Testing of Phosphatidylcholine and Triton X-100 Solutions by Chromogenic LAL Method Using the Endosafe® Multi-Cartridge System: Inhibition of Recovery at High Concentrations of Detergents.

Stanislav Yefimov, Ph.D
stanislav@pharmetriclab.com

Wayne DeHaven, Ph.D.
wayne@pharmetriclab.com

Pharmetric Laboratory
St. Petersburg, FL

Abstract

Upon completion of work performed on the Endosafe® Multi-Cartridge System (MCS), it was discovered that some amphiphilic, micelle-forming substances such as phosphatidylcholine (PC) and Triton X-100 inhibit Limulus Amebocyte Lysate (LAL) chromogenic assay at high concentrations. It was hypothesized that the presence of micelles or liposomes in the solution are responsible for the inhibition. This assumption was tested on Triton X-100 solutions, and it was shown that the Triton X-100 at concentrations less than its Critical Micelle Concentration (CMC) did not inhibit the assay, but at the concentrations above the CMC it did have an inhibitory effect. Minimum Detergent Dilution (mDD) is the threshold quantity that tells us how many times the sample must be diluted to avoid inhibition. We concluded that the Maximum Valid Dilution (MVD) should be greater than mDD to prevent inhibition of recovery. If the inequality MVD > mDD is not true, the simple dilution will unlikely eliminate inhibition.

Introduction

One of the most time-consuming aspects of endotoxin testing using LAL is pretreating samples to overcome assay inhibition and enhancement [1]. Agents such as EDTA and heparin are known to affect the assay if they are present in sufficient concentrations. All assays, independent of methodology, are standardized using endotoxin in water. Therefore, unless the sample is water, some components of the solution may interfere with the LAL test such that the recovery of endotoxin is affected. If the product being tested causes the endotoxin recovery to be less than expected, the product is inhibitory to the LAL test. Products which cause higher than expected recovery values are enhancing. Overcoming the inhibition and enhancement properties of a product is required by the FDA as part of the validation of the LAL test for use in the final release testing of injectables and medical devices [2].

Method and Materials

Limulus Amebocyte Lysate (LAL) chromogenic assay, the most sensitive and robust assay

available, was used for the endotoxin tests [3]. The assay was performed on the Endosafe® Multi-Cartridge System (MCS), which is used for endotoxin testing of the finished product (USP <85>) [4]. The cartridges offer a 15-minute endotoxin assay utilizing an FDA-licensed compendial-compliant kinetic chromogenic testing solution. The basic operating principle of the Endosafe MCS is as follows: Polystyrene cartridges contain pre-calibrated reagents that have known reaction times with reference standard endotoxin (RSE). Cartridge-specific spectrophotometers are used to read from the cartridges. An archived standard curve was created and referenced for each cartridge from the known RSE values so that preparation of daily standard curves is unnecessary.

We used cartridges for two ranges of working endotoxin concentrations (λ). Endotoxin concentrations are calculated as Endotoxin Units (EU) per milliliter (mL). One range was from 0.005 EU/ml to 0.5 EU/ml, and the other range was from 0.05 EU/ml to 5 EU/ml. Samples were diluted to Valid Dilution (VD), which is calculated as **VD=EL/λ** where EL (EU/ml) is Endotoxin Limit, or the maximal allowed concentration of endotoxin (EU) in the injectable drug solution (tested samples) [5]. The Maximum Valid Dilution (MVD) in this case was: MVD = EL/0.005 and Minimum Valid Dilution (mVD) was mVD = EL/5. Phosphatidylcholine (PC) 35mg/ml solution was provided to us by our client to test for the presence of endotoxin, and Triton X-100 was supplied by Sigma-Aldrich.

Results

We detected the presence of endotoxins in samples containing Phosphatidylcholines (PC) at concentration 35mg/ml (0.046M) or approximately 5E7 in Critical Micelle Concentration (CMC) units if CMC of PC was taken as 1nM [6]. The Endotoxin Limit (EL) was 3.5 EU/ml and Maximum Valid Dilution (MVD) was as low as 700 [7].

We did not obtain satisfactory results because the spike values and recoveries were zero (Table 1). We noticed that the PC concentration in the samples was so high (>>CMC) that the PC was in an aggregated form of micelles and liposomes. We encountered a similar problem before when we tried to use Triton X-100 to enhance extraction of endotoxins from oil.

