The Truth of Endotoxin Values

Points for Consideration During Investigation of Aberrant BET Results

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Introduction

Bacterial Endotoxin Testing (BET) is mandatory for detection of Endotoxin/Lipopolysaccharides (LPS) in parenteral drug products. This is important as LPS is one of the most potent stimulants of the human innate immune system. Over the last decades the Limulus Amebocyte Lysate (LAL) test has been established as the gold standard for the detection of LPS. In order to qualify a test sample, the endotoxin activity is determined based on a standard curve using reference or control standard endotoxins. BET is described in international pharmacopoeias (e.g., Ph.Eur, JP, USP). Thus, the test does not need to be validated per se, however product specific verification with tests for interfering factors is required.

In routine, most samples are analyzed in duplicate using one verified dilution of the product. In order to exclude test interference (i.e., inhibition/enhancement of the enzymatic reaction) positive product controls (PPC) are performed in addition. When all system suitability tests fulfill provided acceptance criteria a result is considered to be valid. In case not all acceptance criteria are fulfilled or unexpected results are obtained, further investigations are needed. Sometimes, these aberrant BET results are hard to understand.

Within a quality-controlled BET laboratory, operator and laboratory errors are often investigated relatively fast. However, there are additional challenging aspects which can influence a test result. In order to better understand inconclusive BET results the following points are often discussed:

- Variation in LAL reagents
- Variation in standard curve
- Representativeness of standard endotoxins
- Activity of endotoxin
- Alteration of detectable endotoxin
- Effects of (1→3)-ß-D-glucans

Is a Difference in LAL Reagent Results Possible?

Yes. The LAL reagents are derived from horseshoe crabs and are therefore of biological origin. It has been described that the lysate is a relatively crude mixture and is not a single purified enzyme. This means that the enzyme activity cannot be determined exactly for each lot of lysate manufactured. Furthermore, the manufacturing process includes the addition of buffers and detergents which contribute a further source of variability.[1] A reduced variability can be achieved by using recombinant Factor C reagents.

Can a Variation in the Standard Curve Effect the Test Result?

Yes. To quantify bacterial endotoxin, a standard curve is prepared in order to determine the endotoxin activity of a sample. Therefore, the quality of the standard curve is the basis of quantification. Using a linear standard curve, a change of only 1% in y-intercept can result in a change of up to 35% in measured endotoxin activity.[1]

In Figure 1, Y-intercept (Y-Achsenabschnitt) as a function of number of analysis (Analysennummer) from trending analysis is shown. All data points (full diamonds) fulfill the standard acceptance criteria. However, the typical observed variations may lead to increased/decreased test results depending on the y-intercept as small variations can lead to relative high variations in measured EU/mL.

Is Reference Standard Endotoxin Still Representative for BET?

Yes. Reference Standard Endotoxin (RSE) is the benchmark and allows comparability of test methods. Due to the heterogeneity of endotoxin, standardization of bacterial endotoxin tests was very challenging in the early time of BET. Only the introduction of RSE was the key factor to control the quality of BET, since *Limulus*-based approaches are ultimately biological assays, the lysates are intrinsically variable.[2] Moreover, recent challenges like LER and the implementation of recombinant tests brought up again discussions about Naturally Occurring Endotoxins (NOE). Advocates of NOE in the field of LER are refusing NOE when it comes to the comparison of test methods. It has been stated that NOE more closely mimics a real life contamination event,[3] but on the other hand it has been communicated that NOEs grown in laboratory are not representative of what occurs in nature. This contradictoriness clearly reflects the incongruous application of undefined endotoxin spikes during testing.

Can a Sample Composition Alter the Detectability of Endotoxin?

Yes. There have been a lot of publications about Low Endotoxin Recovery (LER) and endotoxin masking which can lead to underestimation of endotoxin contents.[2,4–6] Due to the presence of certain excipients or active pharmaceutical ingredients or combinations thereof, endotoxin can be masked. An example for the detectability of endotoxin in a typical LER matrix is given in Table 1. Thereby the detectability decreases although the endotoxin is not degraded and potentially hazardous.

Table 1. Detection of endotoxin over time in a typical LER matrix

Low Endotoxin Recovery Study	[EU/mL]
M1 Time Point 0 days	64.0
M2 Time Point 1 days	27.8
M3 Time Point 2 days	17.6
M4 Time Point 3 days	7.3
M5 Time Point 7 days	4.8

The data is sourced from Low Endotoxin Recovery - Masking of Naturally Occurring Endotoxin[6]

In order to reveal these effects so called LER studies are mandatory.

Therefore, undiluted samples are spiked with endotoxin and held for a certain period of time. More guidance for LER including strategies for demasking is found in the Technical Report No. 82 from PDA.

Do (1→3)-ß-D-Glucans Affect the Endotoxin Test Result?

Yes. The LAL test includes per se the Factor G reaction pathway which is described to react with (1→3)-ß-D-glucans.[7] This reaction pathway

