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Hot Topics in Endotoxin Testing 2016

Trends and Insights from Lonza's Second Global Endotoxin Testing Summit

Building on the success of last year's event, Lonza held its second Global Endotoxin Testing Summit in May 2016 – a unique forum for sharing innovative ideas and thoughts on current and future practice. Delegates from across the global bacterial endotoxin testing (BET) community once again met in Annapolis, MD, USA to discuss the most pressing issues affecting the field.

It is a particularly dynamic time for the industry, with a number of challenges to be overcome. Furthermore, in many cases, there is no obvious, single way forward. This year's summit touched upon a wide variety of such topics, including the ongoing search for the best possible testing standards, the need for enhanced efficiency and predictability across test sites via more standardized methods, and the growing role of automated lab systems in improving test reproducibility and throughput. There was also an update from leading experts on our growing understanding of low endotoxin recovery (LER), including exactly when and how the phenomenon is likely to occur, as well as an active discussion around the best sample preparation methods currently available to overcome its effects.

Finally, a trip to Pickering Beach (DE, USA) and a session with Glenn Gauvry, founder of the Ecological Research & Development Group (ERDG), further reminded all the attendees of how important it is to responsibly manage the conservation of horseshoe crabs. As if to mirror this sentiment, a presentation from Jay Bolden of Eli Lilly and Company provided compelling evidence that the recombinant Factor C (rFC) assay is a sustainable and viable test method that, in many cases, can replace the need to use traditional Limulus Amebocyte Lysate (LAL) assays as the test-of-choice in pharmaceutical quality control.

What follows is an executive summary of the hottest topics discussed at the Summit.

The LER Debate Continues

The phenomenon of low endotoxin recovery (LER) dominated the discussion at last year's Global Endotoxin Testing Summit, and the debate showed no signs of slowing down this year. LER occurs when the endotoxin added to undiluted drug products is not subsequently recovered, leading to false-negative results and questionable test reliability. It appears to be triggered when the drug formulation contains polysorbate stabilizers used in conjunction with citrate or phosphate buffers, as is typical for many biologics (e.g. monoclonal antibodies, vaccines etc.). LER is time- and temperature-dependent and dilution-independent, so it cannot be overcome by a simple sample dilution.

Should We Worry About LER?

The discussion at the summit centered on the main question polarizing the industry's response to the LER issue – does LER pose a risk to human health? To date, there have been no adverse reports of pyrogenicity caused by a product that has tested negative using the LAL test. Furthermore, endotoxin is naturally present in the gastrointestinal tract, and is constantly detected and cleared by the body's normal defense systems.

The LAL test in itself is under question, as for some in the audience the test actually detects endotoxin contamination, rather than directly measuring pyrogenicity (unlike other tests, such as the Rabbit Pyrogen Test, where the readout is a direct measure of fever and mortality). As such, this raises important questions about what the LAL test actually measures and whether it is the fever-inducing activity of endotoxin that is the clinically relevant parameter we should actually be testing for.

Regardless of these complexities, by the end of the discussion the delegates seemed to settle upon the idea that the questions around the risks posed by LER are actually somewhat irrelevant to pharmaceutical manufacturers at the current time – put simply, if regulatory bodies such as the US Food and Drug Administration (FDA) continue to insist that manufacturers prove that LER is not an issue for susceptible products, then they must continue to test for it (and overcome it).

The Growing Complexity of LER

Assuming that the FDA and other regulatory bodies will continue to ask pharmaceutical manufacturers to test products at risk of LER, current research continues to hone in on the exact conditions that are likely to

trigger the effect. However, the more research data that is generated, the more complex the LER phenomenon seems to become.

For example, it has been shown that endotoxins from different sources exhibit different masking susceptibilities. Several presenters reported having recreated the LER phenomenon in their own studies in order to better understand some of this complexity. Allen Burgenson, US Manager of Regulatory Affairs at Lonza, shared data from an evaluation of endotoxin recovery using four different types of endotoxin: reference standard endotoxin (RSE), control standard endotoxin (CSE), and naturally occurring endotoxins (NOEs) extracted from both P. aeruginosa and S. marcescens. Allen concluded that, "All four endotoxin types chosen for the study were affected by LER under certain conditions and reacted differently to the buffers used. This shows that the LER issue is a real phenomenon affecting multiple forms of natural and synthesized endotoxins, and that the effect is unlikely to have an all-encompassing solution."

