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Review Article

Endotoxin OOS and the Quest for the Root Cause

Crystal Booth MM*

Regional Manager at PSC Biotech, USA

***Corresponding Author:** Crystal Booth MM, Regional Manager at PSC Biotech, USA.

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Abstract

Out of specification endotoxin results are occasionally obtained. When this occurs, an investigation must be performed. A decision to reject the batch does not remove the requirement to perform an investigation. Finding a root cause to endotoxin contamination early can aid with the control, clean up, corrective actions, and preventative actions that may be required to protect the company, the products, and the patient. This article discusses endotoxin out of specification results and root cause investigations.

Keywords: Endotoxin; OOS; Root Cause

Overview of the bacterial endotoxin assay

The bacterial endotoxins test (BET) is described in the United States Pharmacopeia (USP) <85> Bacterial Endotoxins Test, European Pharmacopeia (EP) 2.6.14 Bacterial Endotoxins, and the Japanese Pharmacopeia (JP) 4.01 Bacterial Endotoxins Test. The majority of these different compendial are harmonized with each other. The portions that are not harmonized, are marked as not being harmonized [9].

The test is used to detect or quantify endotoxins from Gram-negative bacteria using amoebocyte lysate from the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*) [9]. Simply put, coagulogens in the amoebocyte lysate clot in the presence of endotoxins to create a semi-solid mass or clot [11]. The BET assay is often used to test raw materials, water, components, parenteral finished products, medical devices and stability samples.

There are three techniques described in the compendial chapters for the test. The techniques include the gel-clot technique, the turbidimetric technique, and the chromogenic technique.

The gel-clot technique most resembles the first technique discovered and is often relied upon as the referee test in the compendial chapters [9]. The gel-clot test is manually involved. Dilutions are made, mixed with limulus amoebocyte lysate (LAL) in a test tube, and incubated at 37°C + 1°C for a period of 60 + 2 minutes. Following the incubation period, the tubes are inverted and observed for clots. Anything other than a solid clot is considered negative. This test has earned the nickname of the wet-hand test because negative results have the potential to fall onto a technician's hand. One must be cautious when reading this test because the gel clots could loosen if the tubes are jarred too much while analyzing the results.

The turbidimetric technique is based on the development of turbidity after cleavage of an endogenous substrate [9]. This test also involves dilutions and mixing the testing samples with LAL. The test samples, controls, and LAL are typically spiked into a 96 well microtiter plate as opposed to test tubes. A photometric instrument is required to incubate the plate and measure the rate of turbidity change during the assay [9].

The chromogenic technique requires dilutions and mixing the testing samples with a specialized LAL reagent. The test samples, controls, and specialized LAL are typically spiked into a 96 well microtiter plate as opposed to test tubes. The technique is based on the development of color after cleavage of a synthetic peptide-chromogen complex and requires the use of a spectrophotometer. The spectrophotometer is used to incubate the plate and measure the rate of the color change [9].

When setting appropriate specifications for the test, the material should be researched. The compendial monographs may be checked to see if specifications or methods are already established. Individual monographs for specific raw materials may not be harmonized with one another in the various regions. However, utilizing the most stringent criteria from the various regions will allow one method to be developed that is globally compliant.

The USP chapter on endotoxin testing, USP <85>, describes how to calculate endotoxin limits. The calculation is described as the endotoxin limit (EL) being equal to K/M and is expressed as follows: [9]

$$EL = K/M$$

In the equation, K is a threshold pyrogenic dose of endotoxin per kilogram (kg) of body weight. Five (5) endotoxin units (EU)/

kg is used in the calculation for parenteral drugs and two (2) EU/kg is used in the calculation for intrathecal drugs [9]. M is equal to the maximum recommended bolus dose of product per kg of body weight. M can also be the maximum total dose received in a single hour period when the product is injected at frequent intervals or infused continuously [9].

When performing the calculation, the average human weight is typically considered to be 70 kg. However, some countries, such as Japan, utilize 60 kg as the average human weight. Using the calculation, 350 EU are allowed for a 70 kg human per hour. Many companies add an additional safety factor when calculating endotoxin limits to that ensure consumers are safe [4].

It is important to consider all contributing sources of endotoxin when setting a specification. For example, all components of the final product (e.g. water, raw materials, active pharmaceutical ingredient [API], etc.) will contribute some endotoxin and this total amount of endotoxin should not exceed the maximum calculated value.

