Validation Strategies for Gel-Clot Bacterial Endotoxin Testing

Crystal M. Booth, M.M.
Regional Manager
PSC Biotech

Introduction

Bacterial endotoxin testing (BET) utilizing *Limulus Amebocyte Lysate* (LAL) was introduced in the 1970s (The Horseshoe Crab, 2013). By 1980, the Food and Drug Administration (FDA) had developed a draft guideline and procedures for the test. In addition, the United States Pharmacopeia (USP) had developed Chapter <85> Bacterial Endotoxins Test making the LAL test a compendial assay (The Horseshoe Crab, 2013).

The gel-clot assay was one of the first bacterial endotoxin methods introduced to the industry. It established calculations and techniques that are important in properly understanding the assay. Even after 40 years of innovation, principles established with gel-clot testing are important to retain. This article briefly revisits the gel-clot bacterial endotoxin testing concepts required to validate the assay with accuracy and precision.

Gel-Clot Testing Overview

Since the 1980s, newer methods and guidelines for the bacterial endotoxin tests have been developed. As newer methods are developed and accepted, the core fundamentals of the LAL test can be lost in the automated technology process. The loss in technique and understanding of bacterial endotoxin testing could lead to confusion when analyzing the results.

Companies may perform the gel-clot assay for many reasons. These reasons could include the costs of the equipment for the modern assays, or even that the gel-clot assay is required per the compendia. USP <85> states that "in the event of doubt or dispute, the final decision is made based upon the gel-clot limit test unless otherwise indicated in the monograph for the product being tested (USP <85>)."

Gel-clot testing is a manual process. To perform the assay, an endotoxin standard series is made with control standard endotoxin (CSE), LAL reagent water (LRW), and LAL. Lambda (λ) is the sensitivity of the LAL being used for the test. It is also printed on the certificate of analysis (CoA) and on the vial of LAL. The dilutions of the standard curve that are tested is 2λ, λ, ½λ, and ¼λ. The standard curve must be positive within a 2-fold dilution of λ to be valid.

The product dilution that is being analyzed is tested in equal parts with the LAL reagent. Typically, this is 100 microliters (µL) of the product dilution and 100 µL of LAL mixed. Positive product controls are also prepared by making a dilution of the product inoculated to a 2λ level of endotoxin. Negative controls of the uninoculated diluents (e.g., LRW) are also analyzed.

All test tubes are incubated at 37°C. At the completion of a 1-hour incubation plus or minus 2 minutes, the tubes are inverted 180° to look for a solid clot. Anything other than a solid clot is considered a negative result. While performing gel-clot testing, care must be taken to not jar the test tubes because the clot could be knocked loose.

BET Calculations

There are several calculations that are important in endotoxin testing. One of these calculations is the endotoxin limit (EL) calculation. When setting specifications for a product, it

Crystal M. Booth, M.M., *is a Regional Manager a PSC Biotech and has over 20 years of experience in pharmaceutical microbiology. She obtained her master's degree in microbiology from North Carolina State University. Crystal is a seasoned award-winning technical writer and author of Method Development and Validation for the Pharmaceutical Microbiologist. During her career, Crystal has worked in microbiology, consulting, quality assurance, CDMOs, R&D, and quality control laboratories. Crystal has developed and validated numerous microbial methods and has worked with many different product types.*

is wise to consult the compendial monographs first. If a product will be available in many countries, use the most stringent monograph EL as the specification to be compliant in various regions of the world. If a monograph is not available, the EL calculation described in USP <85> may be utilized to calculate the specification.

The EL calculation is used to determine if endotoxin levels in product are safe. It is calculated by using the formula EL=K/M where "K" is a threshold pyrogenic dose of endotoxin per kilogram (kg) of body weight, and "M" is equal to the maximum recommended bolus dose of product per kg of body weight. Typically, "K" is set to 5 endotoxin units (EU)/kg unless intrathecal or radioactive drugs are to be tested (USP <85>).

