De-mystifying Low Endotoxin Recovery (LER)

Allen L. Burgenson, Associate Director, Testing Solutions and Global SME, Lonza Walkersville

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Abstract

Low Endotoxin Recovery (LER) is described as the inability to recover a known concentration of Control Standard Endotoxin (CSE), or Reference Standard Endotoxin (RSE) over time. It is a two-part reaction requiring a chelator and a surfactant. The chelator strips away the divalent cations causing the Lipopolysaccharide (LPS) aggregates to disassemble. Since Lipid A of LPS is hydrophobic, polysorbate encapsulates the LPS in a lipid micelle. This form of endotoxin is unable to react with Factor C of the Limulus Amebocyte Lysate.

The FDA and EMA require all new biologic drugs submitted for approval to be assessed for the presence of the so-called LER phenomenon. However, it should be noted that there has never been a recorded case where a product was released into commerce with a passing LAL test, and then subsequently was found to be pyrogenic in the field due to endotoxin contamination. Nor has there been a case of a health hazard attributable to the LER phenomenon.

Organisms used to produce RSE/CSE standards are grown under high nutrient conditions including divalent cations, then purified. The divalent cations allow the organisms to develop "salt bridges" between adjacent lipopolysaccharide molecules. However, organisms in purified water systems are not exposed to divalent cations in their environment and adapt to this environment via the PhoP/PhoQ system on the bacterial surface, holding the outer membrane together. These adapted organisms' endotoxin is not affected by the chelator, and the aggregates remain intact. This is the endotoxin that will contaminate the product if a breech in the water purification system happens. This presentation discusses the use of a calibrated autochthonous endotoxin in LER hold time studies, as well as mitigation strategies to differentiate assay interference from the LER phenomenon.

Background

In 2012, The US Food and Drug Administration retired the long-standing "Guidance on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices (1) This document was replaced with the Guidance for Industry: Pyrogen and Endotoxins Testing: Questions and Answers (2). This document set the expectation in Question 3: "Is sample handling and storage important?" that the level of assayable endotoxin (activity in products that are naturally contaminated) in a sample be the same after a hold period before testing as it was at the time of manufacture. This led analysts to use a control standard endotoxin (CSE) or USP reference standard endotoxin (RSE) as a surrogate because their products were not naturally contaminated with "assayable endotoxin", to establish the initial concentration from which any potential reduction in endotoxin recoverability could be assessed. However, these standards are not representative of the endotoxin that may contaminate a product. The potential differences of recovery between CSE or RSE and natural contamination were specifically discussed in the FDA Q&A document, stating "Protocols should consider the source of endotoxins used in the study, bearing in mind that purified bacterial endotoxins might react differently from native sources of endotoxins."

Natural or autochthonous endotoxin is the unpurified endotoxin that results from the growth of Gram-negative organisms, mostly from the water systems in the manufacturing environment. When water systems work as designed, they work very well. However, when they break, due to lack of appropriate maintenance and repairs, wear and tear on critical components, or inadequate sanitization after repairs, they do not.

For example, the water systems used to manufacture pharmaceutical products are a significant cause of endotoxin ingress into manufacturing processes (3) if they fall into disrepair. Such water systems may be breached via:

1. Leaking valve in production caused by improper/incomplete repair of a valve causing contamination of the water system
2. Malfunctioning heat exchanger in WFI loop, such as pinholes in a heat exchanger using mains (potable) water to cool WFI loop
3. Improper return from shutdown, such as improper completion of maintenance operations after shutdown. Additional tasks performed outside of the scheduled maintenance tasks

The Control Standard

The current Reference Standard Endotoxin (RSE) was developed for use as a calibration standard in water for the Limulus Amebocyte Lysate Assay (4) standard curve and was not designed for direct product inoculation. Its use as a positive product control is to determine if there are any assay interferences that cause the PPC to act as if it is not in water. Any interferences affecting PPC recovery are mitigated through dilution or sample treatment(s) as illustrated below.

The RSE is derived from *E. coli* strain 0113:H110K(-), while the CSE distributed by manufacturers is either 0113:H110K(-), or *E. coli* strain 055:B5, and are formulated with lactose, polyethylene glycol, and other excipients not found with native endotoxins.

"Is the current standard acceptable for this use as a surrogate for natural contamination?"

FDA personnel have stated that the endotoxin used as the recoverable analyte must be highly characterized when used in this application without an explanation as to why. This appears to be a dogmatic approach that seems to contradict longtime FDA expectations related to microbial testing, such as using representative organisms from a manufacturing facility when determining media growth promotion suitability in the USP <71> Sterility Test, or <61> Microbiological Examination of Nonsterile Products. Further, this requirement to use a well characterized CSE does not allow for the fact that the CSE or RSE do not occur in nature and have significant differences from the facility's autochthonous endotoxin which would contaminate a product.