When performing the assay, invalid results may be obtained from time to time. Invalid results are different than out of specification results (OOS) and may be handled differently. However, there should not be an overabundance of invalid results. Frequent invalid results may require an investigation to determine the root cause of the invalidity. Common causes of invalidity include the following:

- Pipetting errors [10]
- Incorrect well selection [10]
- Subpotent endotoxin standards (particularly the lowest endotoxin concentration.) [10]
- Dilution errors [10]

The Food and Drug Administration (FDA) Guidance for the Industry document "Guidance for the Industry-Pyrogen and Endotoxins Testing: Question and Answers" provides information on commonly asked questions. The document states that "when conflicting results occur within a test run, firms should consult USP Chapter <85>, Gel Clot Limits Test, Interpretation, for guidance on repeat testing. As specified in Chapter <85>, if the test failure occurred at less than the maximum valid dilution (MVD), the test should be repeated using a greater dilution not exceeding the MVD. A record of this failure should be included in the laboratory results. If a test is performed at the MVD and an out-of-specification (OOS) test result occurs that cannot be attributed to testing error, the lot should be rejected" [6]. When OOSs are obtained in the laboratory, they should be properly investigated to determine the root cause and to prevent reoccurrences whenever possible.

Investigating the unexpected data

Handling OOS results are discussed in many regulatory documents and proper investigations are expected. Several observations have been written regarding improper investigations of out of specification results. In addition, the Code of Federal Regulations (CFR) 21 CFR 211.192 states that "any unexplained discrepancy (including a percentage of theoretical yield exceeding the maximum or minimum percentages established in master production and control records) or the failure of a batch or any of its components to meet any of its specifications shall be thoroughly investigated, whether or not the batch has already been distributed. The investigation shall extend to other batches of the same drug product and other drug products that may have been associated with the specific failure or discrepancy. A written record of the investigation shall be made and shall include the conclusions and follow up" [3]. If an OOS is obtained, it must be properly investigated even if the batch is rejected and the investigation must look at other batches to examine the possible impact. The investigation must be properly documented and discuss the conclusions, corrective and preventative actions, and any effectiveness checks that may be required.

The FDA Guidance for Industry from October 2006 for Investigating Out-of-Specification (OOS) Test Results for Pharmaceutical Production provides expectations on investigating OOS results. The document describes the investigation process consisting of two phases, Phase 1- the laboratory investigation and Phase 2- the full scale OOS investigation.

The first phase of the investigation (Phase 1) is a short preliminary investigation of the laboratory. The goal of during this phase is to look for and rule out any obvious errors that may have occurred [5]. Checklists are often helpful during this stage to help investigate data, equipment, and analysts. Generally, a re-measurement of the originally prepared sample, standard solutions, or dilutions is permitted during this stage if the supplies are not consumed or expired [5]. Everything that is investigated and observed must be properly documented. If clear evidence of an error is identified, the laboratory testing results may be invalidated. If meaningful errors are not discovered that could explain the root cause or if the results of the investigation are unclear, the investigation must proceed to a full scale investigation, Phase 2 [5].

The objective of Phase 2 is to identify the root cause and establish preventative and corrective actions. This part of the investigation should include manufacturing, process development, production process review, review of production sampling procedures, maintenance, engineering, and additional laboratory testing when applicable [5]. Retesting and re-sampling are permitted as part of Phase 2 but the maximum number of retests should be specified in advance to avoid the appearance of testing into compliance.

If laboratory error is identified, the retest results may substitute for the original test results [5]. However, all of the data must be retained and properly explained. If the OOS is confirmed and a root cause is identified, the product should be rejected. If the investigation is inconclusive, it is wise to err on the side of caution

when making batch release decisions [5]. During the investigation, every action and decision must be documented and the investigation should be expanded to examine the impact of the OOS results on other batches [5].

OOS investigation write-ups are often written similar to quality deviations. Some companies have quality managements systems designed to document OOS investigations, while other companies attach OOS investigations to deviations (or non-conformances) in their existing quality management system for tracking purposes.

When entering into Phase 1 of the OOS investigation, the first steps usually include notifying management, notifying QA, and initiating the investigation documentation. The event should be described properly with a problem statement. The problem statement should be written to encompass who, what, where, when, and why [2]. It is important to perform the investigation with an open mind and have no preconceived assumptions as to what may have caused the unexpected endotoxin result [5].

Whenever possible, the original test preparations should be kept to aid in the investigation [5]. If the batch has already been distributed, a field alert report (FAR) should be submitted to the FDA within 3 working days of any OOS [5].