When the product is to be injected at frequent intervals or infused continuously, "M" is the maximum total dose administered in a single hour period (USP <85>). "M" in the United States is typically considered 70 kg for the average human body weight and 60 kg is usually used in Japan when the calculation is performed.

In addition to knowing the EL established by the calculation, some companies may add on a safety factor to their endotoxin limit. For example, a company may calculate that the limit must be not more than 1.5 EU/mL of product. To be safe, the company may choose to utilize 0.3 EU/mL as their EL to ensure the endotoxin levels in the product do not come close to the maximum allowable limit. When establishing limits, it is important to consider all contributing sources of endotoxin such as water, components, and raw materials (Charles River Endosafe Workshop, 2008). The maximum allowable amount of endotoxin a 70 kg human can be exposed to in an hour is 350 endotoxin units.

There are two calculations that are important in understanding how much a product can be diluted and generate valid results. The first calculation of this type is the maximum valid dilution (MVD). To find the MVD, multiply the endotoxin limit by the sample concentration and divide that result by lambda (USP <85>).

$$MVD = \frac{\text{Endotoxin Limit X Sample Concentration}}{\lambda\ (EU/mL)}$$

The next calculation comes in handy when endotoxin toxin limits are expressed in milligrams. This calculation is known as the minimum valid concentration (MVC). To find this value, divide lambda by the endotoxin limit (Charles River Endosafe Workshop, 2008).

$$MVC = \frac{\lambda}{\text{Endotoxin Limit}}$$

The geometric mean is another calculation utilized in endotoxin testing. It is explained more in USP <85>. This calculation is typically used when qualifying a new analyst or performing quality control on new lots of reagents.

Two additional calculations are for finding the results of the assay. In the first calculation, divide lambda by the test concentration. This gives the result of the amount of endotoxin units that are present in milligrams (mg) of product.

$$\frac{\lambda}{\text{Test Sample Concentration}} = EU/mg$$

If both tubes are negative, the result will be reported as less than (<) the calculated value (e.g., <2.5 EU/mg).

The next calculation is for results that are expressed in volume. Simply multiply lambda by the dilution factor. For example, 0.125 EU/mL times 10 for the dilution factor is 1.25 EU/mL.

$$\lambda \text{ X Dilution Factor} = EU/mL$$

The last calculation is useful in finding the dilutions in the standard series for the assay. The calculation can be used to easily find any desired value, such as the 20λ value from the reconstituted CSE vial. It is the known endotoxin value divided by the desired endotoxin value (Charles River Endosafe Workshop, 2008). For example, a vial contains 50 EU/mL and lambda is 0.125 EU/mL. The desired value is 20λ or 2.5 EU/mL. The result 20. This means that a one to twenty (1:20) dilution should be made to get 2.5 EU/mL.

$$\frac{\text{Known Endotoxin Value}}{\text{Desired Endotoxin Value}} = \text{Required Dilution to reach Desired Endotoxin Value}$$

Twenty lambda (20λ) can be used as a hot spike to make positive controls. Make a hot spike by adding 10 µL of 20λ CSE to 100 µL of the product dilution. This creates a 2λ endotoxin spike that must come out positive to be valid.

Twenty lambda (20λ) can also be used to easily prepare the endotoxin standard series. A 1:10 dilution can be made from the 20λ dilution to achieve the 2λ dilution. Following the preparation of the 2λ dilution, a series of 1:2 dilutions can be prepared from the 2λ dilution for the remaining dilutions of the standard series.

Method Development Considerations

The first step in any method development process is to get to know and understand the product. Work closely with the development scientists to learn product traits such as solubility, potential interference issues, endotoxin limits, dilutions, and the pH.

Perform any trial-and-error testing in a method development notebook. Products need to be screened for any interference issues and a method needs to be established to overcome the issues if they are present. Performing this work in a method development notebook allows for a clear and concise validation protocol package to be created. Once a method is identified, it can be executed for validation neatly in an approved protocol.

