Bacterial Endotoxins Testing of Water-Insoluble Drug Substances by Means of Dispersion with Dimethyl Sulfoxide

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Abstract

Active pharmaceutical ingredients are often poorly soluble in water. The detection of bacterial endotoxins in such substances requires a homogeneous aqueous suspension, which presents difficulties in ensuring uniform dispersion of the sample through the mixture. We demonstrate a technique for sample preparation in which the drug substance is dissolved in dimethyl sulfoxide (DMSO), followed by dilution in water. This results in a more uniform suspension than can be achieved by direct addition of sample to water. This method allows testing for endotoxins via the chromogenic technique, which results in a 15-fold increase in method sensitivity compared to the gel-clot technique. The increased sensitivity allows for testing of higher sample dilutions, increasing the likelihood that sample interference can be overcome.

Introduction

Bacterial Endotoxins Testing (BET) ensures the safety and purity of parenteral drug products by detecting and quantifying endotoxins from Gram-negative bacteria. The test relies on Limulus amoebocyte lysate (LAL), an enzymatic detection system derived from the horseshoe crab, *Limulus polyphemus*.[1]

Like all biological systems, the LAL enzyme complex has evolved to operate in an aqueous environment. Since many active pharmaceutical ingredients and excipients are poorly soluble in water, this can present technical challenges for the BET assay.[2] One common remedy is to prepare the insoluble compound as a homogeneous suspension in water, which allows any endotoxins present to migrate to the aqueous phase and interact with the LAL enzymes. However, in some cases the nonpolar nature of the test article can make achieving a uniform suspension difficult, either due to precipitation or aggregation on the surface of the liquid.

Mechanical homogenization with glass beads is possible, often with the aid of a surfactant such as polysorbate 80, but this method presents a number of technical challenges. It does not always yield a uniform suspension: sample may settle quickly, or float to the surface, or the grinding action of the beads may not be sufficient to break up agglomerations of sample. Emulsions prepared in this manner are generally opaque, and must therefore be tested for endotoxins using the gel-clot technique, which is slower and less precise than the kinetic techniques. The binary nature of the gel-clot test, which depends on the presence or absence of a firm gel after incubation, does not allow for a quantitative measurement of endotoxin content; all that can be said is that the endotoxin is above or below a certain limit, equal to the sensitivity of the reagent multiplied by the dilution factor of the sample solution. The gel-clot assay also offers little information on the degree to which the surfactant is inhibiting or enhancing the enzymatic reaction with the sample. Method verification must therefore be performed on a series of spiked endotoxin solutions prepared in the sample mixture, which is both time-consuming and labor-intensive.

In order to ensure a more uniform suspension and avoid the potential pitfalls of the mechanical homogenization technique, it may be helpful to first dissolve the test article in another solvent for which it has greater affinity, and then disperse this solution into a larger volume of water. This would separate the molecules of the test article from one another, thus encouraging them to enter aqueous suspension as finely dispersed particles rather than large agglomerations. Such a finely-dispersed suspension would be a better candidate for the kinetic assay than the opaque emulsions obtained through mechanical homogenization with surfactant. For this approach to be useful, however, the solvent chosen must be fully miscible with water, and must not itself interfere with the BET assay. Many common solvents, such as ethanol, 2-propanol and hexane, fail one or both of these criteria.

Dimethyl sulfoxide (DMSO) presents several distinct advantages as a dispersant: it dissolves both polar and nonpolar compounds; it is fully miscible with both water and a wide range of organic solvents; and it is far less volatile than ethanol and 2-propanol, so solutions of stable concentration can be prepared and tested under ambient conditions.

We set out to assess the suitability of DMSO as a dispersant for the BET kinetic assay. Since homogeneous sample suspensions are generally turbid, we chose the chromogenic variant of the assay rather than the turbidimetric form, since it was less likely that the suspended sample would interfere with the spectrophotometer's detection of the distinctive yellow chromophore.

