Challenges on performing LAL test in oil products

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• Founded in 1945. (70 years)
• More than 250 different products (drugs, parapharmaceuticals and dentals)
• QC Microbiology
  ▪ Microbiology of non-sterile products
  ▪ Sterility
  ▪ **LAL test (since 1989., 1200 tests/year)**
  ▪ Microbiological assay of antibiotics, heparin and protamine sulfate
  ▪ Disinfectants effectiveness test
  ▪ Efficacy of antimicrobial preservation
  ▪ “In-house” and contract testing
Background of endotoxin examinations

Pyrogens are substances that produce elevated body temperature. Pyrogens are usually bacterial products and remains or decaying products of the bacterial cell walls, but can also be non-microbial.

Endotoxins are heat-stable, resistant to proteolytic enzymes, immunogenic, can not be converted to toxoides and are produced by Gram-negative bacteria. They are lipopolysaccharides that can be devided into 3 distinct regions: The lipid A, the R-core and the O-specific chain.
Background of endotoxin examinations

The R-core is an oligosaccharide and can be divided into two subunits: a so-called "backbone" unit (inner core) and an external unit (outer core). The inner core has the same composition in all Gram-negative bacteria, containing rarely occurring components (heptose (Hep) and 3-deoxy-D-manno-2-octulonic acid (Kdo)) and is linked to the lipid A part. The outer core is made of up to six hexose components, and is linked to the O-specific side chain.

The outermost element of the LPS structure is the hydrophilic O-specific chain, which is built up from 1-40 repeating units (usually consisting of 2-7 sugar components) resulting in diverse composition. The repeating units are unique and characteristic for the bacteria.

The lipid A part is a hydrophobic structure directed towards the interior of the cell, which is responsible for the toxic biological effect (Watson and Kim, 1963).

Regarding its structure, it is a specific glycosphospholipid (usually a bis-phosphorylated disaccharide backbone acylated with fatty acid chains), which is an integral part of the cell wall. It has a conservative structure, all Gram-negative bacterial lipid A parts are similar to each other (Rietschel and Brade, 1992). Lipid A it is found responsible for the endotoxin activity of the endotoxins. The free form of lipid A, extracted from endotoxins by acid hydrolysis, has almost the same spectrum of biological activities as the endotoxin itself.
Overview of pharmacopeial bacterial endotoxin tests

EP describes several different techniques, using amoebocyte lysate from the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*) for bacterial endotoxins (BET) detection or quantification. There are 3 techniques for this test:

- **the gel-clot technique**, which is based on gel formation;
- **the turbidimetric technique**, based on the development of turbidity after cleavage of an endogenous substrate; and

These tests should be carried out in a manner that avoids endotoxin contamination. All the apparatus used in BET testing has to be free of detectable endotoxins.
Overview of pharmacopoieal bacterial endotoxin tests

Based on afore mentioned techniques, EP describes 6 available methods for BET testing:

- Method A. Gel-clot method: limit test
- Method B. Gel-clot method: quantitative test
- Method C. Turbidimetric kinetic method
- Method D. Chromogenic kinetic method
- Method E. Chromogenic end-point method

EP allows using any of these 6 methods. But, in the event of doubt or dispute, it is stated that the final decision should be made based upon method A unless, otherwise indicated in the monograph (EP, 2.6.14., page 194).
Overview of pharmacopoeial bacterial endotoxin tests

GEL-CLOT TECHNIQUE (METHODS A AND B)

The gel-clot technique is based on clotting of the lysate in the presence of endotoxins. The minimum concentration of endotoxins required to cause the lysate to clot under standard conditions is the labelled lysate sensitivity (EP, page 195).

TURBIDIMETRIC TECHNIQUE (METHODS C AND F)

This technique is measuring the increase in turbidity. The kinetic-turbidimetric test (Method C) is a method to measure either the time (onset time) needed for the reaction mixture to reach a predetermined absorbance or transmission, or the rate of turbidity development. The end-point-turbidimetric test (Method F) is based on the quantitative relationship between the endotoxin concentration and the turbidity (absorbance or transmission) of the reaction mixture at the end of an incubation period.
Overview of pharmacopoieial bacterial endotoxin tests

CHROMOGENIC TECHNIQUE (METHODS D AND E)

This technique is used to measure the chromophore released from a suitable chromogenic peptide by the reaction of endotoxins with the lysate. The end-point-chromogenic test (Method E) is based on the quantitative relationship between the endotoxin concentration and the quantity of chromophore released at the end of an incubation period. The kinetic-chromogenic test (Method D) measures either the time (onset time) needed for the reaction mixture to reach a predetermined absorbance, or the rate of colour development.
Overview of pharmacopoieal bacterial endotoxin tests

- In common for all the methods:

- Samples for the BET test should be dissolved or diluted in the water for BET or other aqueous solutions. It is important, if necessary, to adjust the pH of the test solution (or dilution thereof) so that the pH of the mixture of the lysate and test solution falls within the pH range specified by the lysate manufacturer, usually 6.0 to 8.0.

- Determination of the maximum valid dilution, MVD.

- Check for interfering factors!
Overcoming Interference

- **Dilution**
- pH adjustment
- Heating
- Surfactant/Dispersing Agent
- Cellulase or Glucan Blockers
- Divalent Cation Addition
- Ultrafiltration
Sample preparation of oil products

- Interference may be overcome by suitable validated treatment, such as filtration, neutralization, dialysis or heat treatment. To establish that the treatment chosen effectively eliminates interference without loss of endotoxins, repeat the test for interfering factors using the preparation being examined to which the standard endotoxin has been added and which has then been submitted to the chosen treatment (EP, page 196).

- **Q:** How to handle interference in oil products? **A:** extraction!

- **Why?**
  - Assumption is that bacterial endotoxins will transfer to the aqueous phase.
Sample preparation of oil products

- How?

  ✓ 0.5 ml oil product + 5 ml LAL reagent water
  ✓ Vortex 5 times for 30 sec (30 sec time intervals between)
  ✓ Allow phases to separate
  ✓ Use water phase for analysis (1:10 product dilution!).
Method validation for chosen oil product

1. BET inoculation of oil
2. BET inoculation of finished product

- Sample + BET
- 0.5 ml of prepared sample + 5 ml LAL water
- Vortexing procedure
- LAL assay
Method validation for chosen oil product, Results

- The compendial requirements are provided for a large number of medicinal products (EP, USP) as well as manufacturer’s requirements for bacterial endotoxins limit concentration (ELC) in parenteral products. If the requirement for ELC in the tested preparation is unavailable, that ELC needs to be calculated.
- Based on the assessed ELC value of testosterone enanthate (0.88 EU/mg) and the content of testosterone enanthate in the product (250mg/ml), the ELC was calculated (220 EU/ml).
- In accordance with obtained ELC value, we decided to inoculate samples with 220 EU, 22 EU and 2.2 EU.
Method validation for chosen oil product, Results

<table>
<thead>
<tr>
<th>BET inoculated (EU/ml)</th>
<th>BET result (EU/ml)</th>
<th>Recovery (%)</th>
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<tbody>
<tr>
<td></td>
<td>Product</td>
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<td>2.2</td>
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</tbody>
</table>
Literature

- Ph. Eur. 8.0.
- Arambašić M., Extraction from and assay of bacterial endotoxins in oil parenteral preparations. ii: Progesteron depo (hydroxyprogesterone caprate) and Testosterone depo (testosterone enanthate) injection.
Thank you for your attention!
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