EtoxiClear™: A new adsorbent for the efficient removal of endotoxin from biopharmaceuticals

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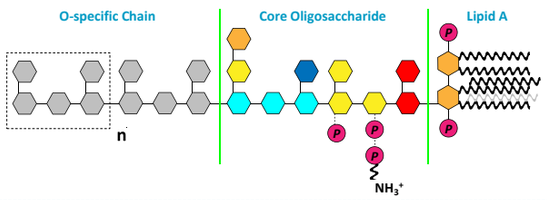
Introduction

Endotoxin or lipopolysaccharides (LPS) (Figure 1[1]) are highly toxic components of the cell wall of Gram-negative bacteria and are often present in significant amounts in bacterial cell culture expression systems such as *E. coli.*

FIGURE 1

Schematic representation of Gram-negative bacterial endotoxin (LPS).



A number of methods have been adopted for the removal of endotoxin based on adsorption, in particular ion-exchange chromatography.

Although downstream processing can significantly reduce endotoxin levels in the product, efficient and cost-effective removal of residual endotoxin from biopharmaceutical preparations remains a challenge.

This technical poster addresses the issues of removal of endotoxin from biological preparations. Specific reference will be made to a new synthetic ligand affinity adsorbent, EtoxiClear™ (Figure 2), which exhibits high affinity for endotoxin, low protein binding and can be depyrogenated using sodium hydroxide.

FIGURE 2

EtoxiClear™ is available in a range of disposable column sizes (5 mL, 50 mL and 500 mL all with a standard bed height of 10 cm).

The bi-dentate ligand, attached to ProMetic BioSciences Ltd (PBL's) proprietary base matrix – PuraBead®, binds in a spatially selective and optimal manner to the LPS molecule with a binding capacity for endotoxin in excess of 1,000,000 EU/mL of adsorbent in a flow through chromatography mode (5 minute residence time).

A number of biomolecules with different isoelectric points have been used to demonstrate efficient protein recovery and clearance of residual endotoxin across the pH range. Protein recoveries in excess of 95% are achievable with endotoxin clearance to below 0.1 EU/mg protein.

Binding Capacity

EtoxiClear™ has a high dynamic binding capacity for endotoxin in the presence of a wide range of biological buffers. The dynamic endotoxin binding capacity is shown in Table 1.

TABLE 1

Endotoxin Binding Capacity	Endotoxin (EU/mL of adsorbent)
Dynamic	>1,000,000*

* Linear flow rate 120 cm/hr, 5 minute residence time

Product Comparison

EtoxiClear™ was compared against two commercially available competitor endotoxin removal products (IEX and affinity based membranes), using the manufacturers instructions. A neutral pH HSA solution, containing 2000 EU/mL of endotoxin, was loaded onto each product at a flow rate of 1 mL/min to determine endotoxin clearance and protein recovery (Table 2).

TABLE 2

Comparison of EtoxiClear™ versus other commercially available endotoxin removal products.

Product	Protein recovery (%)	Endotoxin level (EU/mg)	Endotoxin clearance
EtoxiClear™	≥98	<0.06 (below LOD)	>3.7 log
IEX endotoxin removal membrane	84	<0.06 (below LOD)	>3.7 log
Affinity endotoxin removal membrane	98	>21	<1.0 log

Protein recovery vs endotoxin concentration

IgG protein solutions (~5.5 mg/mL) containing either **low** (110 EU/mL) or **high** (3300 EU/mL) levels of endotoxin were loaded onto EtoxiClear™ disposable columns (5 mL column volume (CV)). Each column was loaded with similar total amounts of endotoxin (>10,000 EU) and protein recoveries were measured (Table 3).

TABLE 3

Protein recovery and endotoxin clearance results.

	Initial endotoxin concentration (EU/mL)	Volume loaded (mL)	Total endotoxin loaded (EU)	Load [Protein] mg/mL	Protein recovery (%)	Final endotoxin concentration (EU/mg protein)
IgG Solution (1)	110	100	10,950	5.5	100	**0.08**
IgG Solution (2)	3322	5	16,610	6.0	87	**0.13**

Results show that the EtoxiClear™ adsorbent gives excellent endotoxin clearance (~0.1 EU/mg protein) and high protein recoveries, for protein solutions containing either low or high starting concentrations of endotoxin.

Effect of protein concentration on endotoxin removal and protein recovery

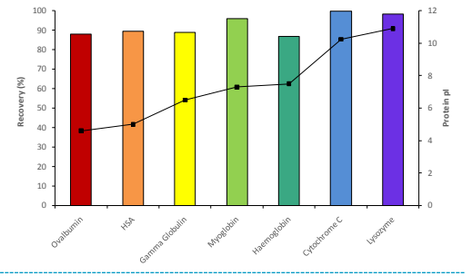
Increasing $F(ab')_2$ concentration (from 0.8 to 8 mg/mL) resulted in increased target protein recovery (from 80 to >95% respectively) when loading 24,000 EU/mL of endotoxin onto EtoxiClear™ at neutral pH. Results indicate that increasing protein concentration has no significant effect on the level of endotoxin clearance obtained.

Protein Binding

EtoxiClear™ has low protein binding and a wide range of proteins can be processed independent of their iso-electric point achieving high protein recoveries. Figure 3 indicates that typically >90% recovery is achieved for various model proteins spanning the pI spectrum.

FIGURE 3

Typical protein recoveries at neutral pH.



Endotoxin removal from purified antibody fragment

EtoxiClear™ was used to remove residual endotoxin from an antibody fragment from an *E. coli* lysate partially purified using Fabsorbent™ F1P HF as a capture step (Table 4).