Materials and Methods

We performed bacterial endotoxins testing using the kinetic chromogenic technique. The DMSO used was 99.9+% purity and ACS Reagent Grade. LAL reagent, control standard endotoxin (CSE) and lysate reagent water (LRW) were all qualified prior to use.

To determine the noninterfering dilution of the DMSO, we performed a screen test on dilutions of 1:10, 1:20, 1:50 and 1:100 in LRW. Sample dilutions were tested in accordance with USP <85> and LAL manufacturer's instructions, with a four-point CSE standard curve using endotoxin concentrations of 5, 0.5, 0.05 and 0.005 EU/mL. A log-linear regression was calculated, and samples were tested with four unspiked sample replicates and four positive control replicates spiked to contain 0.5 EU/mL. A dilution was considered to be noninterfering if the spike recovery from the positive controls fell within the accepted range of 50-200% (inclusive).

For the dispersion test, five different test articles were evaluated (Table 1). All of these samples had proven to be insoluble in water during initial method verification work, and mechanical homogenization with surfactant was either not uniform or yielded an opaque mixture unsuitable for kinetic testing. The two liquid test articles, products I and II, were diluted ten-fold in DMSO as an initial step. For the solid test articles, products III through V, a convenient initial solution was prepared by weighing the sample into tared glassware and then adding DMSO to dissolve. All DMSO solutions were vortexed until completely dissolved before proceeding to the dispersion step.

For products I, II, IV and V, the sample/DMSO solution was dispersed into water by adding 100 µL of solution to 9.9 mL LRW, then immediately vortexing at high speed until a uniform suspension was observed. For product III, the very low endotoxin limit required adding 200 µL of solution to 9.8 mL LRW. The resulting dispersions contained 1% DMSO (or 2% DMSO, in the case of product III), which was within

Table 1. Non-polar test articles evaluated for DMSO dispersion.

Product	Description	Endotoxin Limit	Initial Preparation in DMSO	Aqueous Dispersion Step (mL:mL)
I	A lipid excipient used in the preparation of lipid nanoparticles for mRNA-based drug products. Liquid at ambient conditions.	NMT 10 EU/mL	1:10 dilution	0.1:9.9
II	A branching organic compound with multiple aromatic rings and sulfonyl groups. Prepared as a 400 mg/mL solution in a viscous, proprietary solvent. Precipitates on contact with water.	NMT 40 EU/mL	1:10 dilution	0.1:9.9
III	An organic compound with a methanesulfonate group and multiple aromatic rings. Solid powder.	NMT 19 EU/g	0.01 g/mL	0.2:9.8
IV	An organic compound with benzene, isoindole, and piperidine rings. Solid powder.	NMT 1.0 EU/mg	20 mg/mL	0.1:9.9
V	A cyclic organic compound consisting of multiple interconnected rings and aromatic groups. Solid powder.	NMT 0.20 EU/mg	100 mg/mL	0.1:9.9

Table 2. Screen test results for DMSO using the kinetic chromogenic technique.		
Dilution	**DMSO Concentration**	**Spike Recovery**
1:10	10%	9%
1:20	5%	39%
1:50	2%	73%
1:100	1%	104%

the range at which DMSO was shown to be noninterfering (see the "Results" section).

From this initial sample suspension, samples were screened at a range of higher dilutions up to and including the Maximum Valid Dilution (MVD) determined by each product's endotoxin limit. Except where noted otherwise in the "Results" section, higher dilutions were prepared in LRW. To maximize the available dilution range, these screen tests were performed using the chromogenic LAL reagent at maximum sensitivity (λ = 0.001 EU/mL), with a CSE standard curve using concentrations of 1, 0.1, 0.01 and 0.001 EU/mL. Positive product controls were spiked to contain 0.1 EU/mL. A dilution was considered to be noninterfering if the spike recovery from the positive controls fell within the accepted range of 50-200% (inclusive). The lowest valid dilution (LVD) and MVD were tested again by three separate analysts to ensure intermediate precision and repeatability of results.

