Introduction

Endotoxin testing is a vital element of the manufacturing of parenteral pharmaceuticals and medical devices – and without it there would be a significant risk to patient health. Since its discovery in 2013, low endotoxin recovery (LER) has sparked controversy within the endotoxin detection community.

This phenomenon is the masking of endotoxin in undiluted materials, and is thought to be attributable to combinations of specific excipients. This differs from the inhibition or interference of endotoxin tests caused by pH, high divalent ion concentrations, chelators, serine proteases and beta-glucans, which can usually be overcome using a pre-treatment such as dilution.

The LER Challenge

The Endotoxin Complex

Endotoxins, a type of pyrogen, are genetically synthesized constituents of the outer membrane of Gram-negative bacteria. This envelope provides protection for the cell and controls permeability. The natural endotoxin complex contains many cell wall components including phospholipids, lipoproteins, and lipopolysaccharide (LPS), which is the biologically active component of endotoxin (Figure 1, next page).
LPS provides Gram-negative bacteria with structural integrity and, importantly, provides a virulence marker for virtually all metazoans. It has long been known to activate both the classical and properdin pathways of complement (triggered by the Lipid A and polysaccharide regions respectively).1

The biological activities of LPS include pyrogenicity and immunogenicity and therefore, on introduction to the human cardiovascular and/or lymphatic systems, can cause severe clinical symptoms and even death.2 For this reason, any contamination of parenteral pharmaceuticals with bacterial endotoxin poses a significant health risk. To protect patients from this, all products labeled as sterile or pyrogen-free must be tested to ensure they are negative for endotoxin. For medical devices, this includes everything ranging from simple fluid catheters to complex knee replacements.

Given the ubiquity of Gram-negative bacteria, their ability to proliferate in nothing more than clean water, and the difficulty of destroying LPS as a residue of Gram-negative bacteria, endotoxin control is a constant concern for pharmaceutical and device manufacturers.

**Pyrogenicity Testing**

There are two primary methods currently available for detecting pyrogenicity in parenteral drug products:

- **Rabbit Pyrogen Test (RPT)** – This involves the injection of a small amount of batch test material into a rabbit’s bloodstream, and monitoring for temperature increases. This test is required for parenteral biological products by law in the US [21 CFR 211.167(a) and 21 CFR 610.13(b)]. However, the RPT requirement can be waived provided that an alternative method is shown to be equivalent or superior.3

- **Limulus Amebocyte Lysate (LAL)** – This test is a bacterial endotoxin test (BET) derived from the blood cells of the horseshoe crab, *Limulus polyphemus*. Factor C is the first enzyme (the biosensor) within the enzymatic cascade that forms the basis of this test. The binding of Factor C to the hydrophobic lipid component of the LPS molecule initiates an enzymatic cascade that results in the formation of a blood clot, which envelopes the invading endotoxin.4 The LAL assay is reliable, sensitive and specific – and is now used almost exclusively in place of the RPT for the testing of endotoxins in biotechnology products, where equivalency can be demonstrated.2

The sensitivity, reliability and relative simplicity of LAL-based assays make them the method of choice in most labs. However, more recently, the detection reliability of LAL tests has come into question through the phenomenon of low endotoxin recovery (LER).

**Low Endotoxin Recovery (LER)**

LER is the failure to detect a known amount of spiked endotoxin in an undiluted product (positive control) over multiple time points. The phenomenon was first reported by Chen and Vinther at the PDA Annual Meeting in 2013 when they described how endotoxin was masked in a sample containing a chelating agent and a detergent.5 Neither dilution of the sample nor magnesium replacement resulted in recovery of endotoxin. Endotoxin was not detected by the LAL test, however, pyrogenicity was detected when injected into rabbits. Many key opinion leaders now believe a similar endotoxin masking effect may have been observed for several decades, although reported cases were rare.

LER is the masking of endotoxin in undiluted materials, thought to be attributable to combinations of specific excipients. This differs from the inhibition or interference of endotoxin tests caused by pH, high divalent ion concentrations, chelators, serine proteases and glucan, which can usually be overcome using a pre-treatment such as dilution.

