Functional Challenges for Alternative Bacterial Endotoxins Tests

Part 1: Attributes for Alternative Tests

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Introduction

USP <85>,"Bacterial Endotoxins Test"(BET) is a harmonized compendial analytical procedure that describes the use of lysates (extracts) prepared from the blood cells (amebocytes) of horseshoe crabs to detect and quantify endotoxins activity in parenteral products (USP 2019a). All of the methods currently described in <85> use reagents that are sourced from the hemolymph of living horseshoe crabs (HSC), *Limulus polyphemus* (LAL) and *Tachypleus tridentatus*, (TAL). BET assays are critical safety tests for parenteral drugs, biologicals, and medical devices, as results obtained using these procedures may be predictive of a febrile response in patients (Greisman and Hornick, 1969; Hochstein et al, 1994).

Alternatives to naturally sourced lysate reagent are commercially available, or are in development. These *recombinant reagents* utilize one or more recombinant zymogen proteases cloned from the natural clotting cascade of horseshoe crabs to detect and quantify endotoxins activity (see below). The benefits these alternatives could bring include:

1. An alternative to sourcing LAL reagents from live HSC.

   While comparable alternatives to the use of animals or animal-derived products is always desirable, manufacturers of lysate reagents in the United States are required by licensing and local jurisdiction to return the animals used solely for biomedical purposes to the marine environment after drawing hemolymph. Although the Asian horseshoe crab populations are dwindling, a 2019 report from the Atlantic States Marine Fisheries Commission indicates that the United States populations are currently stable (ASMFC, 2019).

2. The absence of the glucan pathway that, in the naturally sourced lysate, can result in a non-endotoxin specific enhancement of the test result,

3. The ability to produce reagents through the use of cell culture, possibly enabling a more consistent product and better control over the supply chain.

The central issue for the implementation of alternate methods is comparability of candidate test results to the results using existing compendial methods to assure continuity in product quality and patient safety. Some of the published data on the comparability of endotoxins activity in natural and pharmaceutical waters challenge the assumption that recombinant reagents and natural lysates will detect and quantify these "natural" endotoxins equally. These data will be discussed in more detail in Part 2 of this series. Part 1 focuses on the history and science of alternative methods in pyrogen testing to define the concept of comparability using endotoxins that are autochthonous (indigenous) to parenteral manufacturing facilities.

Background

It has been known for almost a century that endotoxins, derived from the outer membranes of aquatic Gram-negative microorganisms which were once common contaminants of pharmaceutical water systems, correlated with the cause of fever in patients receiving parenteral medications (Seibert, 1923; Seibert, 1925; Bourn and Seibert, 1925; Probey and Pittman, 1945). This knowledge prompted the inclusion of USP <151>, "Pyrogen Test" (otherwise known as the Rabbit Pyrogen Test, or RPT) in USP XII (1942). This test relies on a febrile response in rabbits after injection with a test solution such as a finished drug product, to predict whether the preparation under test would cause fever in humans.

USP Chapter <85> first appeared as an informational chapter in USP XX/NF XV (1980) describing an alternative test to the RPT. The methods described in <85> are not tests for all pyrogens. The BET detects only endotoxin pyrogens from Gram-negative bacteria (GNB), which are the most prevalent and potent pyrogen found in pharmaceutical manufacturing facilities. Although the text of <85> was harmonized with the European and Japanese Pharmacopeia in 2012, its content remains essentially unchanged since its introduction.

The biology of the innate immune response in the HSC is complex. This 400 million year old species has evolved a highly modulated response to the multitude of Gram-negative organisms present in its environment (Jiang et al, 2009). Like many innate immune responses, the HSC amebocyte lysate reaction includes a cascade of zymogen proteases (Figure 1).

In the horseshoe crab and in the *in vitro* lysate reaction shown in Figure 1, endotoxins bind to and convert Factor C to its active form, which in turn activates Factor B, which in turn activates the proclotting enzyme. The activated proclotting enzyme then cleaves the clotting protein, coagulogen, resulting in turbidity or clotting of the HSC blood or laboratory test mixture. The chromogenic assay is similar to the gel clot or turbidimetric assay, but rather than cleaving coagulogen, the activated proclotting enzyme cleaves a chromophore from a colorless substrate, resulting in a yellow color. The extent of turbidity or intensity of yellow color is proportional to the levels of endotoxins activity in the test solution.

