Compounding Pharmacies and the Bacterial Endotoxin Test

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History
Latter 19th Century to Present

- Parenteral solutions cause fevers
  - “Injection Fever”, “Distilled Water Fever”, “Saline Fever”
- Hort and Penfold 1912 publication
  - Filterable substance
  - Stable to Heat
  - Produced by bacteria
- Seibert’s research 1923-1925 proved conclusively
  - Developed method for apyrogenic injections
  - Many hospital pharmacists began to use this process
- Joint work led to the development of the Rabbit test first described in 1925 and in 1942 (USP <151>)
Pyrogens
Physiological Effects

- Small Amounts
  - Increase capillary permeability
  - Fever
  - Altered resistance to bacterial infections
  - Leukopenia followed by leukocytosis
- Larger amounts
  - Possible Septic Shock
  - Multiple organ failure
  - Schwartzman Reaction & Phenomenon
  - Death

Schwartzman Reaction & Phenomenon

**Generalized Schwartzman Reaction**
- Generalized reaction following two intravenous injections of endotoxins
- Given 24 hours apart
- Generalized Reaction
  - Reduced numbers of white blood cells and platelets
  - Renal Necrosis
  - Death

**Schwartzman Phenomenon**
- Localized cutaneous reaction following SQ injection
- Followed 24 hrs later by IV injection of endotoxin
- Reaction at site
  - Hemorrhage
  - Necrosis
  - White blood cell infiltration
Pyrogens vs Endotoxins

- Pyrogens broken down into two major groups
  - Exogenous pyrogens (produced by infectious entities)
  - Endogenous pyrogens (produced by cells in the body)
- Endogenous pyrogens
  - Low molecular weight proteins produced by the body
  - Produce acute phase reaction
  - Eleven identified
  - Usually Cytokines
- Exogenous pyrogens
  - Mediated by Endogenous proteins

Exogenous Pyrogens

- Bacterial Endotoxins
- Other microbial products
- Antigen-antibody complexes
- Viruses
- Synthetic polynucleotides
- Incompatible Blood and Blood products
- Androgen breakdown products such as etiocholanolone
Bacterial Endotoxins

- Outer bacterial wall of Gram-Negative bacteria
  - Regardless of pathogenicity of bacteria

- Consists of components of cell wall as well as polysaccharide and Lipid A
  - Immunogenicity attributed to polysaccharide
  - Toxicity associated with Lipid A

- Pyrogenetic response with as little as:
  - 5 EU/kg Parenteral drug
  - 0.2 EU/kg Intrathecal drug

Pyrogen Testing Required

- High-risk batches (except inhalation and ophthalmic) of >25 identical individual single-dose packages or
- Multi-dose vials for administration to multiple patients or
- Exposed longer than 12 hours at 2°C to 8°C before they are sterilized
- Longer than 6 hours at warmer than 8°C before they are sterilized

- Consider all intrathecal medications as the sensitivity is 1000 times greater via this route
USP <151> Pyrogen Test
Rabbit Pyrogen Test

• 3 similar rabbits
• Same breed
• Healthy with similar starting recorded temperatures
• Injected with known amount of product
• Temperature monitored
• Limits on testing frequency

Advantages

- Works on all types of pyrogens
- Well established parameters
  - Temperature increase of 0.6°C or more in any one rabbit
  - Temperature increase in total for all 3 rabbits of more than 1.4°C

Disadvantages

- Costly
- Rabbit husbandry
- Large facilities
- Uber regulations for animals
- Takes considerable time
- Rigorous training and experience necessary
- Not very quantitative
Limulus Polyphemus

Limulus Amebocyte Lysate

- Hemocyanin not Hemoglobin
  - Blue Blood – copper not iron
- 1956 Bang discovered that a crab died as a result of an infection caused by Vibrio
- Entire circulatory system clotted into a semi-solid mass
- Open system – engulfs bacteria
- Amoebocyte – Limulus Hemocyte
- Release granule which contain coagulogen
- Enmeshes bacteria

Limulus Polyphemus

Research by Fred Bang

- Only Gram Negative bacteria
- Heat treated (dead bacteria) also caused reaction
- Similar to well known endotoxin reaction in mammals – Schwartzman Reaction
Connecting the Dots

- 1960’s Bang joined by Jack Levin – a hematologist
- clotting reaction induced by a 3-step enzymatic cascade that was remarkably similar to the cascade occurring in human coagulation
- Very sensitive method to detect endotoxins in preparations

Process of Lysate Production

- Bleed with large gauge needle
- Up to 30% of blood removed
- Returned to sea within 72 hrs
- Blood volume returns 1 week
- Centrifuge to collect amoebocytes
- Lysed by placing in purified water
- Lysate purified & freeze dried
- Assayed for strength by facility
USP <85> Bacterial Endotoxins Test

