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⟨1228.5⟩ ENDOTOXIN INDICATORS FOR DEPYROGENATION

1. INTRODUCTION

Depyrogenation is defined as destruction or removal of pyrogens (see *Depyrogenation* ⟨1228⟩). For the purposes of this chapter, the terms "bacterial endotoxin" or "endotoxin" refer to a component of the outer cell membrane of Gram-negative bacteria, which is known to induce a febrile response in humans and other mammals. The endotoxin complex contains many cell wall components including, but not limited to, phospholipids, lipoproteins, and lipopolysaccharides. Lipopolysaccharide (LPS) is the biologically active portion of both naturally occurring and laboratory-prepared endotoxin complexes. The USP Endotoxin Reference Standard (which, by convention, is abbreviated as RSE) and control standard endotoxin (CSE) preparations purchased from lysate manufacturers and other third-party vendors are not endotoxins but rather are preparations of purified LPS.

"Endotoxin indicators" (EIs) are tools that are used (where required) in conjunction with physical measurements to analyze the effectiveness of a depyrogenation process. EIs used to determine the effectiveness of dry heat depyrogenation processes are commonly purchased as glass vials that are inoculated with a known level of LPS activity. This chapter expands the definition of EI to include any carrier, including glass vials, inoculated with endotoxin or LPS that is used to challenge a depyrogenation process. EIs can be used to analyze the effectiveness of endotoxin removal by washing, rinsing, cleaning, or by using separation technologies, such as filtration or chromatography. Carriers can be a variety of materials, including rubber stoppers to assess stopper-washing processes, bulk product to assess and validate processing steps, and stainless steel coupons to assess the cleaning of production vessels.

Purified LPS, such as CSE obtained from lysate manufacturers or other third-party vendors, has historically been a convenient choice for use as the analyte used in the preparation of EIs. However, EIs prepared in-house using laboratory-derived endotoxin more closely mimic product contamination, and as a result can provide a more realistic assessment of the depyrogenating capability of various production processes than does highly purified LPS. This chapter provides information on the preparation and use of these more specialized indicators to assure both consistency and comparability of data among method development and validation studies.

2. ENDOTOXIN AND LPS

A bacterial endotoxin is defined in the *Introduction*. LPS is the biologically active portion of the naturally occurring and laboratory-prepared endotoxin complex. Highly purified LPS, extracted from the natural endotoxin complex, is used to prepare the primary compendial RSE or secondary CSE preparations, such as those purchased from *Limulus* amebocyte lysate (LAL) reagent manufacturers. LPS consists of three distinct regions:

1. The structure of the hydrophobic lipid A portion of the molecule is the most highly conserved among Gram-negative species and is responsible for most, if not all, of the biological activity of endotoxins
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

When drug products and devices are contaminated with endotoxin, the contaminant is not purified LPS but rather whole Gram-negative cells and/or cell wall fragments containing LPS. LPS and endotoxin are therefore dissimilar in many respects.

The amphipathic nature of the LPS molecule [i.e., having both a polar (hydrophilic) end and a nonpolar (hydrophobic) end] enables it to form complicated, three-dimensional, aggregated structures in solution. The aggregated forms of LPS have the capacity to adsorb, or "stick", to surfaces, and depending on the LPS formulation and the surface, extraction and detection may prove difficult using conventional

extraction methods (see below). The degree of aggregation of the purified molecule is also affected by the conditions to which the LPS is exposed. Factors such as temperature, pH, salt concentration, divalent cation concentration, chelating agents, and detergents can have a profound effect on the biological activity and stability of LPS in solution. Purified LPS preparations used for depyrogenation studies should not contain any “fillers” or excipients. The excipients that are commonly used in the formulation of CSE have been shown to reduce the heat resistance of LPS (*1*) and may interfere in the recovery of LPS because of a caramelized excipient that has been post-processed by dry heat.

Endotoxins contaminating parenteral products may exhibit greater stability of activity in solution and less surface adsorption than purified LPS. As well, the detection of endotoxin may be less influenced than LPS by aggregation, disaggregation, or other conformations induced by some product matrices. Information on principles to consider when preparing endotoxin in the laboratory can be found below.