The interference (inhibition or enhancement) screen is usually performed by diluting down to the MVD or MVC in serial dilutions, such as 1:2 dilutions. Inhibition occurs when positive controls that should be positive, give negative results or when the endotoxin amount of the dilution cannot be determined. Enhancement occurs when a positive control is a lot higher than it should be or negative products are giving positive results.

In gel-clot testing enhancement is difficult to detect. It must be assumed that the product is giving a positive result unless it can be proven otherwise. The most common ways to get over interference issues is by dilution, balancing a pH if needed, or using an endotoxin specific buffer to eliminate possible Glucan interference. When performing endotoxin testing, the pH of the product dilution and the LAL reagent combined must be between a pH of 6-8 for the assay to work properly (Charles River Endosafe Workshop, 2008).

Method Validation Strategies

Once a method has been developed, clearly describe it in a method validation protocol. The protocol should describe performing an inhibition and enhancement screen once to show that the method of choice works in overcoming any interference issues. This is important for new or tricky products that may be known to interfere with the BET assay.

The protocol should describe which dilution that worked from the method development phase or interference screen. Choosing the 3rd highest positive dilution in the interference screen is a good choice (Charles River Endosafe Workshop, 2008). It is not the only choice, but it is typically a good choice.

During the validation stage, three lots of product are usually used for commercial product method validations. If a product is a clinical product, one lot performed three times may be sufficient until more products become available, and the formulation is finalized.

Recall that the pH of the LAL reagent and the product dilution mixed must be documented to be between 6 and 8. If the pH needs to be adjusted during the validation phase, it will need to be adjust the same way during routine testing (Charles River Endosafe Workshop, 2008).

To perform the method validation, a product standard series is made in addition to the endotoxin standard series that is prepared in LRW. To prepare a product standard series, the production dilution to be tested is prepared. This dilution is inoculated with enough endotoxin to be equivalent to the 2λ endotoxin value. This inoculated 2λ product dilution is then diluted to the λ, ½ λ, and ¼ λ by performing 1:2 serial dilutions in the uninoculated product dilution. Each one of these dilutions of endotoxin inoculated product is tested in quadruplicate. A negative product control (NPC) is also performed in quadruplicate. The NPC is the uninoculated product dilution.

In addition to the product standard series, two replicates are typically analyzed for the standard series of the endotoxin prepared in LRW. Figure 1 demonstrates the setup of test tubes for the product standard series and the standard series in LRW.

Like the endotoxin standard series, the product standard series must be positive within a 2-fold dilution of lambda to be valid.

During the bacterial endotoxin assay, instances of invalid results may arise. Invalid assays are different from out of specification results. However, they may require an investigation to find the cause of the invalidity. Some common causes include, pipetting errors, analyst error, subpotent endotoxin standards, contamination, or dilution errors.

Conclusion

This article reviewed some of the validation strategies and core concepts for gel-clot BET. The gel-clot method is a manual test that utilizes equations and aseptic laboratory testing techniques. Newer methods and guidelines have been developed for endotoxin testing over the years. However, the core fundamentals of the LAL test can be lost when newer technology is adopted if employees are not properly trained.

According to USP <85>, the gel-clot method is required to be used "in the event of doubt or dispute with other endotoxin testing methods, unless otherwise indicated in the monograph for the product being tested (USP <85>)." Some companies may also choose to perform the gel-clot method due to the price of the assays using the modern methods. Knowing the fundamental concepts established from the gel-clot method and the validation can help analysts troubleshoot, analyze results, and completely understand the assay. Having the base concepts of bacterial endotoxin testing and validation strategies are essential for testing with accuracy, precision, and confidence.

References

1. Charles River Endosafe Workshop, August 2008
2. The Horseshoe Crab (2013). Accessed on 10Jun2021 at https://horseshoecrab.org/
3. United States Pharmacopeia (USP) <85> Bacterial Endotoxins Test, USP43-NF38, Currently Official as of 01May2018.

Figure 1. Standard Series Dilutions

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