- Levin and Cooper searched for more timely testing with the advent of radioactive drugs in the late 1960's
  - Needed to be more quickly carried out due to short half life of drug
  - Needed to use a much smaller volume of drug
- Compared LAL sensitivity to rabbit response
- Found correlation between rabbit response and LAL activity (gel time)
- Published paper in 1971 with results

USP <85> Bacterial Endotoxins Test
Types of Testing

- Gel-Clot
  - Limit test
  - Semi-quantitative
- Photometric
  - Turbidimetric
    - Kinetic
    - End-point
  - Chromogenic
    - Kinetic
    - End-point
Interference With Test

- Need to screen for interference with test
- Conditions for interference
  - Beta glucans (False Positive)
  - Low or High pH (False Negative)
  - Monovalent or Divalent cations (False Negative)
  - Endotoxin micelle formation (False Negative)
  - High Concentration of Sample (False Positive or Negative)

Preparatory Testing

- USP <85> has two requirements prior to testing
  - Confirmation of LAL test performance using standard endotoxin but no sample
  - Interference testing – to show that the sample does not interfere with the LAL during the test
- Drug sample dissolved or diluted
- pH must be in range (usually 6.0 to 8.0)
- Test at least in quadruplicate a sample at less than MVD
Interference Testing

- Test a duplicate set of samples with a series of known endotoxin concentrations
- Purpose of test to prove that sample vials have no significant difference than water
- Interference may be overcome by treatment then validating
  - Filtration
  - Neutralization
  - Dialysis
  - Heating

Maximum Valid Dilution (MVD)

- Greatest dilution of the preparation at which the endotoxin limit can be detected.

\[ MVD = \frac{\text{Endotoxin Limit} \times \text{Concentration of sample}}{\lambda} \]

- \( \lambda = \) the sensitivity of the test

- The sensitivity of the gel clot reagents are marked on the packages
- For Photometric tests the sensitivity is the lowest concentration on the standard curve and is flexible
Endotoxin Limit

- Conservatively represents the safe amount of endotoxin that is allowed in a dose of a specific medication
- USP monographs for drugs lists the endotoxin limits for specific drugs in EU/mg
- Route specific endotoxin limits often used

Endotoxin Limit

- \( \text{Endotoxin Limit} = \frac{K}{M} \)
- \( K \) equals
  - Parenteral administration – 5 EU/kg
  - Intrathecal administration – 0.2 EU/kg
    - Intrathecal administration 1000 times more sensitive
- \( M \) equals
  - Maximum dose in milligrams per body weight or milliliters per body weight in kilograms per hour of the drug given
Gel-Clot Method

- Limits Test in duplicates
  - Negative control vials must not react
  - 2λ Positive control must react
  - 2λ Positive Product Controls must react (Product + Endotoxin)

Gel-Clot Method

- Diluted sample injected into a vial containing lysate
- Diluted sample injected into vial containing lysate and known endotoxin
- Incubated per lysate instructions (usually at 37°C for 60 minutes)
- Gently remove from incubation block and invert gently 180°
- Gel formation in Positive control vial indicates no interference
- No gel formation in sample vial indicates endotoxins within acceptable limits
Gel-Clot Kits & Supplies

Analysis

Advantages
- Low initial outlay
- Heating block
- Kits
- Moderately expensive test
- No external shipping
- Training not overly involved

Disadvantages
- Qualitative not Quantitative
- Vibrations can affect test
- Not as efficient as other test modalities
- Performed and documented in about 1.5 hours
- Difficult to validate to higher standards
Photometric Testing

- Turbiometric – reactions with LAL cause turbidity which can be measured with an optical reader which measures the decrease in the amount of light coming through a sample.
- Chromogenic – LAL is combined with another agent which causes it to change with the reaction making it easier to measure the change in light
- End-point testing has fallen out of favor
- Kinetic testing is most used

Kinetic Results

- Measurement of changes in the amount of light over time give a kinetic profile of the changes over time
- Changes over time measured against a known data set stored in the device
- Matching the results with stored data gives a specific quantitative answer in EU per sample (mg or mL)
- Still need interference testing
- Still need the Positive Product Control vial
- Still need Negative controls
- Can run multiple samples at one time
  - 96 reaction tubes in some devices
### Photometric Analysis

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td>Many samples at one time</td>
<td>High initial cost</td>
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<td>Less costly per test with large volume</td>
<td>Reagent media preparation is somewhat difficult</td>
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<td>Less labor intensive overall if used optimally</td>
<td>Low volume compounders may waste materials and time</td>
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<td>Chromogenic about 10-15% more expensive than the turbidometric</td>
<td>Extensive training required in comparison</td>
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### Photometric – Turbidometric Assays