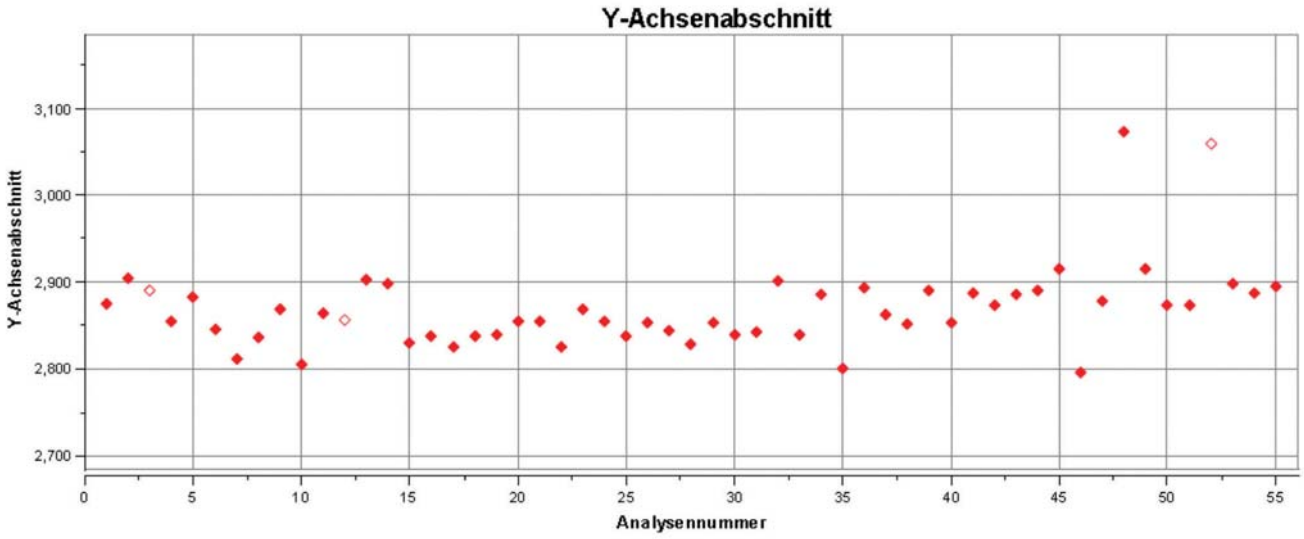

Figure 1. Y-intercept of standard curves from trending analysis

has been identified years ago. Interestingly this pathway is unequally pronounced in different LAL tests. There are also agents available to repress Factor G reaction pathway. Unfortunately, it is neither proven that a) all glucans nor b) that their full activity is blocked. Obviously, glucans are very heterogeneous and present in various aggregation states and can be derived from a variety of sources. Once present in a sample the absolute differentiation between LPS and glucans with LAL is virtually impossible.

In the following example, a routine in-process control sample during drug manufacturing resulted in an unexpected endotoxin test result. With a routine chromogenic LAL test method, 6.5 EU/mL (Table 2, arithmetic mean) was determined. With a turbidimetric LAL test method, 0.8 EU/mL (Table 3, arithmetic mean) was determined. The two test methods obtained valid results but with variations greater than the well-established 50% to 200%. Further analysis of (1→3)-ß-D-glucans revealed that the sample was contaminated by glucans (Table 4) which are most likely the root cause of the inconsistent results.

In order to determine the activity of endotoxin, only a recombinant reagent will allow determination of endotoxin, because of the lack of a Factor G reaction pathway.

Table 2. Analysis of in-process control sample with chromogenic LAL test

Dilution	Measured value [EU/mL]	Endotoxin content [EU/mL]	PPC [%]	Status
1:5	1.470	7.35	143	Valid
1:10	0.675	6.75	118	Valid
1:20	0.271	5.42	175	Valid

Table 3. Analysis of in-process control sample with turbidimetric LAL test

Dilution	Measured value [EU/mL]	Endotoxin content [EU/mL]	PPC [%]	Status
1:5	0.141	0.705	176	Valid
1:10	0.098	0.980	147	Valid
1:20	0.0372	0.744	149	Valid

Table 4. Analysis of in-process control sample with (1→3)-ß-D-glucans test

Dilution	Measured value [pg/mL]	Glucan content [pg/mL]	PPC [%]	Status
1:50	59.799	2990.0	97	Valid
1:100	34.952	3495.2	74	Valid
1:500	5.922	2961.0	89	Valid

Do Measured Activities Allow an Absolute Quantification of Endotoxin?

No. These test methods do not measure the amount of endotoxin/LPS, these tests rather measure activity (Endotoxin Units (EU)). The measurements quantify endotoxin activity which may vary from endotoxin to endotoxin. In the example below, supernatants of bacterial suspensions were analyzed using chromogenic LAL, recombinant reagent rFC and PBMC/IL6-based Monocyte Activation Test (MAT) (Table 5). While all tests were valid (i.e., according to European Pharmacopoeia 2.6.14, 2.6.30, 2.6.32) and manufacturer instructions, LAL and rFC tests resulted in the same order of magnitude, MAT measured values approximately 100 times less in one sample. Although the test results substantially deviate in MAT, this result should not be judged as incorrect. MAT is based on the reactivity of human monocytes (e.g., Toll-like Receptor 4) and LAL/rFC is based on the reactivity of Horseshoe Crab Factor C. Considering this fact, the result from MAT seems to be more relevant regarding the proximity/relevance of the test method to a patient.

Table 5. Analysis of supernatants of bacterial suspension using LAL, rFC and MAT

Sample	LAL [EU/mL]	rFC [EU/mL]	MAT [EU/mL]
Agrogbacterium radiobacter	207,000	242,500	2,000
Burkholderia multivorans	21,000	17,513	10,228

Conclusion

With respect to the examples provided it is difficult to rely on a single value. One single test method might not give the ultimate result. Although these tests methods have been used for decades, this does not imply that they can be used without considering their inherent advantages and disadvantages. Bacterial Endotoxin Tests are biological test systems and require careful interpretation as the relative detectability can vary more than the typical 50% to 200%.

Furthermore, application of the Monocyte Activation Test can be beneficial in providing more dedicated insights regarding the pyrogenic effects of a contamination. Generally, the test methods are only models to recapitulate the human situation. Despite all the challenges, available tests including recombinant tests are fast and sensitive methods to detect minute amounts of endotoxin.

References

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