Results

The DMSO screen test demonstrated noninterference at dilutions of 1:50 and higher (Table 2). The endotoxin value of the unspiked sample replicates was below the limit of detection for all dilutions, demonstrating that the DMSO itself would not add any detectable level of endotoxin contamination to the samples.

All of the tested products yielded valid spike recoveries over a range of dilutions (Table 3). The ratio between the LVD and the MVD ranged from 1:3.8 for product III to 1:40 for product IV. Products I and III required minor procedural modifications to ensure valid results (see notes in Table 3). For product III, suspended particles of sample interfered intermittently with the light beam in the spectrophotometer, leading to a higher-than-usual amount of noise in absorption measurements. Raising the onset optical density (OD) from 0.05 to 0.10 set the threshold for measurement above this noise floor, which allowed the reaction times in the spiked and unspiked sample wells to be determined accurately.

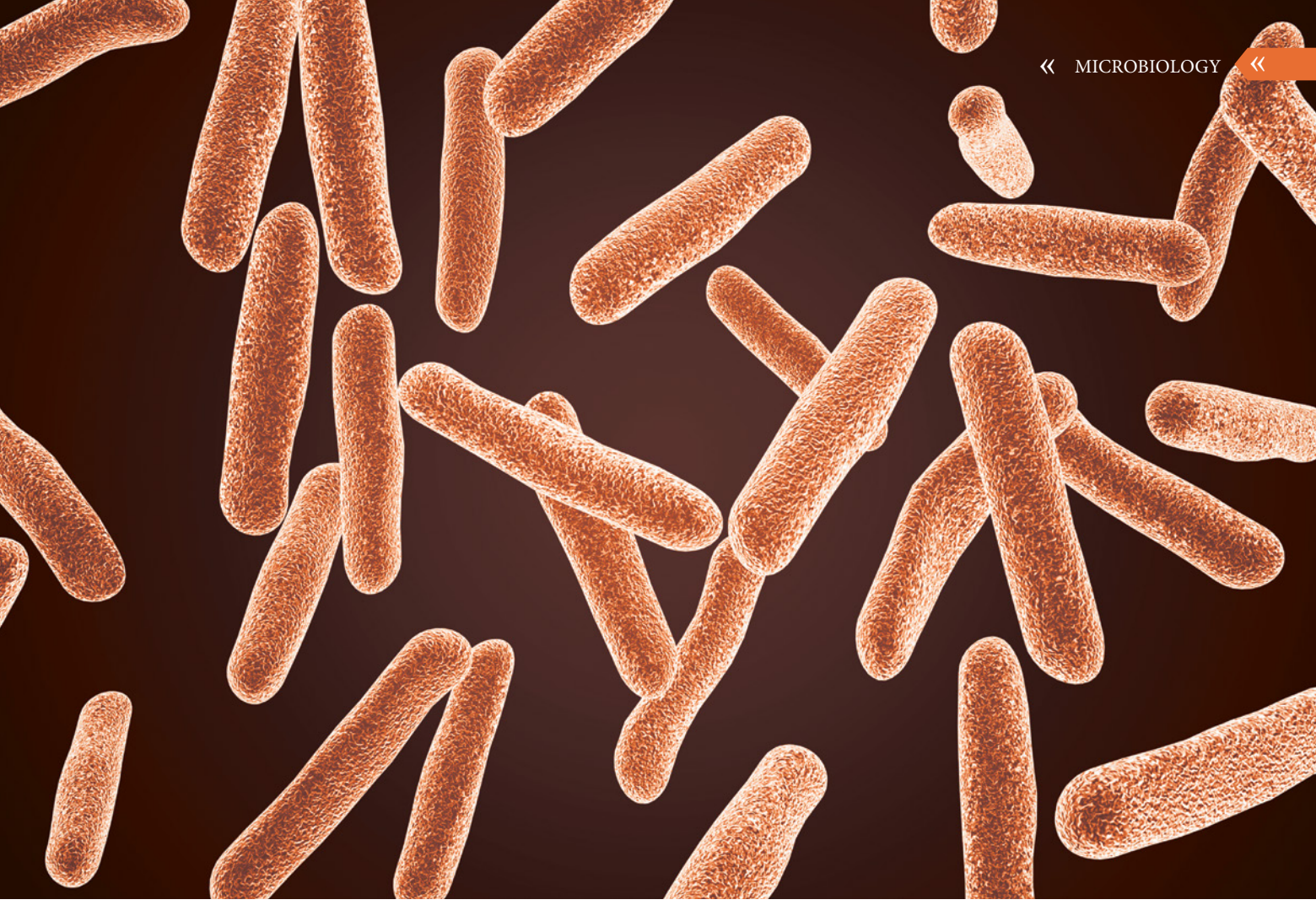
For all five products, the validity of the LVD and MVD was upheld by the three confirmatory analysts (Table 4). All analysts generated spike recoveries for both sample dilutions that were in the 50-200% range specified by USP <85>.