![Photometric – Turbidometric Assays](image-url)
PTS System - Cartridges

- Cartridge system – Kinetic chromogenic reagents and endotoxin controls are dried into 4 channels of a polystyrene cartridge
- Sample diluted according to protocols (see Cooper, JF Endotoxins (Part 3) IJPC V15(1) pp 49-54)
- Cartridge ID entered into device
- Analyst Initials, Drug Name, Drug Name, Specified Dilution Factor, etc entered into device

PTS System - Cartridges

- Add 25 µl of sample to each of 4 channels in cartridge
- Press enter
- Pump moves samples through cartridge (about 15 minutes)
- Optical cells are read kinetically at 395nm +/- 20nm
- Results displayed on screen
- Recovery must be between 50 and 200% for valid test
- Invalid test may require further dilution or buffers
- Many dilutions on record
PTS system Advantages

- Cartridges about $50 each
- Initial device cost reasonable
- FDA-licensed
- Portable, handheld LAL test system
- Fast, quantitative results—anywhere
- Simple, one button operation
- Single step, quantitative LAL test
- Results in about 15 minutes
- LAL test components all included
- Detects between 0.005 - 10 EU/mL
- Data downloadable to a central PC

Endosafe®-MCS System
Five Cartridge Chromogenic System

Figure 1: Multi-cartridge Endotoxin Detection System
Endosafe®-MCS System

Five Cartridge Chromogenic System

- Test multiple samples simultaneously
- Samples run independently allowing for random access
- High throughput for real-time results
- Single step, semi-quantitative LAL test
- Uses FDA-licensed PTSTM endotoxin cartridges
- LAL test components all included in cartridges
- Four levels of sensitivity: 0.005 EU/mL; 0.01 EU/mL; 0.05 EU/mL; 0.10 EU/mL
- Results can be tracked and trended via EndoScan-VTM and Microtrend
- Sophisticated data management and reporting capabilities
- Samples can be traced to the individual spectrophotometer used to perform assay

Contract Laboratories

Decision Factors

- Determine type of testing used as each has advantages and disadvantages
- History of testing for pyrogens
- Types of drugs tested
- Ensure enhancement-inhibition testing is performed
- Note whether the laboratory asks for dosage information
  - Best to use dosage limits
- Frequency of testing types of drugs that you will be submitting
- Can laboratory establish data trends
  - Can notify if trends start varying towards problem area
- Ensure the laboratory reports endotoxin units and not pass/fail
Internal vs Contract

- If decide internal then validate results with external testing with first run of each new preparation
- Consider volume of testing required to get return on investment (10 – 15 per week for DCF)
- Understand the limitations of internal testing
- Not everything can be tested internally and when this occurs, MUST use contract laboratory
- Consider training required for specific devices
- Consider support for testing protocols and endotoxin limits
- Consider labor involved

Bibliography

Marine Biological Laboratory
http://hermes.mbl.edu/marine_org/images/animals/Limulus/blood/bang.html accessed 5-19-14
- Guidance for Industry: Pyrogens and Endotoxins testing: Questions and Answers
Comprehensive QC Solutions for Compounders

Charles River’s Microbial Solutions for Compounding Pharmacies

- FDA-licensed products and cGMP-compliant services to maintain control and consistency in your compounding processes, from in-process testing to product release.
- Gain access to our BET/LAL technical expertise for method development, training support and product offering:
  - Endosafe® Endotoxin Testing: Gel-clot, Kinetic and PTS™ Methods
  - Accugenix® Microbial ID for EM Programs
  - Sterility Testing
  - Microbial Limits Testing
  - Bioburden Testing
Endosafe®-PTS™: Rapid Endotoxin Test System

- Portable handheld spectrophotometer that utilizes FDA-licensed disposable cartridges that are pre-loaded with all test components.
- Delivers accurate, convenient endotoxin results in **15 minutes** at the point of sample collection.
- Simple, one button operation that Detects .005-10 EU/mL
- Eliminates bottlenecks, retests, std. curve errors, improves sample management and, most importantly, assures that your products are free of endotoxin (pyrogen) within limits set by the *Pharmacopeia*.

Educational Resources & Technical Support

- [www.criver.com/compounding](http://www.criver.com/compounding)
- **Building Trust and Stabilizing Quality Control in Compounding Pharmacies Blogs**
  - Compounding Pharmacy Regulation: A Matter of Life and Death
  - Compounding and Unapproved Drugs: What are the facts?
  - Know Your Enemy: Accurate Identification of Microorganisms is the Key to Environmental Monitoring
- **Testing Compounded Sterile Products for Endotoxin Webinars**
  - This webinar details how to test CSPs for endotoxins, as recommended by USP <797> Pharmaceutical Preparations-Sterile Compounding.
- **Microbial Solutions for Compounding Pharmacists Technical Sheet**
- **Contact Information:**
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