Clarified cell lysate was loaded onto Fabsorbent™ F1P HF and the $F(ab')_2$ fragment eluted at pH 5.0. The resulting elution fraction was loaded directly onto EtoxiClear™.

Equipment	Chromatography workstation	
Step	Capture	Polish
Adsorbent	Fabsorbent™ F1P HF	EtoxiClear™
Column parameters	18.4 cm bed height (37 mL CV); 2.6 cm Ø column	3 cm bed height (2.4 mL CV); 1.0 cm Ø column
Equilibration/Wash buffer	50 mM Tris, pH 8.0	50 mM sodium phosphate, pH 7.2
Load	1 CV of *E. coli* cell lysate containing antibody fragment	Fabsorbent™ F1P HF eluate
Elution buffer	50 mM sodium citrate, pH 5.0	n/a

TABLE 4

Endotoxin levels determined using a chromogenic endotoxin assay kit.

Sample	Endotoxin (EU/mL)
Cell lysate	192,000
Fabsorbent™ F1P HF elution fraction	46,000
EtoxiClear™ flow through	19

Fabsorbent™ F1P HF produced a high purity antibody fragment, followed by a 4 log reduction of endotoxin achieved across the process (3.4 log clearance was observed for the EtoxiClear™ column step).

Effect of additives on endotoxin removal and protein recovery

The effect of various additives on target protein recovery (specifically $F(ab')_2$ fragment) and endotoxin removal was investigated. It was observed that additives such as high levels of salt (0.5 M NaCl) and metal ions (Ca^{2+} ,Mg^{2+}, Cu^{2+}) detrimentally affected performance.

However, EDTA (up to 20 mM) positively promoted protein recovery from ~85% to 95% without affecting endotoxin clearance.

Buffer pH

EtoxiClear™ can operate in acidic to neutral conditions (pH 4.0 to pH 8.0) without a reduction in endotoxin clearance and typically maintaining >90% recovery of various proteins (up to 5 mg/mL).

Endotoxin removal from proteins expressed in *E. coli*

Two different proteins, produced in *E. coli* (post initial capture step), were applied to EtoxiClear™ at neutral pH.

Results presented in Table 5 below show high protein recovery and significant endotoxin clearance.

TABLE 5

Protein recovery and endotoxin clearance results.

	pI	Load [Protein] mg/mL	Load EU/mg protein	Protein recovery (%)	Endotoxin clearance
Protein 1	7.3	0.70	7200	85	1.8 log
Protein 2	4.6	0.34	22000	94	1.7 log

Determination of β-D-glucan Interference

There are many commercially available endotoxin detection tests/kits to determine endotoxin clearance from protein solutions. However, if a chromogenic based (LAL) test is used, it is recommended to include Glucashield® buffer to render the reagent insensitive to (1→3)-β-D-glucan interference which may be present in the sample.

HSA (9.1 mg/mL) and IgG (9.6 mg/mL) protein solutions, containing endotoxin, were loaded (1 mL) onto EtoxiClear™ disposable columns (1 mL CV) at 1 mL/min. The flow through fractions were collected and the samples analysed using a chromogenic based test (with and without Glucashield® buffer) and by Lonza Bioscience by LAL Kinetic chromogenic assay (Table 6).

TABLE 6

Endotoxin clearance, from both HSA and IgG protein solutions, determined using a chromogenic endotoxin assay kit (± Glucashield® buffer) and by Lonza Bioscience in their endotoxin testing laboratory.

Sample	PBL Chromogenic Analysis (EU/mg)		Lonza Analysis (EU/mg)
	(-) Glucashield®	(+) Glucashield®	
HSA Load	101.7	45.3	23.6
HSA flow through	7.9	0.14	0.06
Endotoxin Clearance:	92%	99.7%	99.7%

Sample	PBL Chromogenic Analysis (EU/mg)		Lonza Analysis (EU/mg)
	(-) Glucashield®	(+) Glucashield®	
IgG Load	28.2	16.9	8.3
IgG flow through	5.0	0.3	0.09
Endotoxin Clearance:	82.3%	98.2%	98.9%

Inhibition of the β-D-glucan interference allows for more sensitive and more accurate determination of endotoxin removal comparable to the analysis performed externally at the Lonza Bioscience laboratory.

Conclusions

- EtoxiClear™ provides superior endotoxin removal from a wide range of proteins across the pI spectrum, with recoveries that can be in excess of >95%, in a range of conditions from acidic to neutral pH.
- EtoxiClear™ has a high capacity for endotoxin and binds >1,000,000 EU/mL of adsorbent loading at 120 cm/hr, 5 minute residence time.
- EtoxiClear™ shows improved endotoxin clearance and protein recovery in comparison to other commercially available endotoxin removal products.
- EtoxiClear™ gives excellent endotoxin clearance (~0.1 EU/mg) and high protein recoveries for protein solutions containing either low or high starting concentrations of endotoxin.
- Protein recoveries can be improved by increasing the target protein concentration without any impact on endotoxin binding.
- Following the capture and partial purification of an antibody fragment, EtoxiClear™ provided a 4 log reduction of endotoxin across the process.
- Endotoxin clearance of ~1.8 log from purified proteins expressed in *E. coli* was obtained using EtoxiClear™.
- The introduction of Glucashield® buffer to remove interference by β-D-glucans improved the sensitivity of the chromogenic assay and provided a more accurate determination of endotoxin clearance.
- Overall, EtoxiClear™ is ideally suited for use in process development applications or final polishing steps used during cGMP manufacturing of biological molecules.

References

[1] http://www.biozentrum.unibas.ch/~grzesiek/RESEARCH/home04.htm