

# Endotoxin Detection Methods – Where are we now?

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## Abstract

Endotoxins are considered as the major contributors to the pyrogenic response observed with contaminated pharmaceutical products. Products manufactured by bioprocessing are microbiologically controlled to ensure that the end product meets the requirements for release. The test methods used to detect and quantify endotoxin have evolved over the years to provide us with improved sensitivity in detection and opportunities for obtaining results in real time. Present endotoxin detection challenges pertain to the low endotoxin recovery phenomenon observed with biological products. This paper reviews current endotoxin detection methods and those under development for various industrial purposes, and discusses the implications for endotoxin detection in therapeutic products within the context of human response to endotoxins.

## Introduction

Endotoxins are amphiphilic lipopolysaccharides (LPS) located in the outer cell membrane of gram-negative bacteria. LPS activates the toll-like receptor 4 (TLR4) resulting in the release of a range of vasoactive peptides and cytokine mediators<sup>1</sup>. Administration of parenteral products contaminated with pyrogens including LPS can lead to development of fever, induction of inflammatory response, shock, organ failure and death in humans. The Code of Federal Regulations, 21 CFR 211.167(a), requires that any drug product claiming to be sterile and non-pyrogenic be tested prior to release<sup>2</sup>. The necessity to ensure that parenteral products are free from heat stable endotoxins and other pyrogens was recognized early during World War II, when the demand for intravenous fluids was huge. At the time, safety of parenteral products was ensured by conducting the rabbit pyrogen test developed by Florence Seibert<sup>3</sup>. The rabbit pyrogen test (RPT) became part of the US Pharmacopeia in 1942<sup>4,5</sup>. Two decades later, Fredrick Bang and Jack Levin found that amebocytes of the horseshoe crab will clot in the presence of endotoxins<sup>6</sup>. This led to the development of the Limulus Amebocyte Lysate (LAL) test for endotoxin which mostly replaced RPT. However, it has not completely replaced RPT because of the interferences observed with the LAL assay and its inability to detect non-endotoxin pyrogens. The LAL test is referred to as the bacterial endotoxin test (BET) in the compendia (USP<85>)<sup>7</sup>. An evaluation of the specificity of LAL for pyrogen detection suggested that false negative results can occur, requiring assay validation for new products with LAL<sup>8</sup>. For specified biological products, 21 CFR 610.13(b) requires a rabbit pyrogen test<sup>9</sup>. The requirement in 21 CFR 610.13(b) may be waived if a method is demonstrated to be equivalent to the rabbit pyrogen test in accordance with 21 CFR 610.9<sup>10</sup>. Endotoxin detection has an important role in settings besides pharmaceutical manufacturing. The endotoxin detection methodologies developed to address current challenges and meet future needs are reviewed.

## LPS Structure and Characteristics

The LPS structure and characteristics provides insights into the interaction of LPS with product formulation and receptors and aid in the development of detection methodologies. The LPS structure consists of three regions: the Lipid A region, core oligosaccharide region, and O-antigen region<sup>11</sup>. The hydrophobic Lipid A region is the active toxic portion and contains C14 and C12 type acyl chains

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and negatively charged phosphate groups. The lipid A portion within the bacterial outer membrane is important for maintaining structural integrity. The core region consists of oligosaccharides that are less heterogeneous and conserved. The core region is divided into an outer core region composed mainly of D-glucose, D-galactose and N-acetyl-D-glucosamine and an inner core region composed of heptose and 2-keto-3-deoxyoctonic acid (Kdo). The O-antigen region consists of repeating units of glycosyl residue and the structure varies among different gram-negative bacterial strains and serotypes. The polysaccharide part of LPS is hydrophilic in nature. The molecular mass of the LPS monomer varies from 10 to 20 kDa depending on the number of repeating units of glycosyl residues in the O-antigen region. The LPS forms aggregates in solutions due to its amphiphilic nature. The stability of the aggregates is dependent on various factors such as, pH, ions, surfactants, and presence of proteins. The negatively charged phosphate groups in the lipid A and core oligosaccharide can bridge the LPS molecules by interacting with divalent cations, mainly  $\text{Ca}^{2+}$  and/or  $\text{Mg}^{2+}$ . The LPS characteristics and structural aspects at the monomer and aggregate level have been exploited for development of endotoxin detection and removal methods.

