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Comparison of Limulus amebocyte lysate test methods for endotoxin measurement in protein solutions

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ABSTRACT

The measurement of low levels of bacterial lipopolysaccharides (endotoxins) in protein samples can be a challenge due to potential interference from the inherent properties of the protein, its formulation or other substances that may be present. Other factors include the expression system which may have endotoxin species distinct from the standard, as well as different purification bioprocesses. The endotoxin measurement assays also have a number of variables. Those studied include differences between laboratories, reagents and standards, and detection modalities. A variety of protein samples from a range of expression systems was included in the evaluation. Endotoxin levels are relatively stable when samples are stored frozen with test variations between 1 and 38% among different aliquots. Test variation between labs was not significantly different when the same procedure was followed (intermediate precision) by trained analysts. Most testing modalities gave results within a 50–100% variation, a difference generally regarded as within assay variability. However, about 25% of the samples showed significant differences between testing modalities and/or reagents. The sources of these differences were further examined by traditional as well as novel sample treatments. These findings demonstrate that for some samples, endotoxin may be over- or under-estimated and a more thorough pre-treatment or testing modality may be required.

1. Introduction

Endotoxins, also called lipopolysaccharides (LPS), when introduced into mammalian blood circulation, can initiate a variety of health consequences, including the release of cytokines, which may lead to fevers and other more serious inflammatory responses [1,2]. Accordingly, endotoxin detection and quantitation is a required quality control test for all parenteral drug products and is regulated by health authorities [3,4]. The Limulus amebocyte lysate (LAL) test is the most widely used method for bacterial endotoxin tests. It is a simple and highly sensitive biochemical assay in which endotoxin binding to factor C starts the coagulation cascade [5].

The number of natural and recombinant biological molecules developed as drugs for human use has increased greatly in the last few decades. Typically these are administered as sterile solutions through parenteral routes, notably intravenously and subcutaneously. This broad array of drugs, generally referred to as "biologics" is more susceptible to degradation than small molecule compounds, due to the inherently labile nature and higher order structure of proteins, compared with small molecules. As a consequence, bioprocesses generally do not include harsh (e.g. strongly acidic or basic) processing conditions during chemical synthesis and purification. Bioprocesses, notably microbial, can have very high starting levels of endotoxin. The "gentle" protein purification that is required can make full removal of endotoxin challenging in bioprocesses [6–9]. For example, protein products expressed in *Escherichia coli* (*E. coli*) expression systems may require several logs of endotoxin removal during purification to reduce them to acceptable levels. The similarity of endotoxins to proteins in complexity, combined with potential interference from added formulation components, makes endotoxin measurement an essential yet challenging field of study [10,11].

The variability in outcome of the endotoxin assays have been addressed by many researchers in the past [2,12,13]. This variability comes from differences in assay sensitivity, surfactant and buffer conditions from different manufacturer's reagents; different polysaccharide compositions and chain lengths of the LPS [10]; and different detection systems: gel–clot is an end-point method while turbidimetric and chromogenic methods are monitored kinetically. The laboratory and analyst also exhibit variability due to accessory differences such as test tubes [14] and microplates and procedure differences [15]. As a result of this variation, the endotoxin test allows a 2-fold error (50–200%) and an acceptable spike recovery of 50–200% [3,4]. This spike recovery analysis is routinely done for all

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samples in the test plates as a matter of process, to rule out the presence of interfering substances, including association of LPS with proteinaceous or lipophilic substances. This practice adds value, but absolute accuracy for low levels is challenging and has been reported to be difficult to detect [16].

In order to assess these variations, in this study we measured endotoxin in 14 samples, including proteins from different expression systems, various purification stages, and different formulations. These were selected to contain a wide variety of types of natural lipopolysaccharide species. The testing protocol included different laboratories, sample handling, days of test, LAL reagents, and endotoxin detection methods. Commercially available endotoxin assay reagents were used. In a small portion of the samples, some pre-treatment was required for sample analysis, otherwise simple dilution was used. Our data show that for most of the testing conditions, endotoxin measurements are within a 2-fold range. Samples that have special origin such as yeast production may need to be pre-treated before putting on test.

2. Materials and methods

2.1. Samples for endotoxin testing

Thirteen protein solutions from Pfizer in-house product candidates and one control solution were chosen for evaluation. The protein solutions include proteins isolated from different expression systems, namely, *E. coli*, yeast and mammalian cells. Samples were included from various stages of purification, such as harvest, column loads, column eluates, filtrates, and proteins in different formulations. Endotoxin levels had been previously estimated by the kinetic turbidimetric method and these results were used to establish dilutions in Water for Injection (WFI) that produced results within the range of the standard curve. In most cases, samples were pre-diluted to approximately 1 EU/ml prior to testing. Endotoxins in these samples are either naturally occurring or spiked LPS (data not shown). Aliquots of these samples are stored in 2 ml polystyrene screw cap tubes in order to remove sample dilution as a variable. Samples were thawed and tested "as is" without further dilution. The control solution was simply control standard endotoxin (CSE) added to water at 1 EU/ml.

