Use of Naturally Occurring Endotoxins (NOE) for the release of No-Biological Drug Product affected by Low Endotoxin Recovery (LER) in LAL kinetic assay

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Abstract

Low Endotoxin Recovery (LER) studies are intended to determine any potential endotoxin masking effect of sample matrices tested using the Limulus Amebocyte Lysate. The failure in endotoxin recovery from drug products (DP) may lead to endotoxin contamination not detected at release and pyrogenic products distributed for commercial uses. According to PDA Technical Report 82, LER study is performed spiking DP with Reference Standard Endotoxin (RSE) as preferred standards. Naturally Occurring Endotoxins (NOE) may be used as further assessment. According to the regulation LER study is mandatory only for biological DP. In the present study a no-Biological DP showed LER phenomenon. The product spiked with RSE showed a spike recovery below 50% at 24 hours. The most common mitigation strategies (i.e. use of $MgCl_2$, $MgSO_4$ and dispersing agent) were found to be not suitable for LER troubleshooting. Further investigations on this LER effect have highlighted that the masking activity was related exclusively to the Active Pharmaceutical Ingredient. No LER effect has been observed spiking the DP with NOE at time zero, 24 and 48 hours. LER effect observed in no-Biological DP and the use of NOE as concrete investigation option might lead to a batch release in a non-regulated scenario.

Introduction and mitigation strategies

All you need to know

The term “Low Endotoxin Recovery” (LER) describes the failure to detect spiked endotoxins in some sterile biological drug products when tested using the internationally harmonized compendial Limulus Amebocyte Lysate (LAL) assay.

A sterile Drug Product (DP) made of a formulated (EDTA and Mannitol in WFI) solution of a chemically synthesized antiemetic agent was analyzed for Bacteria Endotoxins testing, using kinetic turbidimetric assay.

Experimental conditions:

- In the Initial Trial, product dilution was conducted using LAL Reagent Water (LRW). Subsequent trials were carried out to troubleshoot the issue encountered in the initial trial at the time point 24h. During the first Additional Trial, various concentrations of $MgSO_4$ were used for sample dilution. In the second Additional Trial, product was dilution using Pyrosperse™ (dispersing agent) at 2% and $MgSO_4$ in two different concentrations. Finally, the third Additional Trial a dilution of product using a 4.4 mM concentration of $MgCl_2$ was done.
- During the Initial Trial and Additional Trials n°1 and 2, the undiluted product was spiked with a theoretical RSE of 17.5 EU/mL and tested at 1:1400 dilution. The standard curve in EU/mL used included the following points: ST1: 0.0015625, ST2: 0.00312, ST3: 0.00625, ST4: 0.0125, ST5: 0.025, ST6: 0.5, and ST7: 0.1.
- In the Additional Trial n°3, the theoretical RSE spike was increased up to 35 EU/mL, and the product was tested at 1:700 dilution using a standard curve in EU/mL with the following points: ST1: 0.005, ST2: 0.05, ST3: 0.5, and ST4: 5.
- Recovery calculation for each checkpoint: Spike recovery (%) = $\frac{(Endotoxin\ content\ spiked\ sample\ at\ time\ X)}{LRW\ spiked\ at\ time\ zero}\ X\ 100$
- All the samples were stored at 20-25 °C during the study and the reaction mixture (sample solution + LAL Lysate) were found within the required pH limit of 6-8.

Initial Trial: Kinetic Turbidimetric assay results on DP sample solution applying a theoretical RSE spike of 17.5 EU/mL

Sample (Finished DP)		Checkpoints					
		Time zero		24 Hours		48 Hours	
		Result (EU/mL)	Recovery %	Result (EU/mL)	Recovery %	Result (EU/mL)	Recovery %
Finish Drug product (vial)	Unspiked	<2.1875	79	<4.375	0	<2.1875	0
	Spiked	16.8		Not detected (below the last point of the standard curve)		Not detected (below the last point of the standard curve)	
	Spiked LRW	21.28					

