Determining Endotoxin Levels and Inhibition and Enhancement of the Endotoxin Reaction

Using NovaSeptum AV (Accurate Volume) Sampling Units

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Endotoxins are high molecular weight complexes associated with the outer membrane of gram-negative bacteria and are the most significant source of pyrogens in the biopharmaceutical and pharmaceutical industry (1). Due to their ubiquity, relative heat stability, and ability to cause profound physiological changes when administered parentally, their detection and elimination are of paramount concern to manufacturers of parenteral products.

A very sensitive assay for detecting the presence of endotoxin is the *Limulus* amebocyte lysate (LAL) assay, using aqueous extracts of amebocytes from the horseshoe crab. Very low levels of endotoxin induce a clotting cascade of several enzymes. This complex set of reactions can be inhibited or enhanced by certain products, leading to a false interpretation of endotoxin concentration in a test sample.

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In addition, 21 CFR 211.165(a) states that "equipment shall be constructed so that surfaces that contact components, in-process materials, or drug products shall not be reactive, additive, or absorptive so as to alter the safety, identity, strength, quality or purity of the drug product beyond the official or other established requirements" (2). Therefore, the presence of endotoxin levels in products used for sampling or processing an injectable drug product must be accurately quantified.

We used the kinetic turbidimetric method for this study. It measures the increase in turbidity produced by the clotting of the LAL reagent in the presence of endotoxin. The kinetic turbidimetric technique measures either the onset time needed to reach a predetermined absorbance of the reaction mixture or the rate of turbidity development (3).

The lot-release endotoxin specification for NovaSeptum AV (accurate volume) sampling units is less than 2.15 EU/device. Although NovaSeptum AV is not a registered medical device, the specification was developed based on the strictest guidelines for medical devices. The limit of devices in contact with cerebrospinal fluid is 2.15 EU/device (4).

The goal of this study was to determine the endotoxin level for NovaSeptum AV devices used for sampling of injectable drug products or process fluids used in producing drug products. We also determined the effect of the sampling devices on inhibition or enhancement of endotoxin reaction. We tested samples for the presence of endotoxin after sterilization. We also tested them for inhibition and enhancement of the endotoxin reaction by spiking sample units with endotoxin. Endotoxin recovery values were determined after extraction at room temperature for 24 hours, 48 hours,

Product Focus: Injectable drug products and process fluids

Process Focus: Production, assay development

Who Should Read: Analytical, assay development, process engineers, cell culture engineers

Keywords: *Limulus* amebocyte lysate (LAL), pyrogens, endotoxins, kinetic turbidimetric method

Level: Intermediate

and 72 hours. Samples tested for endotoxin were all below detectable limits (<0.5 EU/device). Samples tested for inhibition or enhancements were between 50 and 200% of the spike concentration at all time points.

Materials and Methods

The materials used are listed in the box on this page. Our testing was in accordance with USP–NF 27 using the kinetic turbidimetric LAL assay.

Inhibition enhancement testing complied with the current USP–NF General Chapter <85> *Bacterial Endotoxin Test* and other pertinent pharmacopeia chapters including those from the *European Pharmacopoeia* (EP), and the FDA *Guideline on Validation of the* Limulus *Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products and Medical Devices* (5).

We prepared sample test units by adding LRW to each sample unit. After 60 minutes, samples were collected aseptically and tested for endotoxin content using the kinetic turbidimetric LAL assay. We prepared additional units for inhibition and enhancement by adding LRW and endotoxin at a concentration of ~1.0 EU/mL (referred to here as a "spike"). After the appropriate time at room temperature, the spiked samples were then collected aseptically and tested for endotoxin content using the kinetic turbidimetric LAL assay.

The LRW used as a negative control and for spike preparation was tested for endotoxin content. The endotoxin solution used to spike the units was tested to quantify its endotoxin content.

In each series of determinations, we used at least three standard endotoxin solutions to generate the standard curve. We prepared serial dilutions from the CSE stock solution in an endotoxin-free microplate. Determinations of standards and samples were made at least in duplicate.