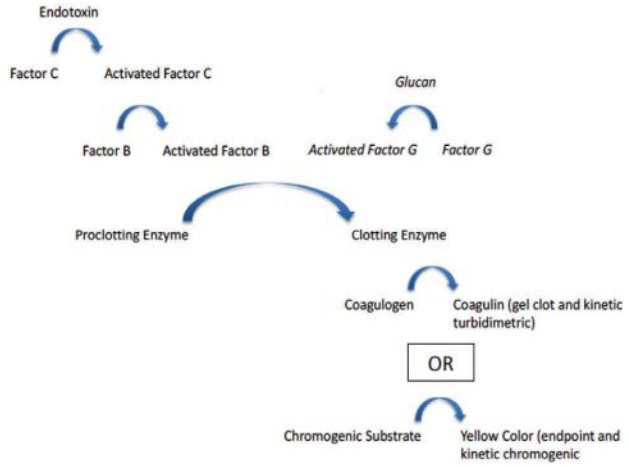

Figure 1. Natural Lysate Zymogen Protease Cascade (after Loverock et al, 2010)

Current Recombinant Factor C (rFC) reagents contain only the recombinant Factor C protease (Obayashi et al, 1985; Loverock et al, 2010). When activated, the rFC protease cleaves a fluorogenic substrate releasing a fluorescent signal that is proportional to the levels of Activated Factor C (Ding and Navas, 1995; Ding and Ho, 2001; Wang et al, 2003; Loverock et al, 2010). A schematic of reaction with current rFC reagents is provided in Figure 2.

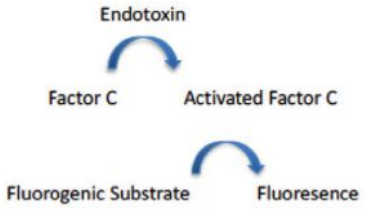

Figure 2. Schematic of the rFC reaction (after Loverock, 2010).

Recombinant Cascade Reagents (rCR) differ from rFC reagents in that they include all three cloned zymogen proteases (Factor C, Factor B and the proclotting enzyme) in their formulations (Mizumura et al, 2017; Muroi et al, 2019). These reagents work like kinetic chromogenic lysate reagents in that the activated clotting protein cleaves a colorless substrate to result in an increase in the intensity of yellow color.

Notably, the recombinant reagents, by design, lack the "Factor G" pathway (Kawabata and Muta, 2010). The Factor G pathway in naturally sourced lysate represents an alternate clotting pathway in the HSC, initiated by the presence of certain levels of β 1,3 D-glucan ("glucans") in the test article. Glucans, if present, can result in the activation of the proclotting enzyme and therefore a non-endotoxin generation of turbidity or color. The presence of glucans, being non-endotoxin specific initiators of the clotting reaction, can result in an

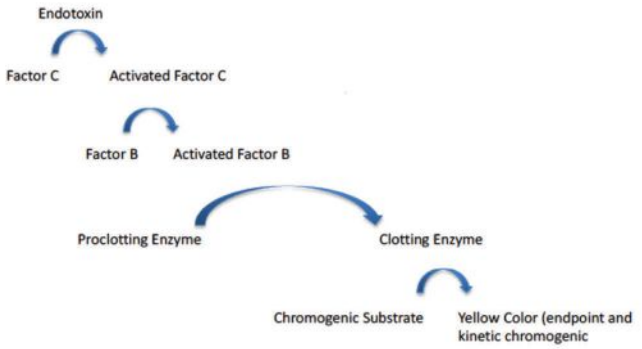

Figure 3. Recombinant Cascade Reagent (rCR) schematic.

inaccurate response, due to overestimation of endotoxins activity in the test article (Roslansky and Novitsky, 1981; Kambayashi et al, 1991). Blocking or eliminating this pathway provides a test result that more accurately measures only bacterial endotoxins activity.

Alternative Tests

Although the instructions for performing the rFC and rCR recombinant methods are essentially identical to the chromogenic endpoint and kinetic chromogenic methods described in <85> respectively, the reagents are not identical to reagents prepared from HSC hemolymph. Therefore, endotoxins testing methods using recombinant reagents are, in compendial terms, *alternative methods*.