The next step is to begin gathering information regarding the endotoxin OOS [2]. An OOS checklist is extremely helpful during this stage. Pertinent information may include the following:

- Product/ Sample Name
- Product Lot Number
- Method Name/Number
- Date of Discovery
- Date of Test
- Result
- Specification
- Analyst
- Analyst Training
- Analyst Interview
- Location of Occurrence
- Areas Notified
- Documentation
- Data Location (e.g. notebook)
- Raw Data
- Calculations
- Testing Controls
- Reagent Information
- Instrument and Equipment performance history
- Instrument ID Number
- Calibration Date
- Calibration Due Date
- Sample Handling
- Sample Labeling
- Glassware
- Consumables
- Certificates of Analysis
- Historical Data/Trends

All of the gathered information should be examined, sorted, labeled, and be readily retrievable for audits. If possible, attaching the information to the investigation is a good course of action. The data should be reviewed promptly for accuracy and to verify calculations [5]. Any hypotheses testing regarding what may have happened should be tested. All observations, decisions, corrective actions, and assignable root cause, if determined, should be explained in detail and documented.

When investigating endotoxin results, it is wise to apply knowledge of the endotoxin assay and how endotoxins behave. This information may include possible endotoxin sources, the behavior of naturally occurring endotoxins versus controlled standard endotoxins, the potential for false positives, the reaction of beta-glucans, the potential for endotoxins to bind to surfaces, interference or enhancement factors, and the use of endotoxin free consumables for the assay.

Phase 2 may begin when lab error is not determined to be the cause and the OOS appears to be accurate. At this point in the investigation, the problem has been identified, the problem statement has been written, and the data has been gathered. Production and sampling procedures should be reviewed for accuracy. Root causes for the OOS result should be investigated from all relevant areas including but not limited to: [5]

- Manufacturing
- Facilities
- Laboratory

Many root cause analysis tools are available. Choosing the best RCA tool for the investigation will depend on the companies' standard operating procedures (SOP), how complex the investigation is, the ease of use of tool for the employees performing the investigation, the ability of the tool to help find the root cause, and how much time and resources are available to perform the investigation correctly. The main goal of using a RCA tool is to guide the user to possible root causes. All root cause categories should be considered when investigating probable root causes [1].

Some available RCA tools include:

- Ishikawa (6Ms, Fishbone Diagram, etc.)
- 5 Whys?

- Is/Is Not
- Failure Mode and Effects Analysis (FMEA)
- Others tools are available (e.g. Kepner-Tregoe Problem Analysis)

Ishikawa is a diagram tool that is also known as 6Ms or the Fishbone Diagram, among other names [1]. The goal of using Ishikawa is to categorize the data and information gathered into categories. These categories typically include Man (Personnel), Method (Procedure), Machine (Equipment), Material, Mother Nature (Environment), or Miscellaneous (e.g. Process Design). This information is plotted onto a "Fishbone Diagram" to look for any information that is out of the ordinary [1].

The 5 Whys is another analysis tool that is commonly utilized in root cause analysis investigations. The question "why" is asked several times to get a deeper understanding of what could have happened to cause the OOS [1]. For example, a question is asked of the problem statement. Then, "why" is asked of the answer the question. Then, why is asked of the answer to that question, and so on until a root cause is identified. It is acceptable to stop asking why before the fifth why is asked if a root cause is identified.

The "Is/Is Not" tool allows a user to compare what the problem is related to what the problem is not [1]. This can appear in a chart form for compare and contrast purposes. Utilizing this tool should allow a user to hone in on all of the impacted components of the investigations and guide the user to a root cause.

The Failure Mode and Effects Analysis (FMEA) tool allows the user to look at the severity of the problem, the probability that the problem may occur, and the probability that if the problem occurs, it can be detected. These risk factors are assigned a numeric number and multiplied to establish a Risk Preference Number (RPN) [1]. This tool is good in helping to identify and eliminate risks.

Additional laboratory testing may be required. This testing will require an investigational test plan prior to the testing being performed. The test plan should describe the method, acceptance criteria, number of replicates, and how the results will be reported [5].