Question 13 of the 2012 FDA Q&A document asks, "Are control standard endotoxins still acceptable for use in running bacterial endotoxins tests?" The response further clarified that any standard must be calibrated to the USP reference standard. Autochthonous endotoxin from a manufacturing facility may easily be quantified and calibrated for use as an analyte CSE.

Organisms in the manufacturing area have adapted to their environment. Such adaptations are the ability to grow under low nutrient conditions via the PhoQ/PhoP two-part regulatory system that senses low nutrient conditions (5) such as a low divalent cation availability in membrane assembly, and rather than linking the phosphate groups of individual LPS molecules with divalent magnesium "salt bridges", a sugar may be introduced instead to bind the phosphate groups together.

"The standard is the standard and is suitable in all applications"

This is a false assumption. In its natural state, endotoxin is an amphipathic entity, possessing both a hydrophilic and hydrophobic portion that allows it to remain dispersed and stable in solution. The USP Reference Standard Endotoxin (RSE) or Control Standard Endotoxin (CSE) provided by LAL kit manufacturers are highly purified entities that do not occur in nature. The hot phenol "Westphal" extraction process removes membrane associated proteins and partially cleaves the polysaccharide "tail" (6), both of which offer stability in aqueous solution and persistence in the sample matrix. The amphipathic nature is significantly affected, and the purified lipopolysaccharide becomes more hydrophobic and susceptible to LER. Placing such an analyte into a "LER matrix" product causes the LPS aggregates to disperse into monomers and become entrapped in a polysorbate micelle. Such effects do not happen when a facility's autochthonous endotoxin is used.

"Analytical standards must be as pure as possible"

This statement not always true, and in many cases impractical. For example:

- residual host cell DNA
- residual host cell protein standards

These materials are minimally purified by design and heterogeneous that are used nonetheless as analyte standards in biological assays in the development and testing of biological products.

The LER Mechanism

As described in PDA TR-82 LER, the LER phenomenon is a two-step process as illustrated below in figure 1. First, the chelator removes the Mg++ from the endotoxin aggregates, causing the LPS monomers to disperse. The LPS monomers are then engulphed in a polysorbate micelle, which is not reversible under physiological conditions.

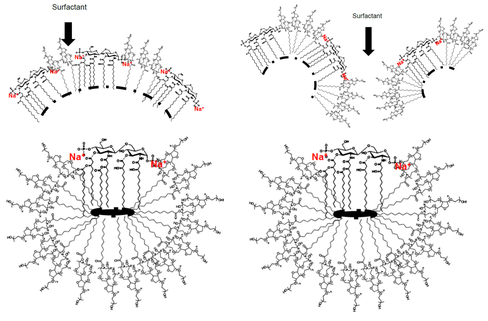

Figure 1. LER mechanism

Differentiating between LER and assay interference

Assay interferences may be confused with the LER phenomenon. However, assay interferences may be overcome using one or more mitigation strategies, while the "LER phenomenon" is not reversible under physiological conditions. The following table lists possible mitigating strategies.

Treatment	Sample type	Notes
Dilution	Most samples	Up to MVD
pH adjustment	(Unbuffered) samples with pH outside range	pH 6-8 in sample/lysate mixture
Heat	Sample containing - active protease - proteins causing endotoxin masking	Variety of times / temperatures described in literature, e.g. 75°C / 30 min
Surfactant/Dispersing Agent (Pyrosperse™)	Oily samples, components binding endotoxin	
Glucan blocker	Sample containing LAL-reactive material/glucans	Possible glucan sources: fungi, cellulose filters…
Divalent cation addition ($MgCl_2$)	Sample containing chelator (e.g. EDTA)	

Table 1. Mitigation suggestions for assay interferences

Putting it into practice

The first step is to develop and execute a LER hold time study as directed by PDA TR-82, keeping in mind the actual hold time from the final bulk formulation step containing the chelator (e.g., citrate) and the surfactant (e.g., polysorbate), to the time it takes to reach the laboratory and is tested. Once LER is determined using the RSE/CSE standard, the most useful exercise is to simulate a manufacturing process breach of the water system that would lead to product contamination. The proper analyte for such an exercise would be the actual endotoxin that would result in product contamination, obtained from the carbon bed upstream water purification system. For the following study, a proper analyte was prepared by obtaining a sample of water at the carbon bed of the pre-purification section of a water system used to prepare Water for Injection quality water. The water was filter sterilized and refrigerated until needed. The sample was treated as an unknown and its endotoxin content determined to be 90 EU/mL using the Kinetic Chromogenic Method (Lonza KQCL method) in multiple replicates. This nominal value was used to determine the proper spiking level of the surrogate product described below.