**Importance to the Manufacture of Biologics**

To date, concern around LER has mainly been associated with certain biological drug products, particularly monoclonal antibodies or therapeutic proteins as they are usually stored in their final form in a matrix of chelating agent and detergent. The scale of this issue is likely to increase as it is anticipated that biologic agents will continue to outpace the overall growth in Pharma spending, representing 19–20% of the total market value by 2017.6

A rabbit pyrogen test (RPT) must be performed on all biologics to determine the drug’s safety prior to administering to a patient. Alternatively, in lieu of the RPT, a bacterial endotoxin test such as LAL, can be used if the chosen BET method is deemed equivalent to the RPT (as stated in 21 CFR 610.9). It is this use of the LAL assay that has made biologics the main target for LER evaluation by the pharmaceutical industry.

The primary concern with LER is the potential for a product to test negative in the LAL assay but positive in the RPT. Such a result could mean the product induces a pyrogenic reaction in a patient and causes a serious clinical outcome. Consequently, some drug manufacturers have had to resort to using the RPT for testing of products shown to be affected by LER when using the LAL method.

To determine whether LER observed *in vitro* actually corresponds to pyrogenicity *in vivo*, biologics manufacturers are typically requested to conduct studies in which the product is spiked with an endotoxin standard, held, and then tested at various time points by the LAL and RPT methods in parallel.
In 2012, the FDA began to request the use of spiking/hold-time studies as part of their Q&A guideline document in order to demonstrate the capability of the LAL BET method to recover endotoxin from spiked samples over time. To achieve this, a known amount of endotoxin is added to undiluted biological products and then tested at intervals to ascertain the recovery over time. It was through these spike/hold-time studies that LER was first detected. It is expected that such results be included in Biologics License Applications (BLA) submitted to the Center for Biologics Evaluation and Research (CBER).

Ever since, LER has remained a controversial topic that has divided the endotoxin community.

Understanding LER

LER Mechanism

The actual mechanism of LER is still poorly understood – partly due to the complex supramolecular structure of LPS, and partly because the LAL enzyme cascade can be influenced by a wide range of experimental parameters. However, we know that LER is only observed during attempts to recover LPS in undiluted drug products or substances, and is time and temperature dependent. It is also well recognized that there is a correlation with the use of the excipients citrate and polysorbate – although, no direct cause or effect relationship has yet been established. Spiking studies from 27 recent BLA submissions were reviewed for LER. From 27 submissions, LER was detected twice (Table 1); once when polysorbate was combined with citrate and once with histidine.

While LER has only fairly recently been identified as a challenge, there is some evidence that it has been in existence for some decades. With the use of biologics on the rise, the importance of better understanding the LER mechanism has become a priority.

Table 1. Detection of LER using spiking studies from 27 recent BLA submissions.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Occurrences of LER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine with polysorbate</td>
<td>1 (from 7 submissions)</td>
</tr>
<tr>
<td>Citrate with polysorbate</td>
<td>1 (from 4 submissions)</td>
</tr>
<tr>
<td>Citrate without polysorbate</td>
<td>0</td>
</tr>
<tr>
<td>Acetate with polysorbate</td>
<td>0</td>
</tr>
<tr>
<td>Glutamate with polysorbate</td>
<td>0</td>
</tr>
<tr>
<td>Succinate with polysorbate</td>
<td>0</td>
</tr>
<tr>
<td>Phosphate without polysorbate</td>
<td>0</td>
</tr>
</tbody>
</table>

LER vs Interference

So, what makes LER different from other sources of interference? The key differentiator here is time-dependence, where LAL test interference is not necessarily time-dependent and can usually be resolved through simple pre-treatment methods such as dilution, pH-adjustment or divalent cation addition. LER, or endotoxin masking as it is also known, is defined as an inability to recover a known spike of endotoxin over several time points. Here, the active form of endotoxin undergoes changes resulting in lower activity. To distinguish between interference and LER, it is recommended to perform time-dependent experiments in addition to a positive control. If recovery of endotoxin reduces over time, masking is occurring.
Aggregation
LPS is an amphipathic molecule (has both hydrophilic and hydrophobic parts) that forms micelles, ribbons, or other three-dimensional conformations in aqueous solution. It is thought that aggregation could be one of the factors determining the biological activity of endotoxin and many believe it to be the most likely cause of LER.