We added the LAL reagent to all samples and standards. The microplate stayed in the microplate reader incubator at 37 °C for 60 minutes. We followed the microplate manufacturer's instructions for performing the kinetic turbidimetric LAL assay.

Materials

LAL reagent water (LRW): endotoxin content < 0.001 EU/mL

Control standard endotoxin (CSE): *Escherichia coli* 0113:H 10, potency 6.36 EU/ng or 3.000 EU/vial

***Limulus* amebocyte lysate (LAL):** appropriate for turbidimetric assay

Pyrogen- or endotoxin-free glass tubes, utensils, pipettes; and Nunclon endotoxin-free polystyrene microplates (www.sigmaaldrich.com)

Microtiter plate reader (ELX808, BioTek Instruments, Inc., www.biotek.com)

Vortex mixer

Table 1: LRW (LAL reagent water) negative control results

Sample #	Result (EU/mL)
LRW - 1	<0.01
LRW - 2	<0.01
LRW - 3	<0.01
LRW - 4	<0.01
LRW - 5	<0.01

Table 2: LRW (LAL reagent water)/endotoxin spike solution results

Sample #	Result (EU/mL)
1	1.33
2	1.32
3	1.38
4	1.43
5	1.63
Average	**1.42**
Standard Deviation	**0.13**
Minimum	**1.32**
Maximum	**1.63**

Assay Acceptance Criteria

The LRW used for negative controls and preparation of spike must be <0.01 EU/mL. We determined that this specification would have no significant impact on measurement of the positive control or any potential endotoxin presence.

The acceptance criteria for the product positive control (PPC) and spiked samples comply with USP and EP standards. The acceptable recovery range is 50–200% of the spiked concentration.

The absolute value of the correlation coefficient for the standard curve should be greater than 0.980 according to the USP standard. For this study, values must be greater than or equal to 0.995.

Table 3: NovaSeptum AV sample units tested for endotoxin content (spiked) after 24 hours at room temperature

Sample #	Result (EU/mL)
2-24-1	1.65
2-24-2	1.73
2-24-3	1.71
2-24-4	1.68
2-24-5	1.61
2-24-6	1.88
2-24-7	1.44
2-24-8	1.39
2-24-9	1.34
2-24-10	1.36
2-24-11	1.38
2-24-12	1.48
2-24-13	1.50
2-24-14	1.57
2-24-15	1.58
2-24-16	1.84
2-24-17	1.69
2-24-18	1.52
2-24-19	1.37
2-24-20	1.39
2-24-21	1.44
2-24-22	1.45
2-24-23	1.44
2-24-24	1.44
2-24-25	1.60
Average	**1.54**
Standard Deviation	**0.15**
Minimum	**1.34**
Maximum	**1.88**

We ran replicate samples to confirm good technique and low coefficient of variation (CV). The CV is 100 times the standard deviation of a group of values divided by the mean expressed as a percentage. The CV of OD (optical density) values in replicate must be less than 10%.

Assay sensitivity is dependent on the LAL formulation and incubation time of the LAL endotoxin reaction. For this study the sensitivity of the test is 0.01 EU/mL, the minimum level of endotoxin that can be detected by the test system. It is defined by several factors in the design of the test system and was verified by the execution of the standard curve in each test.

Table 4: NovaSeptum AV sample units tested for endotoxin content (spiked) after 48 hours at room temperature

Sample #	Result (EU/mL)
2-48-1	1.37
2-48-2	1.36
2-48-3	1.45
2-48-4	1.37
2-48-5	1.42
2-48-6	1.59
2-48-7	1.27
2-48-8	1.18
2-48-9	1.13
2-48-10	1.17
2-48-11	1.19
2-48-12	1.24
2-48-13	1.25
2-48-14	1.29
2-48-15	1.35
2-48-16	1.55
2-48-17	1.34
2-48-18	1.24
2-48-19	1.23
2-48-20	1.2
2-48-21	1.2
2-48-22	1.23
2-48-23	1.19
2-48-24	1.24
2-48-25	1.26
Average	**1.29**
Standard Deviation	**0.12**
Minimum	**1.13**
Maximum	**1.59**

