ENDOTOXIN TESTING OF ALLERGEN EXTRACTS

Rosa Codina Ph.D., Mathew D. Roby, B.S., and Robert E. Esch, Ph.D.
Greer Laboratories, Inc., Lenoir, NC

Abstract

Rationale: While endotoxins are ubiquitous in nature, their role in the immunoresponse is controversial. This study was conducted to optimize an assay to measure endotoxins in allergenic extracts (AE) and determine their concentrations in various AE.

Methods: A chromogenic kinetic assay was optimized to generate a log-log endotoxin standard curve (50 EU/mL - 0.05 EU/mL), which was utilized to calculate test concentrations, including positive and negative controls. Assay performance characteristics including linearity, precision, and accuracy were evaluated. Spike recovery experiments were performed with components typically contained in AE (i.e., 50% and 10% glycerin; 0.4% and 0.2% phenol), and with solutions containing these combined components. The AE analyzed were derived from *Dermatophagoides pteronyssinus* (N=44), *D. farinae* (N=35), pollen (N=49), fungi (N=18), and foods (N=6). Non-parametric statistical analysis was performed.

Results: Negative control values were below 0.05 EU/mL. The standard curve correlation coefficient was >0.998. Assay precision and accuracy were 9.0% and 17.4 %, respectively. 50% and 10% glycerin interfered in the test (average endotoxin spike recoveries: 0.0% and 24.3%, respectively). Interference was overcome by increasing sample dilutions. Endotoxins were selectively detected in most AE, with the greatest concentrations measured in *D. farinae* AE (median = 1,424 EU/mL) and the least in fungal AE (median = 1.2 EU/mL).

Conclusions: An assay for measuring endotoxins in AE has been optimized. Endotoxins are present in AE at various concentrations, generally at levels less than FDA proposed thresholds for parenteral exposure to endotoxins in humans (2,800 – 3,500 EU/70-kg person).

Introduction

The role of endotoxin exposure in the immunoresponse is controversial. While endotoxins can cause adverse health effects upon injection, determination of their presence in allergenic extracts is not currently required. Therefore, endotoxin concentrations in these products have not been defined.

Endotoxins are typically measured using the Limulus Amebocite Lysate test (LAL test), which can be performed using gel-clot, chromogenic, and turbidimetric techniques. Gel-clot techniques are the simplest to perform, and were utilized by the FDA in a pilot study to measure endotoxin contents in allergenic extracts. However, these techniques are semi-quantitative, have a two-fold error, and cannot detect potential enhancement effects of sample matrix in test results. Chromogenic and turbidimetric techniques are quantitative, have a greater resolution than gel-clot techniques, and can detect both inhibition and enhancement effects.

Objectives

- **Optimize an LAL chromogenic kinetic assay to measure endotoxin concentrations in allergenic extracts**
- **Obtain preliminary data on endotoxin concentrations in various Greer extracts utilizing the optimized assay**

Reagents and Supplies

- Pyrochrome® LAL reagent, control standard endotoxin, pyrogen-free supplies, B-glucan inhibiting buffer: Associates of Cape Cod, Inc., East Falmouth, MA.
- Pyrogen-free water and allergen extracts (N = 152): Greer Laboratories, Inc. (tabulated below)

Source*	N (%)
Dermatophagoides pteronyssinus	44 (28.9)
Dermatophagoides farinae	35 (23.0)
Pollen	49 (32.2)
Fungi	18 (11.8)
Foods	6 (3.9)

** The extracts analyzed comprised those for in vivo diagnosis and treatment, in vitro diagnosis, and raw material assessments*

Assay Setup and Design

- *Standard curve*: 50 EU/mL to 0.05 EU/mL
- *Samples*: Serially diluted to overcome interference
- *Controls*:
  - Negative: Pyrogen-free water
  - Positive: 0.5 EU/mL
- *Incubations*: 50 mL samples, standard, and controls plus 50 mL LAL reagent/well.
- *Kinetic reading*: At 37ºC using a Versamax™ microplate reader, equipped with the proper software (Soft MaxPro®, Molecular Devices Corporation, Sunnyvale, CA). Readings were performed at one-minute time intervals for 60 minutes.
- *Kinetic reduction*: Onset time to achieve an optical density (OD) of 0.05 at 405 nm. A log-log standard curve was generated and used to extrapolate sample and control readings to concentrations.
- *Expression of results*: EU/mL of extracts

Assay Validation Criteria (FDA Recommendations)

- Correlation coefficient: Greater than 0.98
- Positive control: The known control value ± 25%
- Spike recovery: 50% - 150% of the added spike concentration
- Assay precision: Percent coefficient of variation of the spiked positive control
- Assay accuracy: Percent coefficient of variation of the spiked negative control