2.2. Endotoxin and glucan testing and reagents

Endpoint and kinetic chromogenic LAL reagents (QCL1000, 50-647U and Kinetic-QCL, 50-650U), PyroGene (50-658U), kinetic turbidimetric LAL reagent (Pyrogent 5000, N384), and beta glucan blocker (N190) were from Lonza Walkersville Inc (Walkersville, MD, USA). Gel–clot reagents (Pyrotell, G0006) and glucan assay reagents (Glucatell, GT001) were from Associates of Cape Cod Inc (East Falmouth, MA, USA). Reference Endotoxin Standard (RSE, 1235503) was from USP (Rockville, MD, USA). All endotoxin assays were carried out according to the manufacturers' instructions.

3. Results and discussion

3.1. Effect of freezing on endotoxin levels

Protein samples are sometimes stored frozen for convenience, and thawed prior to analysis. Because the endotoxin in these samples were present at low levels, stability was evaluated before and after freeze/thaw cycles (−20 °C) as shown in Fig. 1. Duplicate sets of samples were tested using the kinetic turbidimetric method after one freeze/thaw cycle to measure the consistency of aliquots. One set of samples were tested further after two freeze/thaw cycles. The black bars in Fig. 1 represent the endotoxin potencies of these samples after preparation and transferring to aliquots, prior to freezing. All were within 2-fold of the target 1 EU/ml except sample 9 which was 1/10 of expected value, presumably due to the discrepancies of the estimated original amount from ranging studies.

Gray bars are average endotoxin potencies of two tests after one freeze/thaw cycle. The coefficients of variation (CV) ranges from 1 to 38% for the duplicate assays, indicating relatively stable endotoxin content in these frozen stored protein samples. With the exception of samples 4 and 5, all samples frozen once gave results within 2-fold of the original (never frozen) sample, a modest decrease considering the samples were diluted in WFI where non-specific adsorption to the storage container is possible. The results are consistent with an early study [17], which showed that endotoxin in WFI and sterile biologic solution were relatively stable when stored frozen.

Endotoxin structure is comprised of a lipid A portion that is hydrophobic and a carbohydrate chain that is hydrophilic. Charge, ionic strength, surfactants, and other proteins in the sample can alter endotoxin conformation and micelle formation. It has been reported that dispersion of endotoxin aggregation/micelles can help endotoxin recovery in sample testing [18]. Endotoxin has also been reported to stick to container surfaces, which are affected by water quality, buffering condition, the presence of cationic proteins [14,19,20]. In our study, some samples (4, 5, 9, 11 and 12) required dilutions of 2000–5000-fold with WFI, which also diluted the buffering effect of proteins and formulation components present. Bigger assay variations were observed in these samples. In some cases samples were spiked with LPS *in lieu* of the naturally occurring endotoxin. The purpose of this study is not designed to focus on accuracy, so it is expected that LPS redistribution and association with proteins in spiked samples were outside the scope of this study.

The striped bars in Fig. 1 show the endotoxin potencies after two cycles of freeze/thaw. These results showed little change after one freeze/thaw with the exception of sample 9 which lost all signal after the second freeze/thaw and samples 4 and 5 which showed some losses (outside of assay variability). Although these data confirm that most of the frozen samples could be re-frozen after the first thaw, the remaining testing described herein was performed on frozen samples thawed once prior to analysis. All further testing were with samples thawed and tested in the same manner so that this variable is taken out of consideration during evaluation of the others as described below.

3.2. Evaluation of typical assay variables

Typical and expected assay variations (laboratory, day, analyst, and standard) were examined using different aliquots of the same set of samples over the period of a few weeks. The kinetic turbidimetric method and reagents from Lonza were used for this assessment. Two trained technicians in separate labs tested the samples using either linear or power fit standard curves (from the same raw data), different endotoxin standards (Reference Standard Endotoxin (RSE) from USP vs. Control Standard Endotoxin (CSE) in the assay kit [potency standardized by manufacturer to RSE]), and different manufacture lots of LAL reagents. Results from each sample were averaged, and the %CV was calculated as shown in Table 1. All but two samples had %CV < 50% and the ratio of the lowest individual result to the highest test result for each sample ranged from 1.5 to 17.6. Overall the range of results from this study suggested not more than a 2-fold difference in results. Sample 8 had an overall %CV of 13.2%, suggesting the method capabilities across all these variables. By this reasoning, other results with greater %CV values are best explained as sample nature differences.

The data comparing labs (Fig. 2) shows a positive correlations and lack of bias in one lab versus another. The correlation shown