ENDOTOXIN TEST

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INTRODUCTION

- Pharmaceutical products & Medical Devices
- Bacterial Contamination
- Gram negative Bacteria
- Quality of Pharmaceutical product from microbiology point of view
INTRODUCTION

Parenteral products:

Should be sterile because it administered directly into blood circulation

One of important step: Sterilization

Sterile Products sometimes contaminated by ENDOTOKSIN
Why the product can be contaminated by endotoxin?

- In sterilization process (using heating method), Gram negative bacteria, that might be inside the product, will be killed and lysis, the endotoxin will release and will remain in the product.
- The endotoxin is heat-stable
What is Endotoxin?

What is Pyrogen?

- Endotoxin is a toxin produced by Gram negative bacteria.
- Pyrogen is a substance that can cause body temperature raised due to intravenous administration.
- All endotoxin is pyrogen, but not all pyrogen is endotoxin.
- Endotoxin of bacteria consist of Lipopolysaccharide (LPS), which is bound to protein and phospholipid.
- This LPS is a component and located on the outer membrane of Gram negative bacteria.
Structure of LPS

Example: LPS of *Salmonella* consists of Lipid A part which is hydrophobic and bound to a core region contains KDO molecule (2-keto-3-deoxyoctonate)
LPS Structure of *Salmonella*

- Mannose-Abequose
- Rhamnose
- Galactose
- Glucose-N-acetylglucosamine
- Galactose
- Glucose-Galactose
- Heptulose
- Heptulose-P-P-Ethanolamine
  - KDO
  - KDO-KDO-P-Ethanolamine
  - P-GlcN-GlcN-P

O-Side Chains

Core polysaccharides

Lipid A
Effect of endotoxin to human body

- Fever
- Activate cytokine system
- Destroy endothelial cells
- Influence permeability of blood vessels and cause low blood pressure
- Etc.
Development of regulation on pyrogen test

- **Bacterial endotoxin test** (BET) is one of important tests for sterile products and medical devices
- In 1912: pyrogen test was done by Rabbit test
- Rabbit test was used in USP XII in year 1942 for 40 years
- In 1980: new method was introduced: Limulus Amoebocyte Lysate (LAL) test
The **Limulus amebocyte lysate** (LAL) test is an in-vitro test for quantitative analysis of endotoxin.

- Lysate obtained from amubocyte of horseshoe crab (*Limulus polyphemus*)

- 2 Methods of LAL analysis: gel-clot method and turbidimetric method (kinetic and chromogenic/colorimetric method)
The discovery of the horseshoe crab's most significant biological role in recent medicine was made by Frederick Bang in the early 1950's.

Bang discovered that the horseshoe crab's blood cells, called amoebocytes, contain a clotting agent that attaches to dangerous endotoxins produced by gram negative bacteria.

The test was accepted by the United States Food and Drug Administration (FDA) in 1983 as a standard test for endotoxins.

In 1987, the FDA established guidelines for LAL testing of pharmaceuticals and medical devices.
Limulus polyphemus
(horseshoe crab)
Horseshoe crab

African Wolf Spider

Encarta Encyclopedia, Oxford Scientific Films/David Cayless
How to get the lysate

- Big size of Healthy Horseshoe crabs
- Blood of the crab is collected by syringe, centrifuge the blood to separate the amoebocytes from the plasma.
- Amoebocyte is then freeze-dried for further process. The price: One quart of LAL is worth $15,000!
- Three companies in USA producing LAL:
  - Associates of Cape Cod: http://www.acciusa.com
  - Cambrex: http://www.cambrex.com/default.asp
Recommended LAL Methode by FDA – USA

- Gel-Clot Method: LAL + endotoxin ⇒ clotting
- Kinetic turbidimetric Method: rate of gel forming ⇒ concentration of endotoxin
- Chromogenic Method: using synthetic chromogenic substrate, compared with LAL+endotoxin, producing yellow colour and using standard curve
LAL Chromogenic endotoxin assay utilizes a modified Limulus Amoebocyte Lysate and a synthetic color-producing substrate to detect endotoxin presence.

This assay is quantitative and the color intensity developed upon addition of the sample to the LAL supplied with the kit is proportional to the amount of endotoxin present in the sample and can be calculated from a standard curve.

