Developing Endotoxin Limits, Risk Assessment and In-process Testing for CGT Products

Cell & Gene Therapy (CGT) products face a unique challenge in the required pyrogen testing for injectable wares. Since this test was written for conventional pharmaceuticals and medical devices, CGT products face special challenges in applying the endotoxin test to these goods.

Pyrogens are molecules that induce the human immune system to initiate a febrile response. Many of these are cytokines that are part of the immune system. These signalers are called endogenous pyrogens. However, molecules that originate from outside the body that initiate this pyrogen pathway are called exogenous pyrogens.

The most potent pyrogens are the lipopolysaccharide (LPS) molecule components of the outer membrane of gram-negative bacteria. It was known that drugs would frequently cause symptoms of septic shock even when sterilised. In 1942, the United States Pharmacopeia (USP) initiated USP <151> as the first pyrogen test requirement for parenteral and intrathecal injections.[1]

In 1972, it was discovered that the same LPS components that caused the human febrile response were responsible for the clotting of Atlantic Horseshoe Crab hemolymph in response to Gram-negative bacteria.[2] This led to the 1980 publication of USP <85> Bacterial Endotoxin Test (BET), which allows for the usage of this extract (Limulus Amebocyte Lysate – LAL) to be used in place of the pyrogen test.[3]

In 2024, the USP published USP <86>, which allows for the recombinant protein found in LAL to be used to detect endotoxin in products. This allows for horseshoe-crab-free test reagents to be used for endotoxin testing.[4]

As we reach the realm of specific therapies that are personalised for each individual patient, lead times change the ability to perform the appropriate tests on the product. Although this is a much bigger issue in terms of sterility testing, endotoxin testing can still reach a time limit that can be pressing on the needs of the user.

Specifically, many users will have outsourced their QC tests, which may not be acceptable in terms of lead time for the needs of the therapeutic. To bring the testing in-house, an internal testing environment needs to be set up. However, this can be an issue as CGT samples are not simple and require various considerations for testing. Some of these considerations include determining the correct endotoxin limit for the product, overcoming interferences, non-traditional lot sizes and testing needs in non-conventional QC lab settings.

Determining the Endotoxin Limit of the Product

First, the acceptable endotoxin limit of the product needs to be determined. This is different from other forms of QC testing, such as sterility testing, that already have a predetermined set limit, or the limit is to show that the contaminant is not detected. Originally, the need for an endotoxin limit came about as the BET is more sensitive than the rabbit pyrogen test. In the original comparison of the methods, it was determined that most tests reported results consistently between the pyrogen and the endotoxin test. However, there were several cases where the lysate reagent reacted when no pyrogenic response was observed. This led to the need to standardise LAL reagent measurements and correlate them to pyrogenicity. This resulted in the adoption of a globally harmonised Reference Standard Endotoxin to provide calibration for each formulation of LAL reagent and the setting of a threshold pyrogenic dose (TPD) to correlate measurements in endotoxin units against the pyrogenicity of the product.[6] In the years since, many harmonised standard levels for pharmaceuticals, excipients,

water and accessory buffers have been set. However, for novel products, the manufacturer needs to consult the TPD in establishing the limits.

The TPD is set to 5 Endotoxin Units per kilogram of body mass per hour of injection or bolus dose (5 EU/kg/hr).[3] Based on a 70 kg patient mass, this leads to a maximum bolus/hourly pyrogenic allotment per patient of 350 EU. Dividing this by the maximum bolus/hourly dose volume will result in the endotoxin limit for the product. For example, a dose of 10 mL would have a calculated endotoxin limit of 35 EU/mL. This provides the maximum endotoxin limit that should be used for the product. Care should be taken to examine what other products will be given with the product that could also add to the endotoxin given to the patient at this time. Although not a requirement, it is good practice to accept a more stringent endotoxin limit based on these considerations.

Mitigating Product Interference and Choosing the Correct Endotoxin Test Method

Once the endotoxin limit of the product is set, the determination of the endotoxin detection method to be used can begin. USP <85> outlines methods from simple endpoint qualitative techniques with a sensitivity of 0.25 EU/mL to quantitative techniques with a sensitivity down to 0.0005 EU/mL.

Choosing the correct test method will not be as simple as comparing the endotoxin limit of the product to the LOD/LOQ of the test method. Cellular materials, proteins, pH, lipid complexes, and certain ions and detergents cause known interference with the BET method.

Dilution is the most reliable method to mitigate interference. The Maximum Valid Dilution (MVD) can be found by dividing the endotoxin limit of the reconstituted sample to be tested by the sensitivity of the product. For example, a product with an endotoxin limit of 10 EU/mL being tested with a test method with a lowest endotoxin sensitivity of 0.001 EU/mL would have an MVD of 10000. This value indicates how much the product can be diluted and still be applicably tested by the chosen BET test technique.[7]

Most product interferences can be mitigated by product dilution. However, this dilution factor may be significant. Finally, sensitive test reagents are valuable not just for testing to low endotoxin limits but also to accommodate testing highly diluted products to overcome test interference.