Other speakers, including Cheryl Platco, Principal Scientist at Merck Research Laboratories, reminded delegates that purified lipopolysaccharide (LPS) and native endotoxins are not the same material, especially for the attribute of stability of the analytes, "LPS is only one portion of the endotoxin molecule, whereas what we are actually measuring when we test products are the pieces and parts of a gram-negative bacterial cell that has broken down. For the most part you will not find plain LPS molecules naturally." She went on to focus on the LER vs LLR (low lipopolysaccharide recovery) controversy, which hinges on the theory that only purified LPS (e.g. CSE, RSE) is affected by LER, while NOE is relatively stable. In short, the source of the endotoxin has a direct effect on whether LER occurs, bringing into question exactly which endotoxin test standards offer the best tool for validating endotoxin tests and overcoming LER.

The Search for a Consistent and Relevant Standard Goes On

Currently, there is some confusion about exactly what types of studies should be used to test for LER. The FDA presently requires that manufacturers determine the stability of 'assayable endotoxin' in their products. As most products have no 'assayable endotoxin' or native endogenous endotoxin at the time of testing, many are confused about whether there is a compelling reason for the testing laboratory to demonstrate the 'stability of assayable endotoxins' over time when a product is found to have no 'assayable endotoxin' at time zero.

The confusion regarding which studies should be used to test for LER also encompasses the debate as to whether deliberately contaminating a product with a purified LPS, such as CSE or RSE, is a truly relevant experimental design, as these conditions do not mimic contamination by the natural LPS molecules shed from the cell membranes of gram-negative bacteria.

It has since been suggested that the original intent of the FDA was not to artificially contaminate samples with endotoxin to demonstrate stability of 'assayable endotoxins', as explained by Cheryl Platco in her presentation: "The initial meaning of the FDA statement was to determine whether endogenous endotoxins found in products exceeded pyrogenicity levels at the end of the shelf life, not to contaminate the product to see if the endotoxins are stable."

Therefore, many pharmaceutical companies appear to have begun evaluating a naturally occurring endotoxin to spike the product, in an effort to better reproduce what 'real' contamination from bacteria present in the pharmaceutical manufacturing environment might look like. This therefore poses the complex question of which endotoxin standard should be used.

Before discussing standard types, it is important to review how an endotoxin is defined. In USP Chapter <85>, a bacterial endotoxin is defined as a component of the outer cell membrane of gram-negative bacteria. The natural endotoxin complex contains many cell wall components, including phospholipids, lipoproteins, and LPS, which is the biologically active component of endotoxin. As such, purified endotoxin is chemically defined as a form of LPS, and is the basis for the RSE and CSE spike-in standards commonly used in endotoxin testing labs.[2]

However, purified LPS does not exist in nature, so when products are contaminated with endotoxin during manufacturing, the contaminant will not be purified LPS, but rather whole cells or cell wall fragments, as would be expected when the contamination is caused by bacteria. Given this information, purified LPS (used in laboratory standards) and native endotoxins, or NOEs, are two terms that should not be interchanged. The use of native endotoxin as a control material in testing studies may be a better consideration, because a native endotoxin preparation better reflects operational reality.

This sets the basis for reviewing if RSE, CSE and NOE controls are truly equal. The simple answer is: no. RSE and CSE are highly purified entities that are not found in nature, while NOE is what occurs in the environment and could possibly contaminate the pharmaceutical manufacturing process in a typical lab. The phenol extraction process used to manufacture RSE and CSE strips away the associated proteins and cleaves the O-specific side chains. As a result, this type of purified LPS is even further away from being considered a 'natural endotoxic factor'.

Not only are RSE, CSE and NOEs different, but natural LPS molecules are also highly variable depending on the species they are shed from, as Allen Burgenson discussed in his presentation: "We've all come to assume that LPS molecules are all the same, but that is not true – LPS as an entity is highly variable. The basic structure may be conserved but the lipid length and the number of acyl groups may vary, which in turn results in highly variable pyrogenic activity." In fact, this is in keeping with one of the FDA's original reasons for favoring RSE and CSE over NOEs (i.e. NOEs are inherently variable and cannot be easily standardized). However, the variable behavior of LPS means RSE and CSE may not be truly standardized either, perhaps strengthening the suggestion of using NOE as a standard instead.