### Traditional Endotoxin Detection Methods

Traditionally, the *in vitro* LAL assay and the *in vivo* RPT have been used to detect and quantify endotoxin in pharmaceutical products. The LAL test has occasionally also been used to screen cerebrospinal fluid for presence of gram negative organisms associated with meningitis. The RPT involves monitoring for an increase in temperature or a fever after intravenous injection of the pharmaceutical into rabbits (USP<151><sup>5</sup>). For specified biological products, 21 CFR 610.13(b) requires a rabbit pyrogen test<sup>9</sup>. The requirement in 21 CFR 610.13(b) can be waived if an alternate method is shown to be equivalent to the RPT in accordance with 21 CFR 610.9<sup>10</sup>. The European Directorate for the Quality of Medicine (EDQM) recommends that a risk assessment be performed when using the LAL test as a pyrogen test to address the potential contamination of product by non-endotoxin pyrogens. The LAL test can be performed using 3 techniques: (a) the gel clot assay, (b) turbidimetric assay, or (c) chromogenic assay<sup>6</sup>. The requirements for the LAL test include optimal pH, ionic strength, temperature, and time of incubation. The turbidimetric and chromogenic LAL can be performed as an end-point or kinetic assay. The gel clot is considered as the referee

method when there is uncertainty, or a disagreement is observed in the turbidimetric or chromogenic assays<sup>12</sup>. The coagulation of LAL by endotoxin is through activation of Factor C while B-glucans responsible for false positive results in the LAL test activate Factor G in the clotting cascade<sup>13</sup>. The LAL assay is more sensitive than the RPT<sup>14</sup>. Parenteral products (small volume parenterals, amino acids, various antibacterials) have demonstrated interferences, enhancements or precipitation when tested by LAL<sup>15</sup>. Test interferences/enhancements could be due to a variety of reasons such as adsorption or aggregation, cation concentrations, protein modifications or presence of LAL reactive materials<sup>16</sup>. Proteins with positive charge (basic;  $\text{pI}>7$ ) can interact with negative charge in the LPS and interfere with endotoxin recovery. Acidic protein ( $\text{pI}<7$ ) are also known to interact with endotoxins in low ionic strength solutions. The study evaluating interaction of endotoxin with polypropylene and polystyrene tubes used in sample collection and storage suggested the need to test sample containers prior to use<sup>17</sup>. Borosilicate glass and stainless steel are among materials that adsorb endotoxin. The observed interference/enhancements with LAL test led to the development of tools to resolve this problem. The tools include use of B-glucan blocking reagents, cationic dispersing agents, divalent cations, and pretreatment methods (chemicals, heat or ultrafiltration) when testing with LAL. The test for interfering substances is part of assay qualification and suitable validated treatments can be used to overcome the interference. Rapid portable testing systems have been developed using LAL test principles to obtain real time data on endotoxins in raw materials and in-process samples<sup>18</sup>.

### Alternative Test Methods for Detection of Endotoxins

The activation of Factor C by endotoxin and conservation efforts for horseshoe crabs prompted development of new detection methods using recombinant Factor C. The recombinant factor C-based assays are alternative tests to the LAL and RPT and should be appropriately validated prior to use<sup>19</sup>. Another assay developed to detect pyrogens including endotoxins based on release of cytokine mediators by monocytes, is the monocyte activation test (MAT)<sup>20</sup>. In the MAT test, the test drug is incubated with monocytes isolated from the human peripheral blood and pro-inflammatory cytokines released into the supernatant are measured (Eu. Phr 2.6.30)<sup>21</sup>. Pyrogens other than endotoxins are also detected by this assay. The whole blood IL-6 assay is another variation of this test. Detection of endotoxin in cytotoxic agents by the MAT assay may be challenging due to inhibition. The FDA guidance also considers MAT test as an alternative test when appropriately validated<sup>19</sup>.

### Endotoxin Detection Methods Under Development

Rapid detection of endotoxin is a topic of interest to many besides biopharmaceutical manufacturing. This need has fueled research in the area of biosensors for endotoxin detection. The natural host biosensors and their dynamic interactions with endotoxins are being exploited to develop new techniques for endotoxin detection and to better understand host-specific responses<sup>22</sup>. Endotoxin detection using optical, electrochemical, and mass based biosensors are being explored but these detection systems are still in the research phase<sup>23</sup>. A single stranded DNA aptamer with high affinity for endotoxins was used to develop an impedance sensor for the detection of endotoxin<sup>24</sup>. In preliminary studies, the aptasensor had a broad detection range (0.01 to 10 ng/mL) with a detection time of 10 minutes. Cell based assays using the endothelial/E-selectin system are being explored for detection of endotoxins in biomaterials<sup>25</sup>. Additional tools such as hTLR-transfected cell lines are being explored to detect endotoxin in the broader



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context of product impurities that induce immune response<sup>26</sup>. These techniques will likely meet the future need for endotoxin detection in the therapeutic manufacturing and clinical settings.