The molecular weight of the LPS monomer varies from 10,000 – 20,000 Da and aggregates from 300,000 – 1,000,000 Da. Aggregates are the biologically active form of LPS and can be (reversibly) dissociated by deoxycholate (DOC) into monomers. In addition, the degree of LPS aggregation is also known to vary in different salts.

The pyrogenicity of LPS is affected by the size of the aggregates. Where pyrogenicity has been lost following the addition of DOC, it is possible to reverse this through dilution or dialysis, thereby reducing the concentration of DOC. This suggests there is a minimum size of LPS aggregates required to cause pyrogenicity.

The presence of citrate and polysorbate also affects the aggregation of LPS; destabilizing the aggregates causing them to break down into biologically inactive monomers. In fact, it is likely that citrate removes divalent cations from within the LPS aggregates and the detergent disperses these weakened complexes. However, in this case, the aggregation is not easily recoverable through dialysis.

In studies, the presence of human plasma seems to prevent the recovery of LPS potency via DOC, and pyrogenicity is not observed. As such, some members of the endotoxin testing community believe that the potency of LPS in LER conditions is unlikely to be recovered in vivo, so there is no risk to patient health.

Sample Preparation
It has been shown that sample preparation procedures can greatly influence the ability to recover LPS from spike/hold-time studies. After all, the morphology of LPS can be affected by physical (temperature, sonication, lyophilization) or chemical (pH, or the presence of detergents or metal cations) conditions. This problem is compounded by the lack of standardized procedures for spike/hold-time studies and few peer-reviewed papers that describe the experimental details. It has also been determined that endotoxin adheres to plastic surfaces more strongly than to glass surfaces, therefore, it is recommended that only glassware is used in the preparation of samples and endotoxins. In some cases, certain plastics may prove to be acceptable as sample containers, however, it is suggested that it the users are going to store samples for any period of time prior to testing, they should validate the sample storage approach and verify that any endotoxin present in the sample will be recoverable after storage.

Bolden et al. demonstrated that without consistent sample preparation, such as mixing time and sampling scheme, significant batch-to-batch variation in endotoxin testing can occur. They concluded that it is essential for spike/hold-time studies to be performed in full alignment with the verified BET method, including mixing time and sampling scheme, in order to effectively mitigate potential safety risks associated with LER.

There are currently no established principles and conventional procedures for how to design and execute spike/hold-time studies.

Sources of Endotoxin
While the requirement to spike samples with endotoxin is well understood, there is disagreement within the endotoxin community regarding the source of this endotoxin. In USP Chapter <85>, a bacterial endotoxin is simply defined as a component of the outer cell membrane of Gram-negative bacteria. The FDA has never defined the term ‘assayable endotoxin’. There are three potential sources of endotoxin for spike/hold-time studies:

- **Reference Standard Endotoxin (RSE)** — Contains LPS that has been extracted and purified from *E. coli* O113:H10. It does not and cannot exist in nature or as a contaminant in parenteral products. The USP Endotoxin Reference Standard is one such RSE and has a defined potency of 10,000 USP Endotoxin Units (EU) per vial.

- **Control Standard Endotoxin (CSE)** — These are endotoxin preparations other than the international or national reference standards, which are traceable in their calibration to the international reference endotoxins standard. CSEs may be secondary or tertiary standards and are usually manufactured and certified by an LAL reagent manufacturer for use with a specific lot or reagent under defined assay conditions. CSE is an economical alternative to RSE, is widely used for endotoxin testing, and may be a purified extract from the same strain used for the RSE.

- **Naturally Occurring Endotoxin (NOE)** — The natural endotoxin complex contains many cell wall components, including phospholipids, lipoproteins, as well as LPS, the biologically active component of endotoxin. As such, NOEs are crude preparations of endotoxin extracted from a growing culture of Gram-negative bacteria. NOEs have not undergone a purification step, unlike the reference standard or control standard endotoxins, and can lead to varying and confounding recovery results.