3. APPLICATION OF ENDOTOXIN INDICATORS

The choice of an EI should be relevant to the process being validated. For physical depyrogenation, such as dry heat, the carrier material for the EI may be a surface such as a glass vial or appropriate coupon material with documented heating characteristics similar to the materials being processed, and onto which a known quantity of LPS or endotoxin has been inoculated. For stopper washing/depyrogenation studies, stopper carriers are inoculated with known levels of LPS or endotoxin. For raw materials or process intermediates that are inherently contaminated with assayable levels of endotoxin activity, there may not be a need to add LPS or endotoxin to validate endotoxin reduction in the manufacturing process, as the level of contamination may be sufficient to accurately measure activity upstream and downstream of the depyrogenating step(s).

For processes using raw materials or for upstream intermediates that are not contaminated with endotoxins, the use of either the USP RSE or CSE, which are both highly purified preparations, may not reflect the actual removal or reduction potential of the product stream depyrogenation step(s) under challenge. For these purposes, endotoxins harvested from Gram-negative cultures may be more suitable for depyrogenation processes typically found in biopharmaceutical product streams. The cell wall fragments and outer membrane constituents associated with these endotoxins represent realistic challenges to process operations such as ultrafiltration, affinity chromatography, and the use of charged media membranes or columns.

Challenge studies for LPS or endotoxin removal in process streams should be conducted at the laboratory or pilot scale so as not to introduce high levels of endotoxin or LPS into the actual production environment.

4. PREPARATION AND USE OF ENDOTOXIN INDICATORS

4.1 Methodology to Create a Laboratory-Prepared Endotoxin: Principles to Consider

Glass vial EIs purchased from third-party vendors do not need further preparation before use. These indicators are labeled with a nominal value of inoculated LPS, and the label claim should be confirmed upon receipt to assure that there is sufficient activity (endotoxin unit, or EU) available for the study.

- There is not one “best” or “standard” method for preparing endotoxin in the laboratory, but one example of a published method for the preparation of laboratory-prepared endotoxin may be found in Bowers and Tran (*2*). Regardless of the methodology for preparation, the following recommendations should be considered to properly and consistently produce, identify, and maintain laboratory-prepared endotoxin for use as a tool for depyrogenation studies. An appropriate Gram-negative bacterial strain from a recognized culture collection is a good choice for preparing a laboratory-derived endotoxin. Alternatively, a Gram-negative organism isolated from a facility, water system, raw material, or product that is identified to the species level, that has been shown to be genetically stable and that is properly maintained, may also be considered. Establishing the identity and baseline genetic fingerprint of an environmental organism will assure that subsequent preparations are consistent.
- The laboratory should create detailed procedures or laboratory work instructions for culture maintenance and endotoxin preparation to assure consistency between batches of endotoxin. For example, endotoxin may be isolated from a culture of Gram-negative bacteria, according to the method of Bowers and Tran (*2*) or a well-documented variation of that method. Whatever the methodology for growth and endotoxin isolation that is developed by the laboratory, the methodology should be documented and used consistently.
- Consistent with good microbiological practice, the culture and maintenance of the cells used to produce a laboratory-prepared endotoxin should be consistent with *Microbiological Best Laboratory Practices* ⟨1117⟩. Instructions on 1) the proper maintenance of the organism; 2) growth conditions, including any requirements to prepare media, nutrient requirements, and time/temperature of incubation; 3) methods for cryopreservation or lyophilization for master cell banks and working cell banks; 4) storage of the endotoxin, once prepared including concentration, vessel type, and volume; and 5) master batch production records to assure consistency in subsequent studies should be written, managed via change control, and followed.
    - Once isolated, the relative activity of the endotoxin preparation should be established by comparing its activity to a known LPS standard such as RSE, or a CSE that has been standardized against the RSE. Determination of activity involves diluting the endotoxin preparation and assaying the dilutions against an LPS standard curve such that the result of the dilution falls within the range of the referenced standard curve. As with the CSE standard used in the bacterial endotoxins test (BET) assay from *Bacterial Endotoxins Test* ⟨85⟩, the activity of the endotoxin may vary, depending on the lot of lysate and lot of LPS used for the analysis. It is recommended, consistent with the assignment of potency for the CSE, that activity of an endotoxin preparation be evaluated for each lysate manufacturer, lysate lot, and test method (gel, kinetic turbidimetric, or kinetic chromogenic) in use in the laboratory.

      The activity of the stock endotoxin preparation in EU/mL is reported as:

      (Test result in EU/mL) × (dilution factor) = EU/mL of the starting endotoxin preparation

- Once activity has been determined, and if applicable to the study design, a standard series of dilutions of the newly prepared endotoxin should demonstrate onset times that result in slope and *y*-intercept values that are consistent with the standard curve parameters of the RSE/CSE standard using the same lot of lysate. This demonstrates that the activity of endotoxin preparation dilutes and reacts with the lysate in a manner that is similar to LPS.
- Characterization of the endotoxin preparation should also include data on the stability of the preparation, because stability is critical to the comparison of data from one study to the next. If the endotoxin preparation is stored, storage parameters including the concentration of the preparation in EU/mL, the composition of the vessel, the temperature of storage, and the length of storage, should be defined. An expiration date should be assigned based on determined stability.

