Compatibility of Organic Solvents with the Endosafe® nexgen-PTS™ for Bacterial Endotoxin Testing of Water-Insoluble Pharmaceutical Products

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Introduction

Parenteral products must undergo testing for bacterial endotoxins (BET) per USP <1>. USP <85> and <1085> outline accepted methods for testing the endotoxin content of products using limulus amebocyte lysate (LAL), a product derived from horseshoe crab blood. However, USP <86> will become official in May 2025 and provides manufacturers with the information needed to use non-animal derived reagents via the use of recombinant LAL proteins.

Problem Statement: Due to the assay's reliance on enzymatic activity, aqueous solutions near neutral pH are most suitable. This creates a challenge when testing active pharmaceutical ingredients (API) that are designed to be poorly soluble in aqueous media, such as long-acting injectable formulations.

Published data on the compatibility of organic solvents with the LAL assay is lacking. This study evaluates organic solvent compatibility and provides useful insights for developing sensitive methods for materials with low solubility in aqueous systems.

Materials & Methods

Eight organic solvents (N-methyl-2-pyrrolidone (NMP), dichloromethane, tetrahydrofuran (THF), ethyl acetate, acetonitrile, dimethyl sulfoxide (DMSO), ethanol, and methanol), and five solubilizers (10% KLEPTOSE®, 10% D-mannitol, polyethylene glycol, polysorbate 20, and polysorbate 80) were evaluated for use per USP <85> on a Charles River Endosafe nexgen PTS. Solutions were initially assessed on I/E cartridges to determine the dilution required for acceptable spike recovery. A failing result indicates interference and requires further dilution.

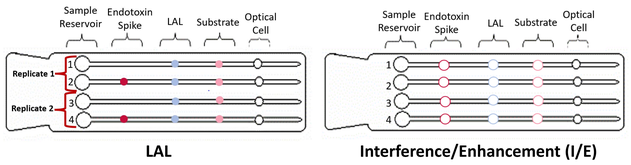
Confirmatory testing was performed on LAL and recombinant Trillium cartridges, thus ensuring that the same dilution worked with both traditional and recombinant reagents. The LAL/Trillium study verifies the accuracy of the I/E study and tests for precision by including replicates, measured by Coefficient of Variation (CV).

Assay acceptance criteria:

1. The CVs of the reaction times for both the sample and the positive product control (PPC) must be <25%
2. Spike recovery of the PPC must be within 50-200%

Materials:

- Endosafe nexgen-PTS with EndoScan-V Software for BET
- Diluent was Limulus Reagent Water (LRW)
- Inhibition-Enhancement (I/E) Screening Cartridge
- Limulus Amoebocyte Lysate (LAL) Test Cartridge
- Trillium™ Test Cartridge



Results

LogP of Solvents and Spike Recovery of Solvents at Different Dilutions on I/E cartridges

Solvent/Solubilizer	LogP	4% 1:25	2% 1:50	1% 1:100	0.50% 1:200	Further evaluation?	Max Concentration
Dichloromethane	1.5	53%	127%	NT	NT	Yes	4%
Ethyl Acetate	0.7	NT	98%	NT	NT	Yes	2%
THF	0.5	NT	33%	49%	**	No	N/A
Sodium Bicarbonate, 1M	0.5	137%	213%	140%	126%	Yes	1%
Acetonitrile	0	15%	106%	NT	NT	Yes	2%
Ethanol	-0.1	NT	35%	63%	91%	Yes	1%
Methanol	-0.5	10%	70%	NT	NT	Yes	2%
NMP	-0.5	NT	0%	6%	**	No	N/A
DMSO	-0.6	NT	47%	83%	95%	Yes	1%
10% D-Mannitol	-3.1	65%	91%	NT	NT	Yes	4%
5% Kleptose	-15	43%	88%	NT	NT	Yes	2%
10% PEG-300	Not available	NT	47%	82%	NT	Yes	1%
Polysorbate 20	Not available	NT	17%	27%	**	No	N/A
Polysorbate 80	Not available	NT	21%	46%	**	No	N/A

NT: Not Tested; pass/fail results are extrapolated based on trend, LogP data sourced from PubChem (https://pubchem.ncbi.nlm.nih.gov/)

