ENDOTOXIN

Naturally Occurring Endotoxin: A New Reference Material Proposed By the US Pharmacopeia

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In response to stakeholder requests, USP (US Pharmacopeial Convention; www.usp.org) is proposing a new reference standard, Naturally Occurring Endotoxin (NOE), to be prepared from cell wall extracts of a well characterized Gram negative bacterium. The proposed reference standard (RS) was developed by analyzing and observing the properties of currently available endotoxin reference materials (purified lipopolysaccharide, or LPS), such as, USP Endotoxin RS as analytes in depyrogenation and hold-time studies. This proposed NOE standard is intended to be used in hold-time studies, depyrogenation studies, and other studies that require, or would benefit from, the use of a "naturally occurring" endotoxin.

It is not the USP's intent that this new reference standard will replace the current USP Endotoxin RS used as a reference standard for *Bacterial Endotoxins Test <85>*.[1]

Endotoxins, LPS

Endotoxins exist in nature as vesicles or "blebs" in the outer cell membranes of Gram negative bacteria.[2] Cell wall fragments (dead bacterial structures) that are shed as part of the normal bacterial life cycle are the *real-life contaminants* which would be present in pharmaceutical raw materials, water systems, in process samples, and finished drug products.

Purified endotoxin is chemically defined as a LPS and the ability of this material to evoke a febrile response is conserved after Westphal extraction and other purification process steps. However, Westphal extracted LPS while often called 'endotoxin' is not found in nature. Also, owing to the amphipathic nature of the LPS molecule [i.e., having both a polar (hydrophilic) end and a non-polar (hydrophobic) end], LPS and native endotoxins are biochemically dissimilar in many respects. As a purified preparation, the LPS, stripped of its cell wall components, will adsorb to surfaces, forming micelles, ribbons, and other aggregate in solution. LPS preparations such as the USP Reference Standard Endotoxin (RSE) have historically been used as calibration standards and positive controls during the development and qualification of the Bacterial Endotoxins Test (BET). Different product formulations can affect the aggregation state of the purified LPS molecules, making them more difficult to detect and therefore causing BET test interference or inhibition. The extent of aggregation of LPS in solution is affected by a host of product formulation attributes, such as temperature, pH, salt concentration, divalent cation concentration, detergents or emulsifiers, and the presence of chelating agents.[3]

Endotoxins and Depyrogenation

Parenteral products not only need to be sterile, but also free from harmful levels of pyrogens, or fever- causing agents. Bacterial endotoxins are the most prevalent pyrogenic contaminants in healthcare products. Depyrogenation is defined as the direct and validated destruction or removal of pyrogens.[3] Although the use of LPS in the use of endotoxin indicators for dry heat depyrogenation has long been the standard for validation of depyrogenating ovens for glass vials, depyrogenation of product streams, particularly complex biological formulations, may rely on ionic attraction, filtration, or other physical or chemical means. In these cases, the use of LPS as a surrogate for native endotoxin that might actually contaminate the product, may be an inappropriate choice, as it is chemically, biologically, and structurally different from natural Gram

negative bacterial cell wall fragments which could be present as product contaminants. Depending on the materials of construction or the formulation of the article to be depyrogenated, the use of a native endotoxin as a challenge material in depyrogenation studies may be a consideration because a native preparation better reflects operational reality. This is particularly true for the depyrogenation of product streams. Also, because LPS molecules in NOE are embedded in cell wall complexes, they may be much less prone to the aggregation and adsorption issues which can be observed when using Westphal extracted and purified LPS.[3]

The new USP Chapter *Endotoxin Indicators for Depyrogenation <1228.5>*[4] includes recommendations for the identification of bacterial strains, along with methodology suggestions for the preparation, use, storage, and documentation of NOE *that more closely mimics "real world" process or product related contamination*. There is not one "best" or "standard" method for preparing NOE in the laboratory, but one example of a published method for the preparation of laboratory-prepared endotoxin may be found in the publication of Bowers and Tran.[5] 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. It is important to note that although the coliform bacteria of the genera Escherichia or Salmonella commonly used over the years in as sources for LPS calibration standards, they are uncommon process, product or environmental contaminants in industry. However, the recovery of non-fermenting Gram negative organisms is routinely reported. Establishing the identity and baseline genetic fingerprint of an environmental organism will assure that subsequent preparations are consistent.
- The activity of the endotoxin preparation should be established by comparing its activity to the USP Endotoxin RS. As with the USP Endotoxin RS 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 used for the analysis. It is recommended, consistent with the assignment of potency for the NOE, 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. It should also be demonstrated 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 Endotoxin Units (EU), 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.