4.2 Inoculation of EIs

To prepare an EI in house, inoculate endotoxin or LPS onto an article (carrier) that will serve as the substrate for the EI. Carriers for EIs can be anything that is subject to depyrogenation such as: vials (for dry heat depyrogenation), stoppers (for stopper washing), stainless steel coupons (for vessel cleaning), or product (for depyrogenation of process streams).

The simplest way to inoculate these indicators is to add a small volume of a highly concentrated solution of endotoxin or LPS to the carrier. The volume and concentration of added endotoxin or LPS should be calculated to add at least 1000 EU to the carrier, although higher or lower concentrations may be justified based on historical data on the endotoxin content of the material. [Note—The remaining discussion assumes a 1000-EU inoculum, but the principles hold for any level of initial inoculum.] For nonliquid carriers, the endotoxin is “fixed” or dried onto the carrier substrate. This fixing step is most easily accomplished by drying in a unidirectional air flow cabinet or hood, although other drying methods including vacuum drying, lyophilization, and other fixation methods could be used. In depyrogenation challenge studies, once a fixing method is chosen, it should not change in subsequent studies to assure comparability of results. Before using the EIs, a recovery procedure, consisting of a reconstitution or extraction method, should be developed and verified for consistency (*3*).

For liquid carriers such as bulk product, the level of inoculation in EU/mL should be justified based on “worst case” challenge for the depyrogenation step under study, meaning that the highest concentration of endotoxin that could be in the upstream product, based on process knowledge and historical endotoxin values, should be used. Such justification should take all contributing factors into account, including but not limited to: Gram-negative bioburden in raw materials and bulk; endotoxin content in raw materials including water, contributions by product contact surfaces; and the effect of hold times, particularly for nonsterile bulk.

4.3 Recovery of Endotoxin from EIs

To use EIs, it is necessary to recover and quantify the activity of the endotoxin or LPS from both unprocessed indicators (controls) and from processed indicators (i.e., those that have been through the depyrogenation process). LPS tends to adsorb to surfaces and may aggregate or disaggregate in some product matrices; therefore, recovery of activity from EIs made with LPS is often not 100% of the nominal or measured spike value. This section addresses the methodology for recovery and possible strategies for addressing recoveries that may be observed in challenge testing.

In the case of commercially available EIs prepared with LPS, the manufacturer’s directions for extraction and recovery should be followed. With such products, there should be little difficulty in achieving recovery within a factor of 2 of the labeled LPS concentration. If recoveries within the specified range cannot be achieved, the manufacturer should be contacted for technical assistance.

For EIs made in-house using LPS, the composition of carriers, such as plastics, can affect recovery or result in inconsistent recovery because of adsorption. For these carriers, there is no prelabeled concentration to verify. In this case, the expected recovery should be based on the measure of the activity of endotoxin or LPS added to the article and the volume of extraction fluid used to recover it. The actual (measured) activity in the extract should then be compared to the measured activity of the endotoxin or LPS added to determine the percentage of recovery.

For example, consider a stock endotoxin or LPS preparation containing a measured activity of 100,000 EU/mL that is used to prepare in-house EIs. If a volume of 50 µL of this preparation is dried on the surface of each of a number of 10-mL vials, the known amount of activity added is 5000 EU. If the recovery/extraction is performed in 5 mL of water for BET, and the recovery is 100%, the expected activity in the extract solution is 1000 EU/mL.

$$\frac{5{,}000\ \text{EU/vial}}{5\ \text{mL extraction solution/vial}} = 1000\ \text{EU/mL}$$

If, however, the measured activity after extraction is 200 EU/mL as opposed to the expected 1000 EU/mL, the efficiency of the extraction method is 20%.

Recovery of endotoxin or LPS from nonliquid EIs prepared in-house can follow recommendations for the extraction of medical devices in preparation for LAL testing. *Medical Devices—Bacterial Endotoxin and Pyrogen Tests* ⟨161⟩ states, “The standard extraction method is to soak or immerse the device or flush the fluid pathway with extracting fluid that has been heated to 37 ± 1.0°, keeping the extracting fluid in contact with the relevant surface(s) for NLT 1 h.” The volume used for reconstitution or extraction should be appropriate for the material, size, and shape of the EI, recognizing that a volume too low may not efficiently recover the endotoxin or LPS and that excessive volumes will unnecessarily dilute the endotoxin or LPS that has been extracted.