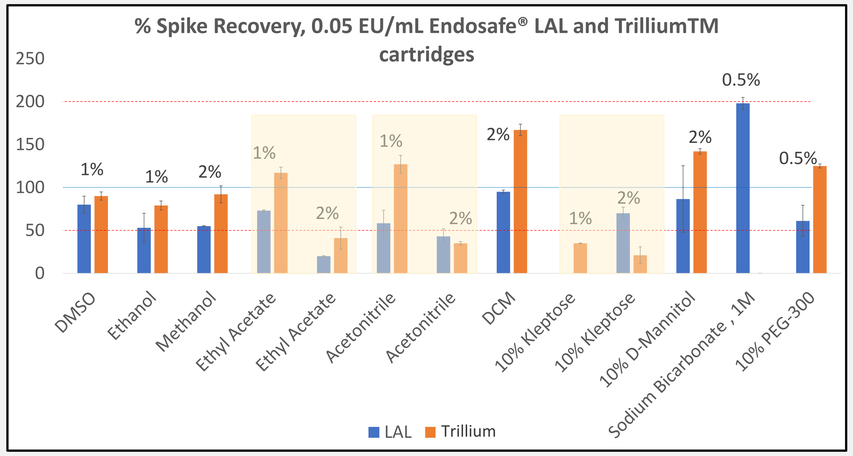
Solvents were screened on I/E cartridges at a 1:50 dilution. If this resulted in 50-200% spike recovery, a more concentrated solution was used to challenge the assay. If it failed, a more dilute solution was used to find the dilution required to meet the spike recovery requirements.

** Solvent excluded from further study:

- NMP and THF: Not widely available in microbiology labs
- Polysorbate 20 and 80: Results exhibited high variability, possibly due to bubble formation during the mixing step

Please note the shaded regions:

- Ethyl acetate: I/E results indicated a 2% solution would meet acceptance criteria, however further dilution to 1% was required
- Acetonitrile: I/E results indicated that a 2% solution would meet acceptance criteria, however further dilution to 1% was required.
- 10% Kleptose®: 2% met acceptance criteria in LAL cartridges, however even at 1% this product did not reach 50% spike recovery.



Application to insoluble pharmaceutical products

Product	logP	Concentration (mg/mL)	Solvent	Dilution	Diluent	Spike CV	Spike Recovery	Assay sensitivity (EU/mg)	Cartridge type
A	2.24	20	DMSO	1:200	LRW	1%	89%	0.5	0.05 EU/mL LAL
B	1.45	50	DMSO	1:300	LRW	N/A	67%	0.3	I/E
C	2.13	20	DMSO	1:200	LRW	3.1%	105%	0.05	0.005 EU/mL LAL

This table shows results of method verification for insoluble pharmaceutical formulations prepared in organic solvent. These examples show that sensitive methods can be developed for insoluble products using organic solvents.

Discussion & Conclusion

Most solvents and solubilizers were compatible with BET at dilutions of 1:25 to 1:200, with the exception of the polysorbates, tetrahydrofuran, and N-methyl-2-pyrrolidone. These solutions produced low spike recovery, are not typically available in microbiology labs, or produced variable results at the dilutions tested and were not evaluated further. Additionally, while a 10% Kleptose® solution met the assay acceptance criteria at a 1:50 dilution on LAL cartridges, this compound did not meet the minimum spike recovery even after further dilution to 1:100 on the Trillium™ cartridges. This is the only solution that did not produce equivalent results between the Trillium™ and LAL cartridges. There are no results for 1M Sodium Bicarbonate on Trillium cartridges, as the results showed positive results for endotoxin possibly indicating that the bottle of reagent had become contaminated.

Not all solvents transferred from I/E to LAL and Trillium cartridges at the dilution initially identified in the I/E study:

- Acetonitrile was tolerated at 2% in the I/E cartridges, but only at 1% in the LAL and Trillium™ cartridges.
- Ethyl acetate was tolerated at 2% in the I/E cartridges, but only at 1% in the LAL and Trillium™ cartridges

Additionally, test materials with spike recovery close to the upper or lower threshold risk not meeting this acceptance criteria due to the inherent variability of the assay. This variability likely stems from the assay's reliance on enzymatic cascades. The LAL reagent is a relatively crude mixture of enzymes and co-factors; not a single purified enzyme[1]. Therefore, there is inherent lot-to-lot variability. For this reason, acetonitrile and ethanol were tested on multiple LAL cartridges to confirm results.

Solvent	%	Spike Recovery, %	Number of cartridges	Number of lots
Ethanol	1	41, 65	2	2
Acetonitrile	1	65, 41, 69	3	3

Mechanistically, it is likely that organic solvents and the solubilizers tested in this study interfere with structure of the enzymatic proteins and/or the ability of LPS to form biologically active macromolecules. Monomeric lipopolysaccharide (LPS) is not thought to be biologically active, and it is well documented that pH, salt concentration, chelating agents, and surfactants all play a role in low endotoxin recovery due to interference with LPS aggregation[2]. This work adds significantly to the body of data on this subject and provides proof of concept data showing the successful implementation of organic solvents to perform BET of insoluble pharmaceutical compounds.

Contact Information

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References

1. Sandle, T. "Variability and Test Error with the LAL Assay." *American Pharmaceutical Review* 17.6 (2014): 22-28.
2. Cao, Yuan, YujI/E Zhang, and Frank Qiu. "Low endotoxin recovery and its impact on endotoxin detection." *Biopolymers* 112.11 (2021): e23470.