As shown, the terms endotoxin, LPS, NOE, RSE and CSE are not interchangeable as each has its own specific definition. In reality, a contamination event would not occur by a purified endotoxin standard but rather an endotoxin excreted by Gram-negative bacteria. This rationale is the basis for why some researchers believe that NOEs are a more relevant analyte for hold-time studies than the control standard endotoxin or reference standard endotoxin.

The theory is that NOEs are recoverable and don’t need demasking agents under ‘LER conditions’. It is assumed that the presence of protein and other cell debris in NOEs allows the LPS to be more robust. However, variability is an issue with NOE as these preparations represent unpurified and uncharacterized standards of unknown potency. In some cases, data has been inconsistent. Without a standardized process for developing an NOE, no real conclusions can be drawn by regulators as to whether NOEs actually provide a useful alternative to the purified LPS standards. Compiled data from several BLAs submitted to the FDA show that regardless of the source of endotoxin, CSE or NOE, some LER samples have shown conflicting results between the LAL and RPT test.
RSE is primarily used by LAL producers to determine the potency of LAL reagents and to standardize CSE reagents used as positive controls in a BET. The experience of the LAL industry is that LPS is a reliable source of positive-control material in a compendial BET, but is more unstable than native endotoxin.\(^\text{10}\)

The chemistry behind each product-specific LER interference might be different, but the effect of those different interferences is the same—an inability to recover the spiked endotoxin from the majority of products when tested in their undiluted form.

**Tackling LER**

**A Range of Approaches**

To date, there is no one solution to overcoming LER. Instead, an approach must be established for each sample product and the method adhered to for further studies.

**Current FDA Recommendations for Performing Spike/Hold-time Studies**

In summary, the FDA recommends to:
- Test at least three different lots
- Spike close to the drug specification (at or below)
- Test for at least 7–8 days (finished product)
- Use RSE or CSE as spike standard

**Considerations for Spike/Hold-time Studies**

There are a number of considerations for conducting a proper spike/hold-time study:
- Directly spike into undiluted drug product, drug substance or in-process solution at a level less than the specification.
- Hold product before testing according to pre-determined parameters (temperature, container type, number of days).
- Product should be tested at specific time-points throughout the hold period (at 4 hours, 24 hours, 72 hours, Day 5, Day 7, for example).
- LER is time-dependent; it can occur over the course of 1 week or very rapidly (within 4 hours) in the presence of citrate or phosphate plus polysorbate. More rapid LER effects have been reported when holding at 20–25°C than at 2–8°C.

**Demasking Solutions**

Various demasking solutions have been evaluated to reverse the effect of LER. However, as each LER case is different, these may or may not work for any specific sample. Demasking involves re-assembly of the endotoxin aggregates and as such requires using sample treatments to push the equilibrium towards the aggregate state. Examples include adjusting the pH to influence hydrogen bonding or adding Mg\(^{2+}\)/Ca\(^{2+}\) to the solution to saturate the chelating agent and prevent destabilization of the LPS aggregates. The addition of metallo-modified polyanionic dispersants, such as PyroSperse\(^\text{TM}\), can also prevent the surfactant from interfering with the LPS aggregation state. Dialysis treatment methods have also proven to be successful in overcoming LER in certain products.

**Reverse Study**

A new recommendation from the FDA is performing a reverse study. In this, samples are spiked with endotoxin on different days and then are all analyzed at the same time to minimize inter-assay variability.

**Support From the LAL Community**

So, what has the LAL community been doing to help drug manufacturers who experience LER? The Parenteral Drug Association (PDA) LER Task Force and Biophorum Operations Group (BPOG) LER Task Force were both formed to investigate the effects of LER and provide viable means of overcoming the effects of LER. These task forces comprise LAL suppliers and end-users working closely with global regulators to investigate and provide recommendations for handling LER-affected products.