If the recovery of added endotoxin or LPS is variable, an alternate extraction method may be developed and validated. This may include agitation or mixing, sonication or alternative extraction solutions. A combination of extraction in 0.01% sodium laurel sulfate, sonication, and vortex mixing is one such approach that has been reported to be more effective than extraction in water for medical devices (*4*–*6*). Other extraction methods are summarized by Bryans et al. (*7*) and in ANSI/AAMI standard ST72:2011 (*8*).

Another situation concerns liquid endotoxin or LPS preparations that are used either to validate a depyrogenation process in a process stream or to investigate the destruction or removal of endotoxin or LPS in a manufacturing process. In these cases, the initial concentration of the stock liquid endotoxin or LPS solution should be measured before it is added to the system or process. If some of this preparation is added ("spiked") to a bulk process solution that is then subject to a particular process or treatment, the activity of endotoxin or LPS in this bulk solution should be measured and recorded as the starting activity. It is important to determine whether changes in the endotoxin or LPS activity of the processed solution are due to effects of the process and not to instability of the LPS or endotoxin in the solution. The stability of the activity of the LPS or endotoxin in these preparations should be verified over a period appropriate to the proposed use of the preparation.

As with the spiking method (choice of endotoxin/LPS and "fixing" process), whichever reconstitution/extraction procedure is chosen should be verified for consistency and should be used for all subsequent studies to assure comparability of results.

4.4 Choice of Test Methodology for the Analysis of EIs

Any of the test methods described in ⟨85⟩ can be used for the analysis of processed and unprocessed EIs. As with the rest of the methodology, it is highly recommended that an assay (kinetic turbidimetric, kinetic chromogenic, or gel clot assay) be chosen during method development and used consistently throughout the initial study and in subsequent studies to assure that data are comparable. The use of alternate assays is permissible, provided that they are validated to assure that they are equivalent to or non-inferior to the standard compendial assays.

5. ANALYSIS OF RESULTS OF DEPYROGENATION STUDIES

To evaluate the effectiveness of a depyrogenation process, the residual activity that is recovered from processed indicators is compared to the endotoxin or LPS activity of unprocessed controls. Typically, the $\log_{10}$ of the endotoxin or LPS activity measured for the processed EI (or solution) is subtracted from the $\log_{10}$ of the measured endotoxin or LPS activity of unprocessed control indicators. The result of the subtraction is the log reduction that is attributable to the depyrogenation process. If there are multiple controls and/or samples of processed material (and there usually are), the most conservative approach is to subtract the highest $\log_{10}$ concentration recovered from the processed EIs (or solution samples) from the lowest $\log_{10}$ unprocessed control endotoxin activity. For example:

- The activities in three unprocessed EIs are 1286, 1000, and 1532 EU/mL
- The activities in three processed EIs are 0.634, 0.512, and 0.496 EU/mL

The log reduction is calculated as:

$$\log_{10}(1000) - \log_{10} 0.634 = 3 - (-0.198) = 3.198 \text{ log reduction}$$

Historically, a ≥3-log reduction has been required by regulatory/compliance guidance. However, depending on the process and historical data, a 3-log reduction may be either excessive or inadequate. For example, for glass vials with a low or nonmeasurable endotoxin content upon receipt, the requirement to continually and repeatedly revalidate with an acceptance criterion of a 3-log reduction of the endotoxin spike of >1000 EU is excessive. Alternatively, a fermentation process with an endotoxin content of $>10^7$ EU/mL in the clarified culture supernatant will require more than a 3-log reduction to achieve safe levels of endotoxin in the drug substance or drug product. Additionally, given the sensitivity of BET assays, it may not be necessary to spike with 1000 EU to demonstrate a 3-log reduction. For example, if the assay sensitivity is 0.005 EU/mL, one may choose to spike with 50 EU and demonstrate an ultimate recovery in the test articles of less than 0.05 EU/mL, which calculates to greater than a 3-log reduction. In any event, the design of experiments including an appropriate specification for the log reduction of processed indicators and required test sensitivity to demonstrate the specified log reduction should be established and justified in a preapproved protocol for the study. The total reduction, of course, may be achieved over several steps in a purification process. Thus, the necessary reduction is often achieved additively over the course of multiple purification steps.

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