Additional Trial (n°1): Kinetic Turbidimetric assay results on sample solution diluting the DP sample with different concentrations of $MgSO_4$ applying a theoretical RSE spike of 17.5 EU/mL

Sample (Finished DP)		Time Point zero							
		10mM $MgSO_4$		15mM $MgSO_4$		20mM $MgSO_4$		25mM $MgSO_4$	
		Result (EU/mL)	Recovery %	Result (EU/mL)	Recovery %	Result (EU/mL)	Recovery %	Result (EU/mL)	Recovery %
Finish Drug product (vial)	Unspiked	<2.1875	0	<2.1875	0	<2.1875	0	<2.1875	0
	Spiked	Not detected (below the last point of the standard curve)		Not detected (below the last point of the standard curve)		Not detected (below the last point of the standard curve)		Not detected (below the last point of the standard curve)	
	Spiked LRW	20.44		20.44		20.44		20.44	

Additional Trial (n°2): Kinetic Turbidimetric assay results on sample solution diluting the DP sample with different concentrations of $MgSO_4$ + 2% Pyrosperse applying a theoretical RSE spike of 17.5 EU/mL

Sample (Finished DP)		Time Point zero			
		2.5mM $MgSO_4$ + Pyrosperse 2%		5mM $MgSO_4$ + Pyrosperse 2%	
		Result (EU/mL)	Recovery %	Result (EU/mL)	Recovery %
Finish Drug product (vial)	Unspiked	<2.1875	0	<2.1875	0
	Spiked	Not detected (below the last point of the standard curve)		Not detected (below the last point of the standard curve)	
	Spiked LRW	19.18		19.18	

Additional Trial (n°3): Kinetic Turbidimetric assay results on sample solution diluting the DP sample with a concentration of 4,4 mM of $MgCl_2$ increasing the theoretical RSE spike up to 35 EU/mL

Sample (Finished DP)		Time Point zero	
		4.4mM $MgCl_2$	
		Result (EU/mL)	Recovery %
Finish Drug product (vial)	Unspiked	<3.5	0
	Spiked	Not detected (below the last point of the standard)	
	Spiked LRW	28.35	

Which component of the drug product formulation causes the low endotoxin recovery effect?

Considering that some excipients in DP formulations have been reported to cause the LER phenomenon in the literature, the effect of specific excipients on LER of the DP formulation was focused by preparing a formulated solution containing EDTA and Mannitol.

Experimental conditions:

- During the test, the undiluted Solution was spiked with 17.5 EU/mL of RSE and tested at a 1:1400 dilution. The standard curve in EU/mL used included the following points: ST1: 0.0015625, ST2: 0.00312, ST3: 0.00625, ST4: 0.0125, ST5: 0.025, ST6: 0.5, and ST7: 0.1.
- All samples tested in the different trials were kept under the same storage condition (20-25 °C) and recovery calculations were performed using the same formulas of initial trial and Additional Trial n°1,2 and 3.

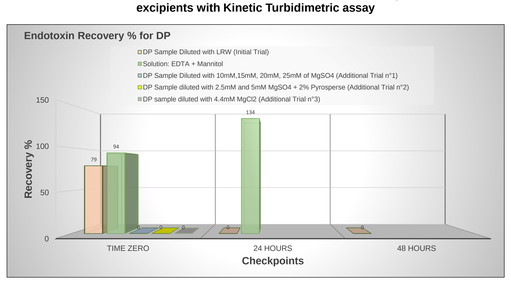
Kinetic Turbidimetric assay results on sample solution applying a theoretical RSE spike of 17.5 EU/mL

Sample		Checkpoints			
		Time zero		24 Hours	
		Result (EU/mL)	Recovery %	Result (EU/mL)	Recovery %
Solution: EDTA + Mannitol	Unspiked	<2.1875	94	<2.1875	134
	Spiked	31.22		44.8	
	Spiked LRW	33.32			

The spiked DP solution showed endotoxin recovery exceeding 50% only at time zero when diluted in LRW. Common mitigation strategies, such as using $MgCl_2$, $MgSO_4$ and dispersing agents, were ineffective in antagonizing LER issues, as no endotoxin recovery was observed even at time zero.