### Challenges in Endotoxin Detection

Product interferences with LAL assay are known to occur. Recently, a “Low Endotoxin Recovery” (LER) phenomenon was reported to occur with undiluted biological products in a matrix containing citrate and polysorbate by Chen<sup>27</sup>. Similar unacceptable time-dependent loss in endotoxin recovery in spiked undiluted drug products has been reported by others<sup>28</sup>. The LER phenomenon has been observed using reference standard endotoxin, control standard endotoxin, and naturally occurring endotoxin. Several studies conducted to understand the mechanism of low endotoxin recovery suggest that surfactant concentrations, divalent cation depletion, disaggregation of endotoxin, and protein binding play a role in this phenomenon but the exact mechanism is not well understood<sup>29,30</sup>. The test for interfering substances does not detect LER and LER varies with product and formulation. To address concerns on underreporting of endotoxin levels in drug products, additional validation studies should be conducted in situations where LER is observed. De-masking strategies are being developed to overcome LER and the strategy will vary with product formulation. Currently, the RPT is used to release product when the LAL test method is unable to detect spiked endotoxin until a more suitable in vitro assay is developed. However, the limitations of the RPT with respect to low sensitivity, use of large numbers of animals for testing, and inability to detect inherently pyrogenic substances continue to encourage development of newer methods of endotoxin detection.

The manufacturing processes for biologics use various expression systems, for example, bacterial (Gram negative), mammalian, yeast, plants, and insects. Endotoxin contamination of biologic products may occur through water, raw materials such as excipients, media, additives, sera, equipment, containers closure systems, and expression systems used in manufacturing<sup>31</sup>. The release of endotoxins is impacted by environmental conditions and occurs during bacterial growth and upon cell death. The risk of pyrogens of microbial origin including LPS in biopharmaceutical process is well understood<sup>32</sup>. The manufacturing process is therefore microbiologically controlled using approaches to reduce and remove

endotoxin and by monitoring raw materials and in-process intermediates at critical steps, in addition to final drug product release testing. In light of the LER issues, the criticality of controlling bioburden and endotoxin contamination in the biopharmaceutical manufacturing processes cannot be emphasized enough<sup>28,33</sup>.

As newer products are developed and new excipients are explored, challenges related to interference and masking will continue to occur and mechanisms to overcome these challenges will be sought. In the case of nanoformulations, interference with the LAL assay has been observed in addition to discrepancies among the various LAL testing methodologies<sup>34</sup>. Decision trees have been explored as possible mechanisms to handle discrepancies and underestimation of endotoxin in product, with verification of the non-pyrogenic dose by RPT.

### Discussion

Current and future treatment modalities include use of drugs in combination, new excipients in product formulations, improved drug delivery systems, nano-pharmaceuticals and novel products. The product formulations and reactivity with endotoxin will need to be well understood and methods developed to ensure that endotoxin test methods are valid. A survey of biopharmaceutical manufacturers conducted in 1990 suggested a need for a comprehensive list of substances that impacted endotoxin recovery or caused interference. A survey of manufacturers of newer clinical products in development could provide more insights into future challenges and methodological hurdles. The current threshold for pyrogenic response is based on studies performed in healthy volunteers. Human exposure to endotoxins and the effects on cardiovascular, pulmonary, metabolic and inflammatory response continue to be of interest. Animal experiments suggest that subclinical levels of LPS can have metabolic and vascular effects<sup>35</sup>. Symptoms and responses observed after intravenous challenge with various doses of endotoxin are used to study inflammatory response and relationship to septic shock, with implications for drug delivery and routes of administration<sup>36</sup>. The implications of the pyrogenic thresholds in critically ill patients and those requiring drug combinations will need to be evaluated. The ongoing research in the field of LPS characterization and function will enhance our understanding of LPS-host interactions, endotoxin tolerance, pyrogenic response, and levels required for metabolic and vascular effects. These research efforts will aid in the development of new endotoxin detection techniques to meet future needs.

### Disclaimer

This article reflects the views of the author and should not be construed to represent FDA's views or policies.

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