The kinetic chromogenic quantitative assay for bacterial endotoxin can be performed at the low assay range (0-5 EU/ml) or the higher range (5-50 EU/ml)
Gram-negative bacterial endotoxin catalyzes the activation of a proenzyme in the Limulus Amebocyte Lysate. The initial rate of activation is determined by the concentration of endotoxin present. The activated enzyme (coagulase) hydrolyzes specific bonds within a clotting protein (coagulogen) also present in Limulus Amebocyte Lysate. Once hydrolyzed, the resultant coagulin self-associates and forms a gelatinous clot.
• LAL test use immune response of Horseshoe crab against the invasion of Gram negative bacteria.
• The Amoebocytes contains protein, factor, co-factor and ion which interact and cause coagulation
• Endotoxin catalyze activation of pro-enzyme in the lysate.
• Initial Rate of activation is determined by concentration of endotoxin
Lyophilized Limulus Amebocyte Lysate must be stored under refrigeration. Care should be taken to avoid exposing the lysate to temperatures above 25°C. Lysate which has been exposed to prolonged periods of temperatures above 25°C or to bright light may turn yellow. Lysate which exhibits such characteristics should be discarded.
Activated Enzyme (coagulase) will hydrolize specific binding in a protein (coagulogen) inside the lysate.
- Limulus produce coagulin.
- Once hydrolysis, coagulin will aggregate to form gel.
Enzymatic reaction on LAL clotting

Alternative way

ENDOTOXIN → FACTOR C → ACTIVATED FACTOR C → FACTOR B

FACTOR G

ANTI FACTOR G

ACTIVATED FACTOR G

PROCLOTTING ENZYME → CLOTTING ENZYME

COAGULOGEN → COAGULIN (GEL)
Samples containing B-Glucans (carbohydrates, mold, starch, plant material, food supplements) will interfere with the pyrochrome and produce falsely elevated endotoxin levels.

Please inform the laboratory if your sample contains carbohydrates or B-glucans. A special buffer is used to resuspend the pyrochrome in order to overcome the nonspecific interference.
■ USP requires 3% of the production lot with a minimum of 3 and maximum of 10 devices to be pooled and tested. For liquid samples, a minimum of 5 ml is required and powder sample require enough material to reconstitute into a minimum of 5 ml pyrogen-free water. Initial validation on 3 lots must be performed in order to validate the method of testing for a given product.
A positive test is characterized by the formation of a solid gel which remains intact after inversion. This should be observed in the positive control vial and in the positive sample control vial.

A negative test is characterized by the absence of solid clot after inversion. This should be observed in the negative control vial. The lysate may show an increase in turbidity or viscosity. This is considered a negative result.

Record positive and negative results for the test sample vials.

A positive result should be observed with all Inhibition Control vials. The absence of gel formation is indicative of product inhibition. If a negative reaction is observed in the Inhibition Control vial with any sample, then the sample's test results are invalid.
Positive result of endotoxin test using LAL method
CALCULATION and MEASUREMENT
Determination of endotoxin limit

- According to USP and EP
- 1983: FDA determine the limit of endotoxin content based on maximum human dose
- In practice endotoxin limit for medicine (except intrathecal): 2.5 EU kg\(^{-1}\) up to 5.0 EU kg\(^{-1}\)
- EU = Endotoxin Unit
Terms

- EL: Endotoxin Limit
- MAEC (Maximum Allowable Endotoxin Concentration)
- ERL (Endotoxin Release Limit)
- ELC (Endotoxin Limit Concentration)
$EL = \frac{K}{M}$

- $K = \text{constant equal to 5 EU or IU per kg of body weight}$,
  $M = \text{maximum human dose administered per kg per hour}$.
- Often it is quoted in:
  Endotoxin limit of 350 EU per hour for average 70 kg man (5 EU kg$^{-1}$)
Endotoxin limit vs volume of preparation per hour

- It should be noted that endotoxin limit should be indicated per hour. The concentration allowed for endotoxin per millilitre of injectable preparation will vary considerably with the volume administered within the hour.

- A single 1 mL injection could theoretically contain 349 EU per mL and still pass the BET, whereas a 1-L infusion would need to contain less than 0.349 EU per mL.
MVD and MVC

- MVD = Maximum Valid Dilution
- MVC = Minimum Valid Concentration
- MVD and MVC are calculated figures that indicate the degree to which a product may be diluted to overcome interference, before the effect of dilution exceeds the ability of the LAL test being used to detect endotoxin in original preparation.
When we use MVD?

- Term “MVD” is normally applied to preparation already in a liquid form where the dose is administered per mL.
- For example: a single 2 mL injection and the endotoxin limit is expressed as EU per mL
When we use MVC?