<table>
<thead>
<tr>
<th>Low recovery of spiked endotoxin (USP BET &lt;85&gt;)</th>
<th>Pyrogenicity in RPT with spiked samples</th>
<th>Release test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>Not tested</td>
<td>USP BET &lt;85&gt;</td>
</tr>
<tr>
<td>No</td>
<td>No</td>
<td>USP BET &lt;85&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>– Tight in-process microbial controls</td>
</tr>
<tr>
<td>No</td>
<td>Yes</td>
<td>USP RPT &lt;151&gt; (interim measure)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>– Tight in-process microbial control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>– Develop suitable release test</td>
</tr>
<tr>
<td></td>
<td></td>
<td>– Risk assessment/in-process testing/in-process microbial controls</td>
</tr>
</tbody>
</table>

Table 2. Outcome of spike/hold-time studies as presented by Dr. Hughes at the Endotoxin and Pyrogen Testing Conference at PharmaLab 2016.\(^\text{11}\)
Regulatory Response to LER

A Cautious Approach
To date, the FDA has taken a conservative and cautious approach when discussing LER and its relevance to drug safety. Although there are no reports or indications so far that LER is a public safety issue, the FDA is concerned that LER could result in endotoxin, not detected by the compendial USP <85> methods, causing a pyrogenic effect in humans.

A Look Back
In 2012, the FDA withdrew a long-standing guideline for LAL testing and replaced it with a series of questions and answers.7 This document stated that ‘for certain biological products, 21 CFR 610.13[b] requires a rabbit pyrogen test. The requirement in 21 CFR 610.13[b] may be waived if a method equivalent to the rabbit pyrogen test is demonstrated in accordance with 21 CFR 610.9 … Bacterial endotoxin assays are subject to a wide variety of interferences related to the physical and chemical properties of the test article. Where such interferences cannot be mitigated through sample dilution (up to the MVD) or other validated means of sample preparation, firms should use the rabbit pyrogen test.’

Also in 2012, the FDA requested spiking studies to determine the ability to detect ‘assayable endotoxin’. This is now interpreted to mean the ability to recover endotoxin in a stored product at the end of its shelf life. It was on the back of these spiking studies that LER was first reported.

NOE
Many BLA applicants have used NOEs from various sources and, in general, no spike recovery issues have been observed – even in cases in which LER was observed with the purified endotoxin standards. However, the FDA has a number of concerns over the use of NOEs, particularly as they are not standards but are uncharacterized and unpurified preparations of different Gram-negative bacteria that can give inconsistent results. At this time, the use of NOE as spikes in spike/hold-time studies is not supported.

Recent Findings
The FDA continues to be concerned about the ability to detect endotoxin in certain formulations of biotech products using the USP BET. It is well recognized that the detection of recoverable endotoxin can be impacted by certain product formulations.

Greater understanding of the LER phenomenon is needed and alternative testing approaches must be developed. All BLA applicants are requested to provide evidence of endotoxin spike recovery data using the USP BET method, conducted in a manner that minimizes any assay variability that could result in errors. Recent findings (2014/15) were consistent with the information above – poor endotoxin recoveries were noted with certain formulations and protein products. In some cases, varying and confounding recovery results were reported due to differences in LAL methods and sample preparation, and/or the influence of different standards. There were also inconsistencies between LER in the LAL assay and pyrogenicity in the rabbit pyrogen test. Some drug manufacturers thought they had LER but it was actually just assay variability, and this was noted as a significant problem. Data was difficult to interpret with respect to LER and the significance to patient safety unclear.

PDA LER Task Force
In early 2015, a PDA LER Task Force was formed with the aims of investigating the root cause of LER, standardizing the experimental protocols for spike/hold-time studies, and identifying the potential safety implications of the LER phenomenon.

During their first LER workshop (2016) several recommendations were agreed:

- If no LER is observed in the final product, it is not essential to perform the spike/hold-time studies with process intermediates.
- Spike/hold-time studies should be carried out in a manner representative of the corresponding QC BET testing with regard to materials, containers, and experimental procedures.
- LER is defined as the endotoxin activity falling below 50% of the spike amount at two consecutive time points.
- Sampling repeatedly from a single-spiked container should be avoided.
- Instead, a reverse assay (spiking independently on different days and testing all the spiked samples on the same day) or a multi-aliquot approach (dispensing the spiked material into individual containers and testing individual containers on different days) is strongly recommended.

Know Your Product
The FDA has been advocating a ‘know your product, know your process!’ approach in order to control and minimize the occurrence of pyrogens in sterile parenteral drug products. They are asking drug manufacturers, ‘how well do you know your process and the sources/origins of pyrogens for your process?’ This kind of information is not only useful for developing risk assessments but also fits well with the Quality by Design (QbD) approach to pharmaceutical manufacturing.

