to material quality attributes.

VALIDATION OF A DEPYROGENATION METHOD

The validation program comprises several formally documented stages to establish that the depyrogenation process is capable of operating within prescribed parameters for process equipment, that independent measurements of critical parameters are possible and accurate, and that acceptance criteria for challenge material removal or destruction are met.

- The development stage investigates and establishes the operating parameters that define the controls to be used for the depyrogenation process.
- The installation qualification (IQ) stage establishes that equipment controls and other instrumentation needed to execute depyrogenation processes and measure the results of the depyrogenation process are properly designed and calibrated. Documentation should be available to demonstrate the acceptability of any required utilities such as steam, water, and air.
- The operational qualification (OQ) stage confirms that the equipment and other processes components function within the defined depyrogenation parameters.
- The performance qualification (PQ) stage of the validation program directly evaluates the depyrogenation of materials or articles. Wherever possible, these studies should employ or simulate the actual conditions of use, including the use of real or simulated product material. Worst case conditions, for example, might include the bracketing of critical parameters such as time/temperature and belt speed for dry heat depyrogenation of glass vials, flow rate for depyrogenation by filtration of solutions, and maximum measured, anticipated, or defined endotoxin loads for any material. "Worst case" should be defined and justified in the validation protocol. Endotoxin indicators may be utilized to support physical measurements in the validation of the depyrogenation process. Although the "rule of three" suggests that three consecutive successful validation runs be executed, perform sufficient replicate studies to demonstrate the capability and efficiency of the depyrogenation process, including the validation of operational ranges of any equipment used in such processes. The number of replicate studies chosen should be scientifically based and justified. At the end of the PQ, a report is written to establish operating parameters.

ROUTINE PROCESS CONTROL

Once a depyrogenation process has been validated it must be maintained in that state to ensure the continued acceptability of its operation. This is accomplished through a number of related practices essential for continued use of the process.

- **Physical measurements:** Data reported by the equipment sensors and recorders must be verified after the completion of each depyrogenation cycle.
- **Calibration:** Any equipment used in the control or quantitative assessment of parameters required for a depyrogenation process must have its measurement accuracy verified against a traceable standard on a periodic basis.
- **Preventive maintenance:** There should be a defined maintenance schedule for each piece of process or testing equipment required for depyrogenation that is consistent with the manufacturer's written recommendation.
- **Ongoing process control verification:** Depending on the specifics of the particular depyrogenation process, there may be additional requirements for ongoing confirmation of process efficacy. These can include the testing of raw materials, water supplies, and in-process sampling. These performance parameters are monitored against assigned limits designed to ensure that finished products meet acceptable endotoxin levels. Monitoring of operating parameters and controls plays an important role in maintaining the depyrogenation process in a validated state.
- **Periodic reassessment:** It is expected that the effectiveness of depyrogenation processes be reconfirmed on a periodic basis. A reassessment schedule should be formalized to assess the potential impact of de minimis or undetected changes to maintain the process in a validated state.
- **Change control:** In order to remain in a validated state, the various material, procedure, and equipment elements impacting the depyrogenation process should be carefully monitored to ensure that changes are properly evaluated for their potential impact on the process. The scope of the change control program must include materials being processed,

process equipment, processing parameters, and process holding time limits. The extent of the effort required to support a change will vary with the potential impact of the change on the process outcome.

- **Training:** Depyrogenation processes rely heavily on scientific principles for the effective destruction or removal of endotoxins. Scientists and engineers well-grounded in the principles of endotoxin removal and testing develop processes to ensure effective depyrogenation. Individuals involved in the development of depyrogenation processes require a background in microbiology, physics, chemistry, and engineering, and they must be familiar with good manufacturing principles and regulations. Depyrogenation is an interdisciplinary activity where the combined knowledge of a group of individuals is generally required for the establishment of a reliable process. In addition to the depyrogenation process development team, individuals responsible for the maintenance and operation of depyrogenation processes must also be trained appropriately to ensure that their actions contribute to success. The operators are often the first to identify changes in process performance because of their intimate involvement with it. Effective training programs should be established and documented. Training programs should emphasize depyrogenation principles, adherence to established processes and procedures, and the importance of documenting deviations from normal operations.

ROUTINE TESTING

Testing is not a control mechanism but rather a tool to assess the effectiveness of control measures. Depending on the specifics of the particular depyrogenation process, there may be additional requirements for ongoing confirmation of process efficacy.

These requirements can include the testing of raw materials, water supplies, and in-process sampling. These performance parameters are monitored against assigned limits based on historical data, and are designed to ensure that finished products meet acceptable endotoxin levels. Monitoring of operating parameters and controls plays an important role in maintaining the depyrogenation process in a validated state.