Although dilution will overcome most interference, there are several factors that may need additional treatment, which may require further consideration. First, proteins that exhibit enzymatic interference on the protein cascade found in the LAL reagent (whether naturally derived or recombinant reagent) will need to be inactivated, as they will cause interference at low concentrations. The easiest method is to perform heat treatment of the product at 70°C for approximately 15 minutes. However, if this causes the sample to congeal, other treatments, such as a digestion buffer or an endotoxin extraction resin, may be utilised. Second, chelating agents and alkali-earth cations can disrupt the binding of the LPS to the Factor C protein of the endotoxin detection cascade. Addition of an appropriate buffer may be required. Finally, the physical insoluble material of a product with high cell material concentration can potentially interfere with the optical readers performing the test. A magneto-optical reader can potentially be used to overcome this interference when dilutions cannot be performed.[8]

Overcoming Time Constraints and Testing Needs in Non-conventional Lab Settings

The next consideration that manufacturers of CGT products will need to consider is how to bring the testing to their site. The BET is a relatively rapid QC test, usually not taking more than 1.5 hours for the result. However, this rapidity is lost if the manufacturer needs to outsource their endotoxin testing needs. Although consideration is greater for sterility testing, as USP <71> requires a 14-day turnaround, leading to sterility testing being done at risk in many cases, I recommend that endotoxin test results should always be known before administration. First, although not a sterility test, the endotoxin test is also a very sensitive indicator of Gram-negative contamination. Second, endotoxin test methods are available that can be performed simply and at the point of use to produce results within 15 minutes. For users of testing services for pyrogen and endotoxin services, I recommend that at least endotoxin monitoring equipment be brought in-house to confirm the products are endotoxin-free before administration. Additionally, robust endotoxin testing can be performed in-house with investment in equipment and training that will pay for itself many times over when the outsourced endotoxin testing can be eliminated.

For a CGT product manufacturer that currently outsources endotoxin testing but is looking to bring the test in-house, a phased implementation is recommended. This phased implementation could resemble the following. First, low-volume, point-of-use endotoxin equipment with FDA-licensed LAL reagent can be brought onsite to monitor the endotoxin content of water sources and the final products in conjunction with the outsourced testing. Second, when the final product testing is shown to match the outsourced testing and the team is sufficiently proficient in the test, new products can begin to be developed, in which the entire endotoxin validation is performed in-house. Third, as more products are tested in-house, higher volume equipment can be purchased with FDA CFR pt.11 compliant software to manage the test results. Finally, all products can be gradually brought to in-house testing as the team's proficiency is complete.

Setting up a Testing Plan for Non-traditional Lot Sizes

Traditionally, endotoxin testing has been performed on at least three samples per manufacturing lot, taking from the beginning, middle and end of the run. However, the FDA and USP have encouraged users to take a risk-based approach to the correct sampling numbers and plan to be used, with emphasis on the need for in-process confirmatory tests.[7,9]

Additionally, traditionally the interference testing for the BET involves testing three lots of each product.

For highly customised CGT products that do not have the traditional manufacturing model of many identical products consisting of one lot, but rather many smaller "lots" that are uniquely manufactured and may not be replicated. For these considerations, one sample of each lot may be what is being

tested to give both interference and endotoxin assurance. In these cases, the regulatory documents highlight the need for the user to demonstrate that the risk of this testing is mitigated by control-process testing that occurs upstream and over time to confirm the manufacturing environment is under control.

It is in these cases that low-cost, frequent, single gel clot tests may give invaluable insight spread over time and over the entire manufacturing process, supporting the final-product testing assurance. Additionally, the in-process testing will most likely have to overcome less product interference than the final product, as the simpler buffers and media will be more accommodating to the LAL reagent.

Conclusion

With the endotoxin test techniques found in USP <85> and <86>, a CGT manufacturer needs to approach their endotoxin needs not as a one-size-fits-all solution, but as several different techniques that can be adopted to give the overall endotoxin sterility assurance. The most information for the lowest cost will be had when low-resource, frequent tests can be performed along the entire production stream that complements the robust final product testing before final product release.

There are several challenges that CGT product manufacturers uniquely face in performing the endotoxin test. But these are not factors that are insurmountable. The overall benefit of bringing endotoxin testing in-house, as opposed to using third-party testing, is that the QC control program can have the tools to take a proactive approach over a reactionary approach to the endotoxin in the product samples. Interested manufacturers are encouraged to reach out, as the reagent manufacturer will be able to provide invaluable insight and validation support in the adoption of the method.

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