- MVC is applied to the preparation where the endotoxin limit is expressed in EU per mg and the dose is expressed in mg/ kg of body weight.
Sensitivity of Lysate ($\lambda$)

- Determination of MVD and MVC is dependent on the sensitivity of the lysate used ($\lambda$).
- The more sensitive the lysate, the higher the MVD or the lower MVC value will be.
Calculation of MVC

\[ MVC = \frac{\lambda M}{K} \]

- \(\lambda\) = sensitivity of the lysate or the value of the lowest standard for quantitative assays
- \(K\) = constant = 0.5 EU kg\(^{-1}\)
- \(M\) = maximum human dose
Calculation of the MVD

\[
MVD = \frac{C \times K}{\lambda \times M}
\]

- \( C \) = concentration of drug
- \( K \) = constant = 0.5 EU kg\(^{-1}\)
- \( M \) = maximum human dose
Example

- An insulin injection of potency 100 unit per mL, the maximum dose of 2 unit per kg and sensitivity of lysate is 0.125 unit/mL, thus:

  \[ \text{MVD} = \frac{100 \times 5}{0.125 \times 2} = 1 : 2000 \]

- MVD value can be calculated from MVC

- MVD = potency of product/MVC
## Price of LAL test kit

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<th>Part Code</th>
<th>Description</th>
<th>Price</th>
<th>Curr</th>
<th>Unit</th>
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<tr>
<td>N189-06</td>
<td>0.06 EU/ml sensitivity, 25 tests</td>
<td>106.00</td>
<td>USD</td>
<td>KIT</td>
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<td>106.00</td>
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<td>KIT</td>
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</tbody>
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LAL test for medical devices

- Level of Endotoxin on medical devices are obtained by an extraction procedure. This involves soaking a number of devices in an extracting fluid, normally LAL reagent water.

- A limit of 20 EU per device was set in addendum of USP 1997, so the permissible endotoxin concentration in extracting fluid (ERL=endotoxin release limit) can be calculated:

  $$\text{ERL} = K \times \frac{N}{V}$$

Where K is 20 EU per device, N = number of devices and V = total volume of extraction solution.
The Gel-Clot Method

- The result could be spot on slide, or in microplate
- The references: Control Standard Endotoxin (CSE)
- All glasses used have to be “de-pyrogenized”
Test Principles and procedure

- 100 ul CSE into depyrogenized glass tube (positive control)
- LAL reagent water (negative control)
- Samples with same volume
- Add 100 ul lysate
- Incubation at 37°C water bath for 1 hour
- Tubes are removed and inverted by 180° in a single, smooth motion to determine if a solid clot has formed at the bottom
Precautions in gel-clot method

- All material coming into contact with specimen or test material must be pyrogen-free. Material may be rendered pyrogen-free by heating at 180°C for 4 hours, or 250°C for 30 min, or other validated method.

- Careful Technique for inverting the tubes: around 2 sec.

- pH samples: 7.0 – 8.0. If required, pH may be adjusted with pyrogen-free acid or base.
Sensitivity of Lysate on Gel-Clot method

- In order to assign significance to positive or negative gel-clot results, it is necessary to determine the minimum concentration of endotoxin which causes gelatination of lysate, known as Lysate sensitivity.
- Expressed in EU or IU.
- Prepare 1 series of endotoksin dilution (in EU/mL) and the test should be performed 4x (quadruplicate).
- End-point dilution is determined as the last dilution of endotoxin which still yields a positive result.
Chromogenic end-point Method

- Chromogenic end point Method offers a fast, relatively low-cost method which allows quantitation and frequently overcomes interference problems that occur with gel-clot method or kinetic turbidimetric methods.
- Suitable for small number of products and infrequent.
- Used for serum on clinical test.
Test Principle and procedure

- Endotoxin will catalyze activation of a proenzyme.
- The activated Enzyme will catalyze the splitting of PNA from colorless substrate Ac-Ile-Glu-Ala-Arg-PNA.
- The released PNA is measured by spectrophotometer at 405 nm.
- The Absorbance value is equal to endotoxin concentration which is plotted to standard curve which is linear in the 0.1 and 1.0 EU/mL range of endotoxin standard.
New Discoveries

- Alternative to LAL test: developed in India (http://www.nio.org) and China. TAL, or *Tachypleus* amoebocyte lysate, with similar function to LAL, is also used to detect gram-negative bacteria toxin.

- Scientists in Singapore is now doing research in cloning gene detecting toxin in horseshoe crab blood. Construction of the gene is in progress.