Today, the FDA’s recommendations for performing spike/hold-time studies include testing at least three different lots, spiking close to the drug specification, testing the finished drug product for at least eight days, and using RSE or CSE. The FDA expects the pharmaceutical industry to devise a way to detect LPS under ‘LER conditions’ and, in the meantime, continues to be concerned about the ability to detect endotoxin in certain formulations of biotech products using the USP BET.
Conclusion

All pharmaceutical manufacturers should be cognizant of the limitations of the LAL test method for determining the quality and safety of the finished parenteral product. If LER occurs, it is important this is recognized and testing methods adapted to avoid it. There is no one solution to this, so multiple regimens of sample preparation and spike/hold-time studies must be conducted to find the best method. Alternatively, if LER issues persist, it may be necessary to employ a rabbit pyrogen test. While some members of the endotoxin community believe that the use of NOE in spike/hold-time studies is the way forward, the FDA is not yet convinced and is concerned the results are too variable.

To meet the challenge of LER, a number of task forces have been developed, bringing together people from the industry, from regulators to drug manufacturers and suppliers of endotoxin tests. One of the main questions to be tackled by these task forces is whether LPS activity that has been lost in LER samples could possibly be restored in vivo with clinical consequences. There is no evidence of this occurring so far, but the FDA is clearly concerned. The task forces will also be looking to standardize the way spike/hold-time studies are performed, as well as standardizing the endotoxin controls themselves.

References

5. Chen and Vinther, Presentation at the PDA Annual Meeting, 2013
11. Hughes, P. FDA’s Current Thinking on LER. Presented at the Endotoxin and Pyrogen Testing Conference at PharmaLab 2016

About Lonza

Lonza has been providing endotoxin detection products and services for nearly 40 years, leading the way in innovation for products, methods, and software. In the 1980s, Lonza developed and commercialized the first FDA-licensed endpoint quantitative Limulus Amebocyte Lysate (LAL) assay. Lonza continued to improve upon the quantitative technologies by launching the Kinetic-QCL™ LAL Assay, the kinetic chromogenic LAL method that revolutionized endotoxin detection testing.

In 2003, Lonza brought to market an endotoxin detection method that does not rely on the use of horseshoe crab blood as the raw material. The PyroGene™ Assay uses a recombinant form of Factor C (rFC), the endotoxin-sensitive protein that initiates the LAL clotting cascade. The recombinant technology is endotoxin-specific, eliminating false-positive glucan reactions. It offers exceptional performance and reliable, lot-to-lot consistency, compared to biologically based products, while reducing the dependency on the horseshoe crab. In 2009, 510(K) submissions were FDA-approved using PyroGene™rFC as a final release test.

Recognizing the importance that software plays in accurate analysis and reporting, Lonza has made significant investments in software and has its own in-house development systems and programmers, dedicated to producing robust endotoxin detection software. WinKQCL™ Software is Lonza’s industry leading endotoxin detection and analysis software offering a fully-integrated solution for quantitative endotoxin detection testing, data management and reporting needs. WinKQCL™ Software meets 21 CFR Part 11 technical requirements for electronic records and signatures, audit trails and database archiving.

Lonza has established an internal LER Task Force to continue investigating the LER phenomenon and its influence on the various endotoxin assays, LAL and recombinant-based. The Task Force offers customers technical and hands-on support through Lonza’s R&D specialists who work closely with customers to develop a method to overcome LER in their product. In addition to the Task Force, Lonza has developed an NOE prep made from Burkholderia cepacia that is available through an Alpha Testing Programme. This NOE can be used as an alternative analyte to reference and control standard endotoxins during an LER investigation.

Global Endotoxin Testing Summit

In 2015, Lonza introduced its first annual Global Endotoxin Testing Summit to create an informal forum for users, vendors and regulators to come together and discuss hot topics within the endotoxin testing industry. Based on user feedback, the summit covers a range of topics from endotoxin testing basics and regulatory updates to current hot-button topics like LER or data integrity.

For further information, visit www.lonza.com/endosummit