During the course of the investigation, multiple trends should be pulled and analyzed. These trends may include:

- Testing of other batches
- Utility Monitoring
- Validation, Calibration, and Maintenance History
- Human Error, Method Error, and Instrument Error

Trending is typically performed periodically and documented in dedicated reports. Any negative trends that are identified during the course of the investigation should be investigated [5]. This may involve opening another investigation or deviation to properly address a separate or related issue to the OOS. Reviewing and analyzing trends could help determine the impact on other products, the impact on the facility, if the problem as occurred before, how often the problem occurs, potential root causes of the problem, and how the problem can be prevented in the future.

The most probable root cause has the fewest assumptions, the simplest assumptions, the most reasonable assumptions, and assumptions that make the most sense.1 After the root cause analysis is complete, an impact and/or risk assessment should be completed on all of the potentially impacted to batches. The impact of the OOS on already disturbed batches should be evaluated [5].

Corrective and preventative actions (CAPA) should be properly documented and performed to contain, correct, and prevent the cause and spread of the endotoxin contamination. These actions should be monitored and effectiveness checks (eChecks) should be performed at a later date to follow-up on the CAPA items to ensure they were effective [2].

A clear summary and conclusion of the investigation should be written describing all of the results, findings, testing, decisions and actions that were taken during the course of the investigation [2]. Everything should be documented, the good, the bad, and the ugly. An OOS report should be written in a clear, easy to follow format. The investigation should be:

- Thorough [5]
- Timely (Usually completed within 30 days) [5]
- Unbiased [5]
- Well-documented [5]
- Scientifically sound [5]
- Supported by facts and data [5]

It is desirable that the OOS investigation to be a standalone document. The investigation should contain a written report that documents everything that occurred in chronological order. This report should start with a clear statement and background information for the reason of the investigation [5]. Then, the report should move to a thorough description of all the details, events, and information in regards to the investigation including logical and detailed descriptions of the decision-making processes. All relevant data or references where the data can be located should be contained in the report [5]. The report should also discuss all of the RCA tools that were utilized during the investigation. In addition, the report should include a summary of all of the potential root causes or the most probable root cause. Any analyses that were performed during the investigation and the results that were obtained should be discussed and compared to the established acceptance criteria [5]. The report should also clearly indicate which results will be reported and provide a recommended batch disposition with rationale. The

findings of the review process including historical trends and any CAPAs that were established during the investigation should be discussed [5]. The report should clearly distinguish between facts and assumptions and contain statements that are supported by facts and data that was gathered during the investigation. Finally, the report should include a summary of the investigation, results, conclusions, impact assessments and any planned effectiveness checks to monitor potential reoccurrences of the identified root cause [5].

Summary

In summary, endotoxin data deviations are investigated like out of specifications (OOS) and the same governing regulations apply. Invalid assays are handled differently than OOS investigations, but may require an investigation as invalid assays should be limited in occurrences. An overabundance of invalid assays may be a signal of other laboratory control problems. Some companies have quality management systems that can track OOSs separately, while others find it beneficial to attach an OOS investigation to a deviation in their existing quality management system for tracking purposes. It is important to note that the rejection of a batch does not negate the responsibility to investigate the OOS result [5].

Phase 1 of an OOS investigation is a laboratory investigation. Checklists in Phase 1 are helpful. Generally, a re-measurement of the originally prepared sample, standard solutions, or dilutions is permitted during this stage if the supplies are not consumed or expired [5]. Everything that is investigated and observed must be properly documented. If clear evidence of an error is identified, the laboratory testing results may be invalidated. If a root cause is not found in Phase 1 and the results appear accurate, Phase 2 is initiated [5].

Phase 2 is a Full Scale Investigation. This phase of the investigation looks to identify a root cause and examine the potential impact on other batches. Phase 2 expands from the laboratory and out into the manufacturing facility where applicable [5]. The main goal of using a RCA tool is to guide the user to a possible root cause [1]. Root cause analysis tools in Phase 2 are helpful and should be chosen according to established SOPs, ease of use for the user, and the ability to identify a root cause. All root cause categories should be considered when investigating probable root causes [1]. Additional laboratory testing may be required and will require an investigational test plan. The test plan should describe the method, acceptance criteria, number of replicates, and how the results will be reported [5]. CAPAs should be established and effectiveness checks should be performed to monitor the corrective and preventative actions [5].

It is desirable for the investigation to be a standalone document in order for the document to be audit ready. Well written and managed OOS investigations should be thorough, timely, unbiased, well-documented, scientifically sound, and supported by facts and data [5].

Finding a root cause to endotoxin contamination early can aid with the control, clean up, corrective actions, and preventative actions that may be required to protect the company, the products, and the patient.

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