Our working hypothesis was that the aggregates of amphiphilic substances (phospholipids, detergents) caused the inhibition or complete elimination of the cascade of enzymatic reaction resulting in yellow coloring. To test this hypothesis we compared the spikes and recoveries for two concentrations of Triton X-100 solutions. The first concentration was 100 times more than CMC and second concentration one was 40 times below CMC, therefore in the second case micelle formation did not occur in the solution. For the first concentrations of PC (Table 1) and Triton X-100 (Table 2) we did not experience any recovery, however in the second Triton-X-100 concentration recovery was almost 100% (Table 2). It appears that the presence of the aggregates (Micelles, Liposomes) in a solution inhibits the chromogenic reaction.

Table 1. Results of Testing a Solution Containing Phosphatidylcholine (PC) on Presence of an Endotoxin. Concentrations of PC are expressed in CMC units.

PC Dilution	Spike Value	Recovery	PC Concentration (CMC)
1:2	N/A	N/A	2.5E7
1:20	<-0.001	<-11%	2.5E6
1:100	<0.000	<0%	5.0E5
1:200	<0.000	<0%	2.5E5

Table 2. Results of Testing a Triton X-100. Concentrations of Triton X-100 are expressed in CMC units.

Triton X-100 Dilution	Spike Value	Recovery	Concentration (CMC)
1:1	N/A	N/A	100
1:4000	0.102	111%	0.025

Discussion

Today, the term 'endotoxin' is used synonymously with lipopolysaccharide (LPS) which is composed of three parts: O antigen, core oligosaccharide, and lipid A. LPS like the PC and Triton-X-100 is an amphiphilic molecule. The hydrophobic fatty acid chains of lipid A anchor the LPS into the bacterial membrane. Likewise, lipid A anchors the LPS into the micelle or liposome. LPS concentration in solution is very low if micelles are present due to the nature that almost all LPS's are bound to the micelles. When incorporated into micelles, LPS's do not trigger LAL dependent enzymatic cascade of chromogenic reactions due to steric restriction. It has been reported that the amphiphilic sodium dodecyl sulfate (SDS) terminates the enzymatic reaction in LAL Nephelometric Method Test [8]. To overcome the negative micelles impact on chromogenic endotoxin testing, the tested solution should be diluted so that the concentration of an amphiphilic component(s) will be lower than its CMC. We call the dilution Minimum Detergent Dilution (mDD). **mDD = [detergent concentration]/ [detergent CMC]**. For example, if the detergent concentration is 1E-3 M, and CMC is 1E-6 M, then mDD = 1E-3/1E-6

= 1000. If, for example, MVD = 10000, then MVD > mDD the sample should be diluted more than 1000 and less than 10000 times. However, if, for example, MVD = 700 then MVD < mDD, and it is impossible to remain in the working range. Therefore to have adequate results in chromogenic endotoxin testing the inequality MVD > mDD must be true.

Conclusion

To overcome the negative micelles impact on chromogenic endotoxin testing, the tested solution should be diluted at least mDD times. It is possible in many cases, but, if the concentration of an amphiphilic component is too high and MVD is low, we can't reach the CMC and the inequality MVD > mDD is not true. In the worst case, another method should be used, such as LAL Gel-Clot Test [9].

References

1. Dawson, M. Ph.D. (2005). Interference with the LAL Test and How to Address It. *LAL Update*. http://www.acciusa.com/pdfs/newsletter/LAL Update Vol 22_No3 rev 001.pdf
2. Guidance for Industry Pyrogen and Endotoxins Testing: Questions and Answers. (2012) *FDA*. https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM310098.pdf
3. Overview of Prominent Lal Tests. (2016). *Barnard Health Care*. https://www.barnardhealth.us/limulus-amebocyte/overview-of-prominent-lal-tests.html.
4. Endosafe - MCS. *Charles River*. http://www.criverindia.com/Endosafe-MCS.htm
5. Dawson, M. Ph.D. (1995). Endotoxin Limits. *LAL Update*. http://www.acciusa.com/pdfs/newsletter/LAL_Vol.13No.2.pdf
6. Critical Micelle Concentrations (CMCs). *Avanti*. https://avantilipids.com/tech-support/physical-properties/cmcs/
7. Tirumalai, R. Bacterial Endotoxin Test. *US Pharmacopeia*. http://www.pharmacopeia.cn/v29240/usp29nf24s0_c85.html
8. Overview of Prominent LAL Tests. (2016). *Barnard Health Care*. https://www.barnardhealth.us/limulus-amebocyte/overview-of-prominent-lal-tests.html.
9. Harmon, P., Cabral-Lilly, D., Franklin, C., Reed, R., & Janoff, A. (2000). Detection of Endotoxin Levels in Liposomes, Lipid Bilayers and Lipid Complexes. https://patentimages.storage.googleapis.com/41/6a/36/65d9ead396c458/US6015716.pdf.