Alternative methods are defined by the USP General Notices 6.30 as follows,

> *"An alternative method or procedure is defined as **any method or procedure other than the compendial method** or procedure for the article in question. The alternative method or procedure must be **fully validated** (see Validation of Compendial Procedures <1225>) and must **produce comparable results to the compendial method or procedure within allowable limits established** on a case-by-case basis" (USP, 2019bc, emphasis added.)*

FDA (2012) commented on the use of an alternate method for testing a compendial article:

> *Question: May a firm use alternative assays to those in the USP for a compendial article?*
>
> *Answer: Yes, firms may use alternative methods and/or procedures if they provide advantages in terms of accuracy, sensitivity, precision, selectivity, or adaptability to automation or computerized data reduction, and in other special circumstances. Such alternative procedures and methods should be **validated as described in the USP General Chapter <1225>,** Validation of Compendial Procedures, and should be **shown to achieve equivalent or better results.** When a difference appears or in the event of a dispute, the final decision is made based upon the USP compendial gel clot method unless otherwise indicated in the monograph for the product being tested. (emphasis added)*

To align with the recommendations of the USP and FDA, we suggest that the validation of alternate methods to assure product quality and patient safety is threefold:

1. Analytical Capability,
2. Suitability or fitness of the alternate method for use,
3. Documentation of Comparable Results.

Ultimately, as described in USP <1223>, "Validation of Alternate Microbiological Methods" the goal is to assure that a candidate method, particularly one with a different signal, provides the same or better product quality and patient safety decisions as a standard compendial method (USP, 2019c).

Analytical Capability is 1) assurance that instruments are properly calibrated and qualified per USP <1058>, "Analytical Instrument Qualification" (USP, 2019d) and 2) the demonstration that the new method is capable of suitably reliable and accurate results as described in USP <1225>, "Validation of Compendial Procedures" (USP, 2019e). For alternative BET assays, not all of the activities required by <1225> necessarily apply, but demonstration of analytical capability should minimally include linearity, specificity, and sensitivity.

Product-specific **suitability** demonstrates that the product under test neither inhibits nor enhances the assay such that test results are altered. For recombinant methods, the demonstration of **suitability** is performed according to current USP <85> "Test for Interfering Factors" (USP, 2019a). *Note: Because recombinant methods utilize reagents that are significantly different in composition and formulation than the natural lysate reagents, there is no expectation that the interference patterns seen with lysate and recombinant reagents will or should be the same. However, a laboratory must verify that the article meets the product-specific endotoxin limit within the confines of the calculated Maximum Valid Dilution (MVD).*

The third and most critical element of validation, given the significant differences in reagent composition and formulation between the recombinant reagents and native lysates are the phrases *"[the method] must produce **comparable results to the compendial method** or procedure within allowable limits established on a case- by-case basis" (USP, 2019b) and, "[the alternative method] should be shown to **achieve equivalent or better results** [to the compendial test]" (FDA, 2012).*

Comparability is NOT the same as suitability. There are several excellent scientific publications that demonstrate analytical capability and suitability in accordance with points one and two above (Loverock et al, 2010; Bolden and Kelly, 2017; Bolden, 2020; Marius et al, 2020). However, these studies do not meet requirements for a demonstration of comparability described in point three above because:

- The unspiked "comparative" data from the publications cited above provided a look at products or materials that contain endotoxins activity below the Limit of Quantitation (LOQ) of the assay. It is not possible to claim comparability when the impurity or analyte that one is measuring, in this case endotoxins activity, is not present in the test article at quantifiable levels.
- The studies compare products spiked with known levels of calibration standard (RSE or CSE) rather than endotoxins that could contaminate the product from manufacturing sources. The use of RSE or CSE is consistent with "suitability" as described in USP <85>, but does not assure that the alternate test will detect contaminating endotoxins from autochthonous manufacturing sources such as poorly constructed water systems, cracks in manufacturing equipment, inadequate manufacturing control or incomplete cleaning procedures (FDA, 2009; EMA, 2011)

If manufactured drug product containing assayable levels of endotoxins activity is not available, the test for comparability

may be conducted by using a diluent during sample preparation (reconstitution and/or dilution of product) that consists of a phylogenetically diverse population of endotoxins activity that could be found in product or the manufacturing process, for example water taken after the carbon filtration step or deionized water that may contain assayable endotoxins activity.

The procedures in <85> are considered to be validated in that they have been shown to meet the requirements of <1225>, they provide for suitability testing, and data have been gathered in the past to demonstrate comparability of test results with USP <151>, "Pyrogen Test", otherwise known as the Rabbit Pyrogen Test (Fleischman and Fowlkes, 1982). Thus one can assume that the data generated using existing BET methods are the standard data against which the data generated from testing the same sample using recombinant reagents should be compared.

The introduction of recombinant technology is not the first time that alternative tests have been introduced into the field of pyrogen testing. It was in the early 1970s when it was initially proposed that the *Limulus* Amebocyte Lysate (LAL) test be considered as a replacement for the rabbit pyrogen test, which had been in the USP since 1942. The BET was not deemed to be comparable to the rabbit test for detecting endotoxins until two critical questions were answered: 1) Could the BET be validated as a procedure that could reliably detect contaminating endotoxins in a pharmaceutical material at levels that could produce fever in humans? 2) Would the BET test allow stakeholder groups in Quality Control and Quality Assurance to make the same (or better) quality decisions regarding product quality and patient safety?