Table 5: NovaSeptum AV sample units tested for endotoxin content (spiked) after 72 hours at room temperature

Sample #	Result (EU/mL)
2-72-1	1.20
2-72-2	1.26
2-72-3	1.22
2-72-4	1.38
2-72-5	1.41
2-72-6	1.09
2-72-7	0.98
2-72-8	0.93
2-72-9	0.94
2-72-10	0.96
2-72-11	0.96
2-72-12	1.02
2-72-13	1.03
2-72-14	1.06
2-72-15	1.30
2-72-16	1.12
2-72-17	0.96
2-72-18	1.02
2-72-19	0.98
2-72-20	0.97
2-72-21	0.89
2-72-22	1.00
2-72-23	0.97
2-72-24	1.02
2-72-25	1.12
Average	**1.07**
Standard Deviation	**0.14**
Minimum	**0.89**
Maximum	**1.41**

Determining Maximum Valid Dilution

The maximum valid dilution is the maximum allowable dilution of a sample at which the endotoxin limit can be determined. The MVD is the limit dilution factor, which may be used in sample preparation for the test to be valid. The general equation is described as

$$MVD = (\text{endotoxin limit} \times \text{concentration of sample solution}) / \lambda$$

where the endotoxin limit for NovaSeptum is 2.15 EU/sample device; concentration of sample solution is given the value 1 because water is used as an extract; and λ is the labeled sensitivity of the LAL reagent (0.01 EU/mL). Therefore,

$$MVD = 2.15/0.01 = 215$$

Table 6: Endotoxin concentration of NovaSeptum AV sample units (unspiked)

Sample #	Result (EU/device)
1	<0.5
2	<0.5
3	<0.5
4	<0.5
5	<0.5

The MVD that can be used for quantifying endotoxin in NovaSeptum sampling units is 1:215.

Results

Five samples (n = 5) of LRW used for the negative control were tested for the presence of endotoxin. All samples were below the detectable limit of the assay (0.01EU/mL). Table 1 shows the raw data.

Five samples (n = 5) of the spike or PPC were tested. We determined the average endotoxin concentration of the spike to be 1.42 EU/mL. Table 2 presents the raw data.

Spike recovery levels must be between 50% and 200% of the added spike; therefore, the spike solution results are acceptable. Based on the average spike concentration of 1.42 EU/mL, the acceptable recovery range of the samples unit tested for inhibition and enhancement must be within 0.71–2.84 EU/mL or 50–200% of the spike concentration. All NovaSeptum AV sampling units at each time point were within the acceptable range. All samples were within 61–132% of the spike concentration. Refer to Tables 3, 4, and 5 for raw data.

Five samples (n = 5) of NovaSeptum AV sampling devices were extracted at room temperature for 60 minutes to determine whether samples were below the endotoxin specification without exceeding the MVD. All samples were less than 0.5 EU/device. Table 6 presents raw data.

The values for the standard curves were all greater than or equal to 0.995. The CV of OD values in replicate were all less than 10%.

Discussion

The endotoxin testing performed on the sampling devices demonstrates that sampling units will not contribute a significant amount of endotoxin to a sample collected in a device. In addition, the components of the sampling units will not contribute to inhibition or enhancement of an endotoxin test when a sample is stored up to 72 hours. Furthermore, we observed a decrease in endotoxin content with an increase in time. Additional testing will need to be performed to determine whether this result is real, within the variability of the test method, or correlated to endotoxin stability over time. The addition of drug products to NovaSeptum AV sampling units may affect the inhibition and/or enhancement of endotoxin results. This effect must be determined case-by-case as part of a comprehensive endotoxin test validation plan. The results demonstrate that NovaSeptum AV sampling units are ideal for sampling and

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storing products that must be tested for endotoxin content.

References

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