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Critical Micellar Concentration is a Criterion for the Inhibitory Effect of Amphiphilic Substances in Endotoxin Analysis Using Limulus Amebocyte Lysate

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Abstract | **Original Research Article**

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) may inhibit Limulus Amebocyte Lysate (LAL) chromogenic assay. It was hypothesized that the presence of micelles in the solution is 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. 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.

Keywords: Limulus Amebocyte Lysate (LAL), Critical Micellar Concentration (CMC), phosphatidylcholine (PC).

INTRODUCTION

The Limulus amebocyte lysate (LAL) test is used to detect contamination with endotoxins. The LAL test is one of the standard methods for detecting bacterial contamination in injectable solutions. The companies that produce LAL tests use the horseshoe crab (Figure 1). The animals are taken from the sea for a short time and brought back after the blood has been taken.

Figure 1: The horseshoe crab - a primeval animal whose blood is used to make LAL

Limulus Amebocyte Lysate (LAL) chromogenic assay, the most sensitive and robust assay available, was used for the endotoxin tests (Overview of Prominent LAL Tests. 2016). Today, the term 'endotoxin' is used synonymously with lipopolysaccharide (LPS) which is composed of three parts: O-antigen, core oligosaccharide, and lipid A (Figure 2).

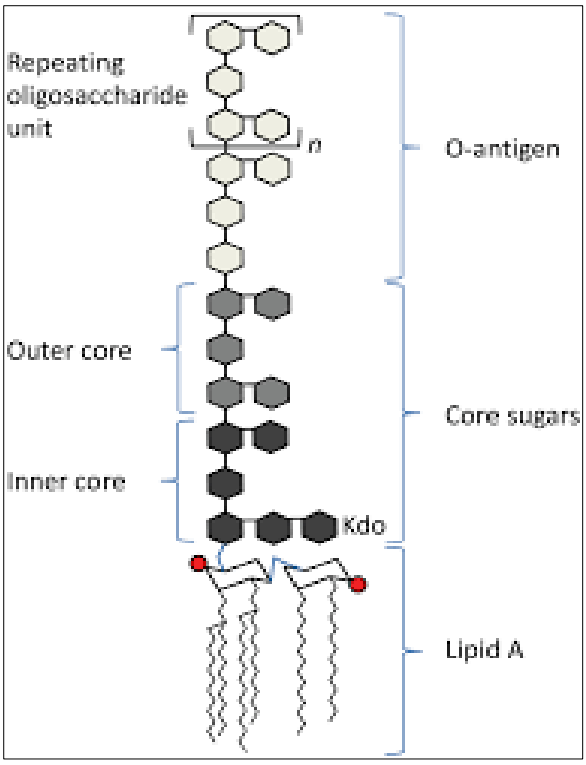

Figure 2: LPS (endotoxin) structure

LPS like the PC and Triton-X-100 are amphiphilic molecules (Figure 3).

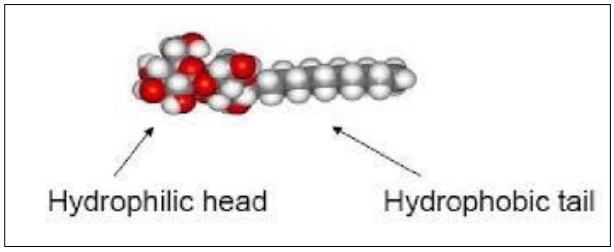

Figure 3: Amphiphilic molecules structure

The hydrophobic fatty acid chains of Lipid A anchor the LPS into the bacterial membrane (Figure 4). Likewise, lipid A anchors the LPS into the micelle or liposome. Micelles (Figure 5) are formed when the concentration of amphiphilic molecules exceeds the critical micelle concentration (CMC). Below the CMC, micelles are absent from the solution.