A representative formulation for evaluation was chosen from an Antibody Therapeutics table of product formulations. (7) In this instance, a 10 mM sodium citrate, 0.02 % polysorbate 80, and 0.5% sucrose solution was chosen as a surrogate product. The solution was filter-sterilized and refrigerated until needed.

Before a LAL assay may be run, the Maximum Valid Dilution (MVD) and Minimum Interfering Dilution must be determined, and steps to mitigate any assay interferences be established. The MVD is defined as the highest level of dilution that may be performed to overcome interferences, yet still able to recover endotoxin at the Endotoxin Release Limit (ERL). In this particular instance, since this was not an actual product dosage form, the endotoxin release limit was arbitrarily assigned a value of 5 EU/mL. The MVD was calculated as:

MVD = ERL / λ (lowest point on the standard curve)

MVD = (5 EU/mL) / 0.005 EU/mL)

MVD = 1000 or 1:1000

The minimum interfering dilution (MID) was determined to be 1:10 when a 10 mM MgCl2 solution was used as a serial diluent to overcome the assay inhibition caused by the chelating effects of the 10 mM citrate of the test solution as per Table 1 above. The test dilution selected was between the MVD and MID at 1:100. A second dilution at the 1:1000 MVD was also tested as a back-up.

5 mL of the surrogate test solution containing the "LER matrix" of citrate and polysorbate 80 was pipetted into each of twenty sterile depyrogenated screw cap borosilicate tubes. The tubes were divided into four groups of five, and labeled T=0, T=8h, T=24h, T=48h, and T=96h. The labeled tubes were then assembled into two equal groups and labeled; one for CSE, and the other for the autochthonous endotoxin from the water system. Five tubes from those groups would be incubated at 5 ± 2C, while the other five tubes would be incubated at ambient room temperature between 20 and 25C.

The CSE group were spiked at T=96h, T=48h, T=24h, T=8h in reverse chronological order over a week with 0.5 mL of the control standard endotoxin for a resulting nominal spike level of 4.5 EU/mL and incubated at either 5C ± 2C or 23C ± 2C.

The autochthonous test group were spiked at T=96h, T=48h, T=24h, T=8h in reverse chronological order over a week with 0.3 mL the autochthonous standard for a final nominal spike concentration of 5 EU/mL and incubated at either 2-8C or 20-25C.

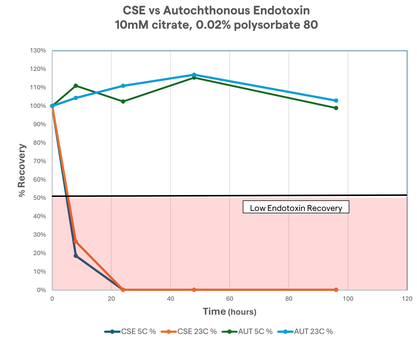

Figure 2. Comparative assay results

A theoretical hold time from the final process step to final endotoxin testing in the QC laboratory was set as 72 hours, and the hold time test ran for 96 hours.

Due to the rapid rate of the LER reaction in these samples, T=0 could not be done in the sample matrix. A 5 mL WFI blank was used for the CSE and surrogate contaminant instead and served as the anchor content for the study.

Upon completion of the incubation periods, all of the 5 ± 2C cohort were tested together on one 96-well plate, using one standard curve to reduce assay to assay variability. The 23C cohort were tested in the same manner.

Results and Discussion

All data were collected and tabulated in a spreadsheet. To account for the differences in spiking levels, all data were compared to T=0 for each respective analyte and reported as a percent recovery. These data are depicted in the graph seen above in Figure 2.

As expected, the products spiked with CSE performed nearly equally regarding the LER phenomenon at either 5 ± 2C or 23C (see above). No CSE was recovered at 24 hours. However, the autochthonous endotoxin cohort showed no reduction in content at either temperature over 96 hours, indicating that the endotoxin from a process breach is easily recovered over the entire hold time.

Conclusion

The highly processed CSE, an entity that does not occur in nature, is not an appropriate analyte for LER hold time studies, as it could never contaminate products during manufacturing. Using the analyte that more accurately represents potential process contaminants to the product during manufacturing demonstrated that if there is a process breech causing contamination of the formulated product, such contamination is readily detectable, and a contaminated batch discarded before it is distributed into commerce.

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