Interference Study

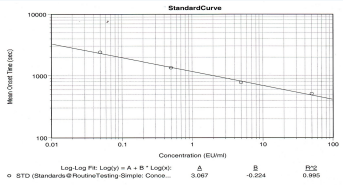
- **Extraction solutions tested:**
  - Coca (0.4% phenol, pH 8.0-8.4)
  - Glycero-Coca's (0.2% phenol, 50% glycerin, pH 7.0-8.0)
  - Modified glycero-Coca's (0.4% phenol, 50% glycerin, pH 7.4-8.4)
- **Single components tested:**
  - Sterile glycerin: 50%, 10%, and 5%
  - Phenol: 0.4% and 0.2%
  - pH: 2 - 12 pH range
- **Spikes**: 5 EU/mL, 0.5 EU/mL, and 0.05 EU/mL
- **Expression of results**: Percent of spike recovery vs sample results without spikes

Statistical Analyses

- **Assay Validation:**
  - Normality tests
  - Parameters analyzed: Positive control, spike recovery of the negative control, B curve parameter (slope), and R^2
- **Allergen Extracts:**
  - Non-parametric: Mann Whitney U test

Results (I)

- Endotoxin standard curves have a Log-Log fit



Analytical Assay Validation Parameters (43 runs)

Parameter	Normally Distributed	Mean	SD
(+) Control (0.5 EU/mL)	Yes	0.533	0.048
(-) Control Spike Recovery	Yes	93.13	16.18
B Parameter	Yes	-0.2225	0.01425
R^2	No	0.998	0.0028

Results (II)

Table 3: Assay Validation Criteria for Variables Normally Distributed

Parameter	FDA Acceptance Criteria	Proposed Analytical Validation Criteria		
		Accept (X ± 2 SD)	Reconsider (X ± 2 SD - X ± 3 SD)	Reject (> or < X ± 3 SD)
B Parameter	NA	-0.196 to -0.251	-0.179 to -0.265	<-0.179 or >-0.265
(+) Control, (0.5 EU/mL)	± 25% (0.375 - 0.625)	0.439 to 0.627	0.392 to 0.674	< 0.392 or >0.674
(-) Control Spike Recovery (%)	50 to 150	60.7 to 125.5	44.5 to 141.7	< 44.5 or > 141.7

Assay Validation Criteria for R^2: 0.987 (minimum value obtained)

Assay Precision: 9.0%
Assay Accuracy: 17.4%

Spike Recoveries of Extraction Solutions

Solution	Dilution	pH	% Spike Recovery		
			5 EU/mL	0.5 EU/mL	0.05 EU/mL
Coca's	Neat	8-9	43.2	93.8	34.0
	1/10	7-8	**83.6**	NA	**66.0**
	1/50	6-7	**87.0**	**95.0**	**100.0**
	1/100	6	**87.4**	**120.0**	**104.0**
Glycero-Coca's	Neat	7-8	0.3	0.0	0.0
	1/10	7	**50.0**	**60.6**	**68.0**
	1/50	7	**69.6**	**88.0**	**58.0**
	1/100	6	**84.0**	**105.4**	**76.0**
Modified Glycero-Coca's	Neat	8-9	0.24	0.0	0.0
	1/10	7-8	**52.0**	48.0	46.0
	1/50	6-7	**86.0**	**103.2**	**72.0**
	1/100	6	**88.0**	**106.4**	**68.0**

Spike Recoveries of Single Components

Solution	Concentration (%)	pH	% Spike Recovery		
			5 EU/mL	0.5 EU/mL	0.05 EU/mL
Glycerin	50	7.3	0.0	0.0	0.0
	10	6.4	41	48	28
	5	**6.3**	**55**	**60**	**56**
Phenol	0.4	**6.0**	**107**	**102**	**74**
	0.2	**6.1**	**76**	**102**	**76**

Note: Spike recoveries at pH levels of 4 – 10 (50-150%) were within FDA acceptance criteria

Results (III)

- Endotoxins were detected in all extracts, with the exception of one *D. pteronyssinus* and five fungal extracts.
- The greatest endotoxin concentrations were detected in *D. farinae* extracts; the least in fungal extracts. Endotoxin levels detected in in vivo testing/diagnosis extracts are tabulated below.

Source	EU/mL			
	N	Median	25th Percentile	75th Percentile
D. pteronyssinus	6	6.3	1.7	13.3
D. farinae	6	1,424	1,002	2,530
Pollen	31	198.4	98.8	1,535
Fungi	11	1.2	0.5	8.0
Foods	5	87.8	53.2	555.8

Conclusions

- This study optimized endotoxin testing in allergen extracts and measured endotoxin content in various types of extracts manufactured at Greer Laboratories, Inc.
- Endotoxins were detected in most of allergen extracts - the greatest concentrations in *D. farinae* and the least in fungi.
- Greer Laboratories, Inc. will continue its studies of endotoxin testing to obtain additional data on the concentrations of endotoxins in allergenic extracts and provide specific custom formulations. The resulting information will be released.

Clinical Relevance

Although extracts are typically diluted ten-fold to provide maintenance concentrations, injection of 0.5 mL or even a full-strenght extract to a patient would generally deliver endotoxin doses ranging from 0.6 EU (fungi) to 712 EU (*D.farinae*). A mix of the five extract types tested in this study would deliver 172 EU.

The proposed FDA limit for intravenous endotoxin exposure in humans to elicit symptoms is 40-50 EU/kg (2,800-3,500 EU for a 70-kg person), greater than the endotoxin levels detected in the allergenic extracts for testing/diagnosis tested in this study.