Another consideration revolves around the influence of LER on this discussion. For example, while LPS preparations such as CSE may be masked under certain conditions, there are some suggestions that the

LPS in NOEs may be protected from the dispersing effect of chelating buffers, as the LPS in these preparations is still embedded in cell wall fragments. However, unpublished data from other hold-time studies performed by some of the delegates at the summit have shown that NOEs are also not immune to the LER effect. Put simply, the argument to use NOEs because they are not affected by LER has yet to be fully proven and more research is needed.

Ultimately there is no clear-cut answer as to which standard should be used. The debates around the factors causing LER and the true purpose of the LAL test (pyrogen or contaminant detector) have triggered deeper questions around which standard should be used across all endotoxin testing. All three discussions are somewhat interlinked, and clarity on all these issues will be needed before rational, data-driven decisions can be made.

Laboratory Automation Can Improve Endotoxin Testing Processes

The pharmaceutical industry continues to face pressure to do more with less, and this is equally true for endotoxin testing laboratories. Laurent Nguyen of Spectra Laboratories put forward a case for more manageable endotoxin testing using laboratory automation. He explained the challenge of scaling their testing process: "We've seen laboratory workload grow over the last seven years, from 330 samples per day to 1,260 – and on a busy day we might receive as many as 1,785 samples." In an effort to reduce manual sample handling, a Tecan Freedom Evo® 150 robotic workstation was installed for scanning barcodes, making standards, pipetting, making dilutions and transferring the assay plate to the reader for analysis.

"As a direct result of the automation, we found we no longer needed to have our more certified staff running the samples and we also decreased the repetitive motion strain on staff," he explained. Over 7 years, the lab's workload increased by 4-5 times while staffing only needed to increase two-fold. We also worked with Lonza and our IT team to integrate WinKQCL™ with our LIMS. This means that instrument recordings are now stored on a server instead of in physical, off-site storage boxes, saving us a lot of money and the effort associated with manual data entry."

Data Integrity Can be Improved Using Digital Systems

It is also important to consider data integrity as well as automation when reviewing and improving laboratory efficiencies. "The regulatory requirements for data integrity – defined as the completeness, consistency, and accuracy of data – are not new and they apply to both paper and electronic data," explained Rob Lutskus, an expert in digital quality control systems who works as a Global Product Delivery Manager at Lonza. "However, the general shift towards electronic systems and automation across the pharmaceutical industry brings increased pressure and scrutiny to these systems." Furthermore, data integrity has become a renewed focus for the FDA, with GMP inspectors receiving extensive additional training in this area. In concert with this, the FDA has just released new data-integrity guidelines and introduced data integrity-specific inspections for high-risk sites. The result is that more and more data-integrity issues are being found and increased scrutiny is being placed on manufacturers.

So why do QC testing labs need paperless informatics tools? Well, for a start, the retention of electronic data is almost always going to be a more economic and efficient option than its paper counterpart. Paperless systems also offer the opportunity to bring together disparate data points and islands of information, thereby improving data tracking, analysis and access.

Meeting and maintaining compliance using an electronic solution relies on designing systems that assure data quality and integrity. This means including controls that are appropriately designed to validate a system for its intended use (including a review of how the software, hardware, personnel, and documentation will interface to produce the desired result). After all, FDA guidance document 21 CFR Part 11 makes data audit trails mandatory as part of any routine data review, so the system should make it as easy and reliable to inspect the data stored, as it is to input the data in the first place.

Ultimately, senior management is responsible for ensuring data integrity within their organization. To manage this successfully, they should ideally build a data-integrity culture that encourages the open reporting of errors, including providing training and raising awareness across all relevant areas of the organization. Put simply, digital systems can help with this goal. As Lutskus concluded in his presentation, "It's time to see data integrity as an opportunity to improve quality overall, and a paperless system, including the practical traceability of sample lifecycle data, provides a wide range of benefits from a quality perspective."