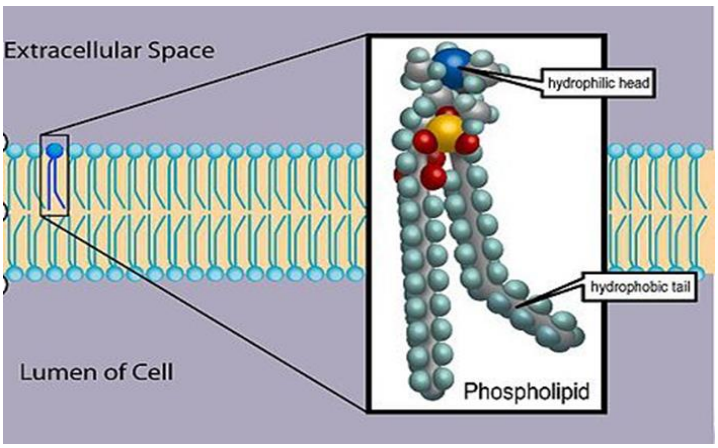

Figure 4: The bacterial membrane structure

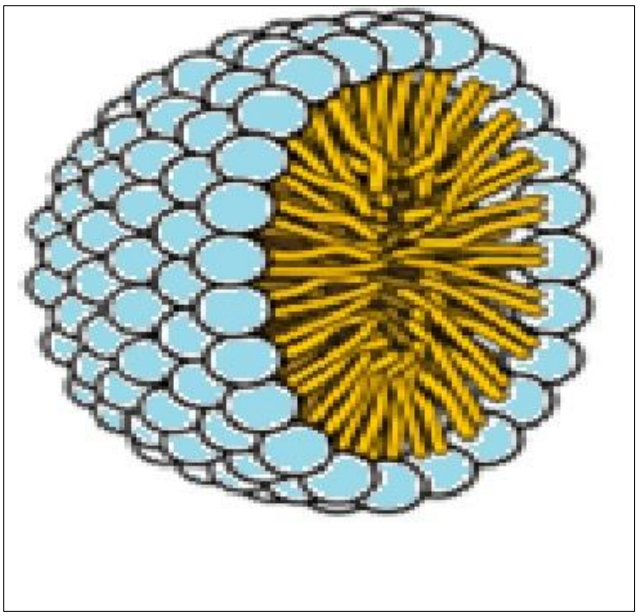

Figure 5: The micelle structure

One of the most time-consuming aspects of endotoxin testing using LAL is pretreating samples to overcome assay inhibition and enhancement (Dawson, M. 2005). 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 that 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 (Guidance for Industry. 2012).

MATERIALS AND METHODS

Limulus Amebocyte Lysate (LAL) chromogenic assay, the most sensitive and robust assay available, was used for the endotoxin tests (Overview of Prominent LAL Tests. 2016). The assay was performed on the Endosafe® Multi-Cartridge System (MCS), which is used for endotoxin testing of the finished product (USP <85>) (Endosafe - MCS. Charles River. 2025). 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 (Figure 6.) 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 the 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.

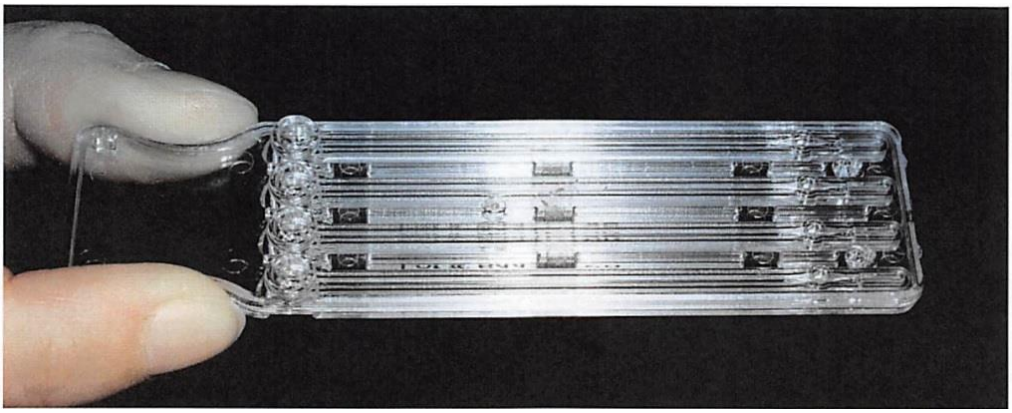

Figure 6: Endosafe® Multi-Cartridge System Cartridge

The cartridge has 4 lanes, 2 lanes for the test solution and 2 lanes in which a certain amount of endotoxin (spike) is added during the manufacture of the cartridge. This "spike" is necessary to assess the extraneous influence of the components of the test solution on the endotoxin analysis result. The Endosafe® Multi-Cartridge System automatically calculates the amount of Spike Value and the percentage of Spike detection. The amount of endotoxin added to the cartridge as the spike (ET spike) can be estimated as: ET spike = Spike Value / (Spike Recovery/100). For example, if the Spike Value <0.0, and recovery is < 0% (Table 1), this means that some components of the solution completely block the detection of an endotoxin. 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 (Dawson, M. 1995). The Maximum Valid Dilution (MVD) in this case was: MVD = EL/0.005 and Minimum Valid Dilution (mVD) was mVD = EL/5. Phosphatidylcholine 35mg/ml solution was provided to us by our client to test for the presence of endotoxin. Triton X-100 was supplied by Sigma-Aldrich.