LER, LPS and NOE

Low LPS recovery has been reported in some undiluted biopharmaceutical formulation matrices containing divalent-cation-chelating buffers and polysorbate.[6] These studies were undertaken to fulfill one company's broad interpretation of Question 3 of the 2012 FDA Guidance on Pyrogen and Endotoxin Testing[7] which requires that assayable endotoxin must remain detectable during sample storage and hold times. Similar observations on the loss of activity of LPS in matrices with chelators and surfactants have been reported earlier.[8,9] The term, "Low Endotoxin Recovery" or LER, was coined to describe this type of interference. However, subsequently and not surprisingly, a number of investigators,[10-14] have reported that a valid recovery of activity as measured by BET was achieved when a laboratory-prepared endotoxin, rather than the LPS, was introduced to the undiluted formulation of protein based products as the challenge material for the same hold-time studies.

While LPS preparations (Endotoxin RS) may be well suited for analytical calibration and in most cases as a spike in recovery studies, it is important to note that these LPS preparations bear minimal resemblance to the real world endotoxin contaminant that might be present during pharmaceutical manufacturing. Neither Endotoxin RS nor CSE are designed or supplied with the intention of chemical or structural fidelity to native bacterial endotoxin. It is therefore scientifically and practically justifiable that a well characterized NOE preparation should be suitable for the performance of these in process hold time and process stream depyrogenation studies.[15,16]

USP and NOE

While a "non-standard" NOE can be prepared by individual laboratories or companies, concerns from some stakeholders have been lack of characterization, possible lack of lot-to-lot consistency, variability in the choice of organism, variability in the growth of the organisms and purification of the endotoxin. Consequently, in response to stakeholders requests, and consistent with its role as a standards setting organization, USP recognizes that a well

characterized NOE material that addresses these concerns can be made available for stakeholder use. The USP intends to distribute a well characterized NOE for use in hold time, depyrogenation studies and other pharmaceutical studies that require or would benefit from the use of a NOE. Toward that end, the USP has launched an initiative to make a naturally occurring endotoxin (NOE) available to the stakeholders.[17]

The proposed NOE standard will have the following characteristics:

1. It will be universally available and distributed by USP.
2. It will be available in large quantities to assure consistency.
3. It will be accompanied by a Certificate stating its activity in EU.
4. To reduce lot-to-lot variability, it will be:
    a. Prepared from a well characterized bacterial species that is considered in industry to be a representative contaminant of pharmaceutical products such as those administered intravenously).[18] A member of the larger family Enterobacteriaceae is consistent with this definition and is closely related to the current purified LPS calibration standards, which are prepared from various strains of *E. coli*. The target organism for the preparation will be procured from a single source to assure consistency and genetic stability. The single source may be a recognized culture collection (e.g. ATCC) or a laboratory strain that is preserved, used, and stored consistent with the principles of good microbiological practice as described in USP <1117>.[19]
    b. Manufactured and vialed under controlled conditions, meaning that it will have a batch record, will be subject to change control, and will have appropriate specifications that will be recorded on the Certificate.

Further, in response to USP's early input process notification on the feasibility of such a standard, stakeholders have indicated strong support for the initiative to make such a NOE Standard available for the demonstration of the stability of assayable endotoxins as well as a control in augmenting manufacturing processes. The stakeholders have also urged the USP to clearly establish the correct terms and definitions for the different forms of "endotoxin" standards consistent with recent developments in the field.

References

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