Horseshoe Crab Conservation Remains as Important as Ever

Just as he did at last year's Global Endotoxin Testing Summit, Glenn Gauvry presented on behalf of the ERDG. He explained that conservation of the world's four horseshoe crab species continues to be a cause for concern, particularly when it comes to Asian TAL species, where numbers are in significant decline. Various initiatives are in place to protect the North Atlantic Limulus polyphemus species (the source of LAL) and its numbers are being managed to ensure sustainability. However, the conservation issues facing the three Asian species is more complex, as their habitats encompass several countries with very different social, economic and environmental priorities. Without multinational cooperation to regulate and enforce harvesting strategies, it's unlikely that the downward trend in these areas is going to stop anytime soon.

For the Atlantic horseshoe crab, the primary threats revolve around being harvested for use as bait by the conch and eel fisheries, and to a lesser degree for biomedical collection for the production of LAL. "In the

US we have a good fisheries management plan and the population is currently stable throughout much of its geographic range. There may even be some modest increases in the mid-Atlantic area. However, the population is continuing to decline in New York and the New England states, which may prompt additional harvesting restrictions," Gauvry emphasized. "With only modest improvements in population stability and growth, it is uncertain whether the Atlantic horseshoe crab can shoulder the projected growth of the global pharmaceutical biomedical industry and its growing use of LAL."

As the global demand for human and animal drugs and medical devices increases, so do the demands for LAL (as well as TAL, the version of the assay derived from a key Asian species of the horseshoe crab). There are several reasons for this growing demand, including increasing global vaccine production and the growing needs of emerging markets. A key question is: Are the world's current horseshoe crab species capable of supplying enough resource material in a sustainable manner to the TAL/LAL industry in order to meet current and growing demands over the next 10-15 years? Gauvry believes the answer to this is no. So what can be done to manage the resource more effectively and/or replace the need for horseshoe crab resources altogether? And most importantly, what role should the biopharmaceutical and medical device industry play to ensure these ancient mariners are protected for future generations?

"As the result of a generous contribution and ongoing support from Lonza, we have been able to expand two areas of the ERDG website – the teachers' toolbox and research database – and also fund an investigation project to search for alternative bait to horseshoe crabs," commented Gauvry. The research database is a repository for any research that in some way link back to LAL/TAL and horseshoe crabs. He urged any individuals and companies to upload their information and data directly to the website. He also had a clear message for the pharmaceutical industry: "You, as the purchasers of endotoxin detection products, can partner with us in making responsible decisions in terms of your supply line ethics to ensure sustainability and conservation of these species. Your purchasing decisions can actually make a profound difference." With synthetic, recombinant versions of the LAL assay finally gaining traction within the pharmaceutical industry and becoming better supported by regulators, there is hope that manufacturers can have a direct effect on the sustainability of horseshoe crab use by moving to tests based on synthetic raw materials or other alternative methods.

Looking Forward to 2017

This year's Global Endotoxin Testing Summit brought together the latest thinking and leading experts from across the world, with a wide range of countries represented throughout North America, Europe and Asia. As well as the presentations themselves, which were of extremely high quality, the open and collaborative nature of the ensuing debate will be necessary to overcome some of the issues faced by the sector. These include trying to come to some sort of conclusions about the best way to deal with LER, as well as fresh concerns around the best way to test for pyrogenic contamination in pharmaceutical products and the most effective standards to use for spike-in controls.

Fortunately, while debate still rages in many areas, there is also good news to report, with rFC finally gaining real traction with manufacturers and regulators alike, and offering real hope that we can start reducing the need for the traditional LAL assays that are derived from the blood of horseshoe crabs. Data integrity also remains a hot issue, but advanced digital systems now exist that can help make traceability and regulatory compliance easier and more cost-effective than ever.

While there is still lots of work to be done in many of these areas, one thing is for certain: next year's summit will be just as full of important debate, data and detailed discussion. To learn more about this active and growing community and potentially attend the 2017 Summit, please visit www.lonza.com/endosummit.

References

1 Kevin Williams, The Emerging View of Endotoxin as an IIRMI, Bio Pharm International, February 2016.

2 USP Chapter <85>, The United States Pharmacopeial Convention

3 Gauvry G (2015) Current horseshoe crab harvesting practices cannot support global demand for TAL/LAL: the pharmaceutical and medical device industries' role in the sustainability of horseshoe crabs. In: Carmichael RH, Botton M, Shin PKS, Cheung SG (eds) Changing global perspectives on horseshoe crab biology, conservation and management. Springer New York, pp 475-481

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