RESULTS AND DISCUSSION

We detected the presence of endotoxins in samples containing Phosphatidylcholines (PC), average molecular weight of approximately 768, at concentration 35mg/ml (0.046M) or approximately 5E4 in Critical Micelle Concentration (CMC) units if CMC of PC was taken as 1.0 mM (Critical Micelle Concentrations (CMCs). Avanti 2025). The Endotoxin Limit (EL) was 3.5 EU/ml and the Maximum Valid Dilution (MVD) was as low as 700 (Tirumalai, R. 2025). 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. We encountered a similar problem before when we tried to use Triton X-100 to enhance the 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 (Figure 7).

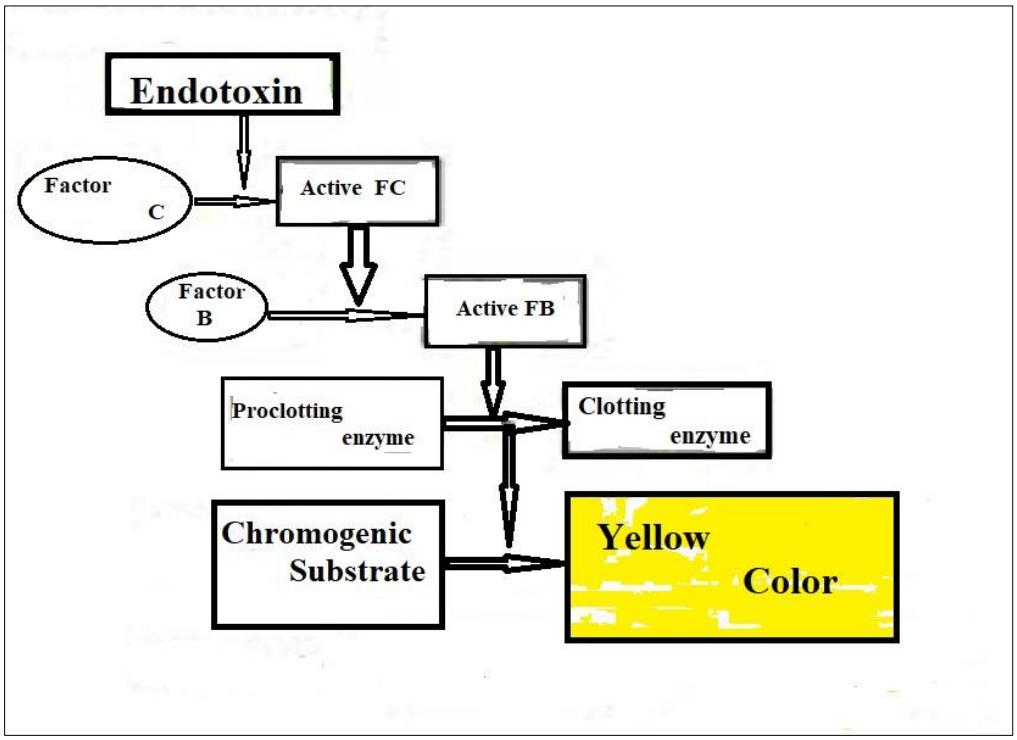

Figure 7: Cascade of enzymatic reaction resulting in yellow coloring

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 the second concentration 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 micelles in a solution inhibits the chromogenic reaction.

Table 1: Results of testing a solution containing phosphatidylcholine (46 mM) for the presence of endotoxin. PC concentrations are expressed in CMC units

PC Dilution	Spike Value	Spike Recovery	PC Concentration in CMC units
1:2	N/A	N/A	25000
1:20	<-0.001	<-11%	2500
1:200	<0.000	<0%	250
1:50 000	0.15	121%	0.92

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

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

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 (Figure 7) due to steric restriction.

It has been reported that the amphiphilic sodium dodecyl sulfate (SDS) terminates the enzymatic reaction in the LAL Nephelometric Method Test (Overview of Prominent LAL Tests. 2016). To overcome the negative micelle's 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

Based on the Spike Test, we concluded that amphiphilic substances could have a blocking effect, but it can be overcome. The key to solving the problem is the critical micelle concentration. 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, the inequality MVD > mDD is not true. In the worst case, another method should be used.

Abbreviations:

CMC - Critical Micelle Concentration
LAL - Limulus amebocyte lysate
EL - Endotoxin Limit
PC - Phosphatidylcholine
MCS - Endosafe® Multi-Cartridge System
RSE - Reference Standard Endotoxin
LPS - Lipopolysaccharide
VD - Valid Dilution
MVD - Maximum Valid Dilution
mVD - Minimum Valid Dilution
mDD - Minimum Detergent Dilution

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