Discussion

The results of the study demonstrate that DMSO is a feasible dispersant for the BET assay and that DMSO-dispersed suspensions of nonpolar substances can be tested with the kinetic chromogenic technique. This approach allowed us to obtain quantitative measurements of endotoxin in samples that previously could only have been tested using the gel-clot technique. The chromogenic assay offers a fifteen-fold improvement in sensitivity compared to the most sensitive gel-clot assay (λ = 0.001 EU/mL versus 0.015 EU/mL), which allows testing at high dilutions that do not interfere with optical measurements via

Table 3. Screen test results for DMSO-dispersed test articles using the kinetic chromogenic technique (λ=0.001 EU/mL).					
Product	**Lowest Valid Dilution (LVD)**	**LVD Spike Recovery**	**Maximum Valid Dilution (MVD)**	**MVD Spike Recovery**	**Notes**
I	1:1000	80%	1:10 000	100%	Subsequent dilutions performed in 1% DMSO to prevent precipitation.
II	1:4000	82%	1:40 000	106%	-
III	1:50	88%	1:190	113%	Onset optical density (OD) was raised from 0.05 to 0.10 to overcome optical interference from sample.
IV	1:500	97%	1:20 000	100%	-
V	1:4000	96%	1:20 000	133%	-

Table 4. Confirmatory testing of lowest and maximum valid dilutions.						
Product	**LVD Spike Recovery**			**MVD Spike Recovery**		
	Analyst 1	**Analyst 2**	**Analyst 3**	**Analyst 1**	**Analyst 2**	**Analyst 3**
I	84%	102%	83%	81%	102%	88%
II	84%	129%	61%	117%	120%	109%
III	88%	105%	100%	116%	122%	109%
IV	96%	99%	103%	110%	119%	99%
V	86%	57%	108%	126%	133%	86%



spectrophotometer. For samples with tighter specifications, raising the onset OD may allow for accurate measurements even in the presence of some optical interference.

This method is likely to be most successful with nonpolar organic molecules of relatively low molecular weight and uniform composition. Product II, in which the active ingredient was suspended in a complex and viscous proprietary solvent, proved to be highly inhibitory for the kinetic assay, with an LVD of 1:4000. By comparison, a 1:100 dilution of this product in a 1% solution of polysorbate 80 yielded a uniform, opaque suspension that did not interfere with the gel-clot test. In this case, the older technique of mechanical homogenization yielded an assay with a lower limit of detection than the kinetic technique (1.5 EU/mL versus 4 EU/mL). For the other four products, however, the DMSO dispersion approach resulted in a satisfactory method with greater sensitivity than could be achieved using the gel-clot test.

References

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Author Biographies

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