On the other hand, Holding Time (HT) studies on the Mannitol + EDTA formulated solutions demonstrated endotoxin recovery above 90% at both time zero and after 24 hours, thus indicating the LER as API-induced (due to some surfactant characteristics of the molecule itself) and no LER effect from excipients.

Summaries of % recovery on DP with and without troubleshooting and on buffer excipients with Kinetic Turbidimetric assay



Conclusion

These studies suggest that it is precisely the API that is responsible for the LER phenomenon, most likely due to its molecular structure interfering with endotoxin recovery when using the internationally harmonized compendial Limulus Amebocyte Lysate (LAL) assay.

⚠ These studies demonstrate that potential LER effects impact not only Biological products but also no-Biological products. These scenarios could represent a future opportunity for regulatory authorities to expand LER concern beyond Biological products context and explore alternative testing pattern (i.e., in process control, use of NOE, Monocyte Activation Test) to demonstrate absence of endotoxins on product matrices.

What if we used different endotoxin?

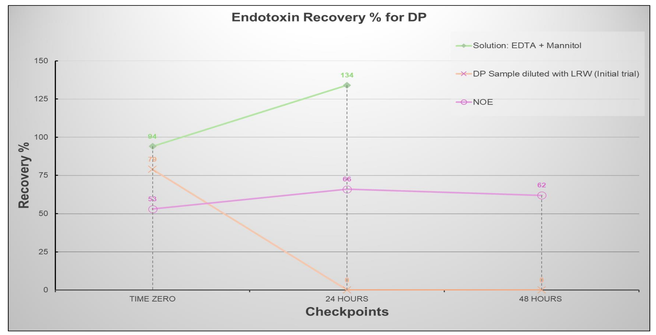
Preparation steps of Naturally Occurring Endotoxin

1. Reconstitution of lyophilized *Enterobacter cloacae* ATCC 7256 with Nutrient Broth.
2. After an initial incubation at 37°C for 24 hours, the culture was then subcultured to obtain a final culture of *Enterobacter cloacae* containing 5% nutrient broth and 95% SWI (sterile water for irrigation). All diluted sub-cultures were incubated at 25°C.
3. An aliquot was aseptically removed and streaked for isolation. and was subjected to bacterial identification by MALDI-TOF method to confirm the purity of the original bacterial culture.
4. Following that, the culture was subjected to redundant sterile filtration, using 0.22 µm filter and the sterile filtrate was aseptically filled into depyrogenated 10mL vials at approximately 3 mL/vial.
5. Verification of concentration of NOE.
6. Performed the HT study with NOE.

Experimental conditions:

- During the test, the un-diluted DP was spiked with 50.1 EU/mL of NOE and tested at a 1:100 dilution with LRW. The standard curve in EU/mL used included the following points: ST1: 0.005, ST2: 0.05, ST3: 0.5, ST4: 5, ST5: 50.
- All samples tested in the different trials were kept under the same storage condition (20-25°C) and recovery calculations were performed using the same formulas of initial trial and Additional Trial n°1, 2 and 3.
- The test was performed using kinetic chromogenic assay.

Summaries of % recovery on DP with different tests



Conclusion

The study using NOE demonstrated a recovery slightly above the 50% permitted by guidelines, indicating that the API itself behaves a significant BET masking effects with both NOE and RSE.

Currently, there are no standardized protocols for NOE production, nor are there official guidelines detailing how and which NOE should be used for such studies.

⚠ Endotoxins are crucial quality parameters in the release testing of sterile products, but unpredictable masking effects can occur with various product formulations. The current unregulated scenario (no LER concern to date reported on regulations for no-Biological products) is believed to require re-evaluation, in the light of evidences provided, and in such circumstances, discretionary resolutions for batch release of specific formulations should be adopted.

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