It took a diverse consortium of stakeholders including FDA, USP, academia, clinicians, and industry nearly a decade to answer these and other pertinent questions regarding the interaction of the LAL reagents with product (Guilfoyle and Munson, 1982, Muller-Calgan, 1982). Testing across the pharmaceutical industry included experiments using carefully controlled "spiking" studies in which measured levels of Westphal extracted *E. coli* lipopolysaccharide were added to products and measured in parallel on both rabbits and LAL. More importantly, industry stakeholders conducted *concurrent testing of regular production batches* that contained varying levels of contaminating endotoxins from autochthonous manufacturing sources (example, Mascoli and Weary, 1979). Comparability between the RPT and LAL tests was demonstrated because the source of contaminants in parenteral products that produced fever upon administration was known to be Gram negative bacterial endotoxin.

Most importantly, in no case was there a "false negative" lysate result meaning a failing rabbit test and a passing LAL test (example, Mascoli and Weary, 1979). Ultimately, the results of these parallel tests demonstrated alignment between the RPT and BET in terms of making equal or better quality product and patient safety decisions using the best methods available at that time.

Discussion

In our evaluation of the analytical data available in the public domain, we have reviewed several studies that have demonstrated alignment with the appropriate sections of USP <1225> and with the suitability requirements of USP <85> (Loverock et al, 2010; Bolden and Kelly, 2017; Bolden, 2020; Marius et al, 2020). In evaluating test data, we have recognized that data were collected from a variety of laboratories associated with different companies or organizations, which may have processed their collected samples with different protocols requiring varying degrees of handling and storage control. Critical analytical variables such as sample mixing, equipment calibrations (particularly fluorometers), analyst training, and whether or not the testing on the two methods was done concurrently all may impact the reported results. In order to truly obtain comparable results between all these evolving BET platforms from different manufacturers using rFC or some other version of genetically engineered reagent we recommend that a comprehensive comparative study, including a protocol with pre-determined acceptance criteria be written to minimize any experimental and analytical variables that may affect the test results. For example, because the recombinant reagents do not have a glucan pathway, we would suggest that laboratories engaging in comparability studies assure that all lysate tests are performed using a glucan blocker as suggested by the manufacturer. Because CSE potency is supplier and lysate lot specific, we also suggest using RSE as the calibration standard and <85> suitability analyte during validation to eliminate differences that may arise from mixing/matching reagents from different suppliers.

The third concern is acceptance criteria. Acceptance criteria for comparability must be scientifically justifiable. We recommend that each test using recombinant reagents recover activity within 50-200% of the reference lysate value. This range is consistent with the Positive Product Control recovery defined in <85>. In no case, however, would we suggest that the recovery range be any less stringent than 50- 200%. Recovery data should be viewed as individual test results and as the aggregate of test results. If a recombinant reagent demonstrates a bias toward the low side of the recovery range (e.g. 50-70%) or the high side of the range (e.g. 170-200%), then the reason for the bias should be investigated. Comparability on the low side may suggest an under-estimation of endotoxins activity in a product, which could be a patient safety risk. Conversely, bias on the high side may suggest an over-estimation of activity in a product, which would be a manufacturers' risk in that "good" product may be rejected.

Another challenge that bears discussion is the fact that the manufacture of these reagents, unlike the lysate reagents described in <85> is not regulated by a competent regional authority. Since the manufacturers of these reagents may or may not follow conventional drug, biological, or medical device CGMP, assurance of critical manufacturing control for the consistency and accuracy of the recombinant reagent will become the responsibility of the laboratory user. We would suggest that the reagent manufacturers be treated as critical suppliers in the users'Quality System to assure consistency and accuracy of test results.

Conclusion

Introduction of recombinant reagents as alternatives to naturally sourced LAL should meet the same standard of evidence of comparability as was achieved when LAL was being considered as an alternative to the RPT. Endotoxins that are found in product are more likely to be from a diversity of genera of non-fermenting Gram negative bacteria than enterics (Reid, 2019). In addition, organisms found in manufacturing plants have adapted to their environments, possibly changing the fine structure of their LPS (Morita, 1985; Bonnington and Kuehn, 2016). The ability to detect and quantify the LPS from these adapted organisms requires a level of confidence in our alternative test methods that if products were contaminated with endotoxins autochthonous to manufacturing facilities, the endotoxins activity would be detected and accurately quantified.

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