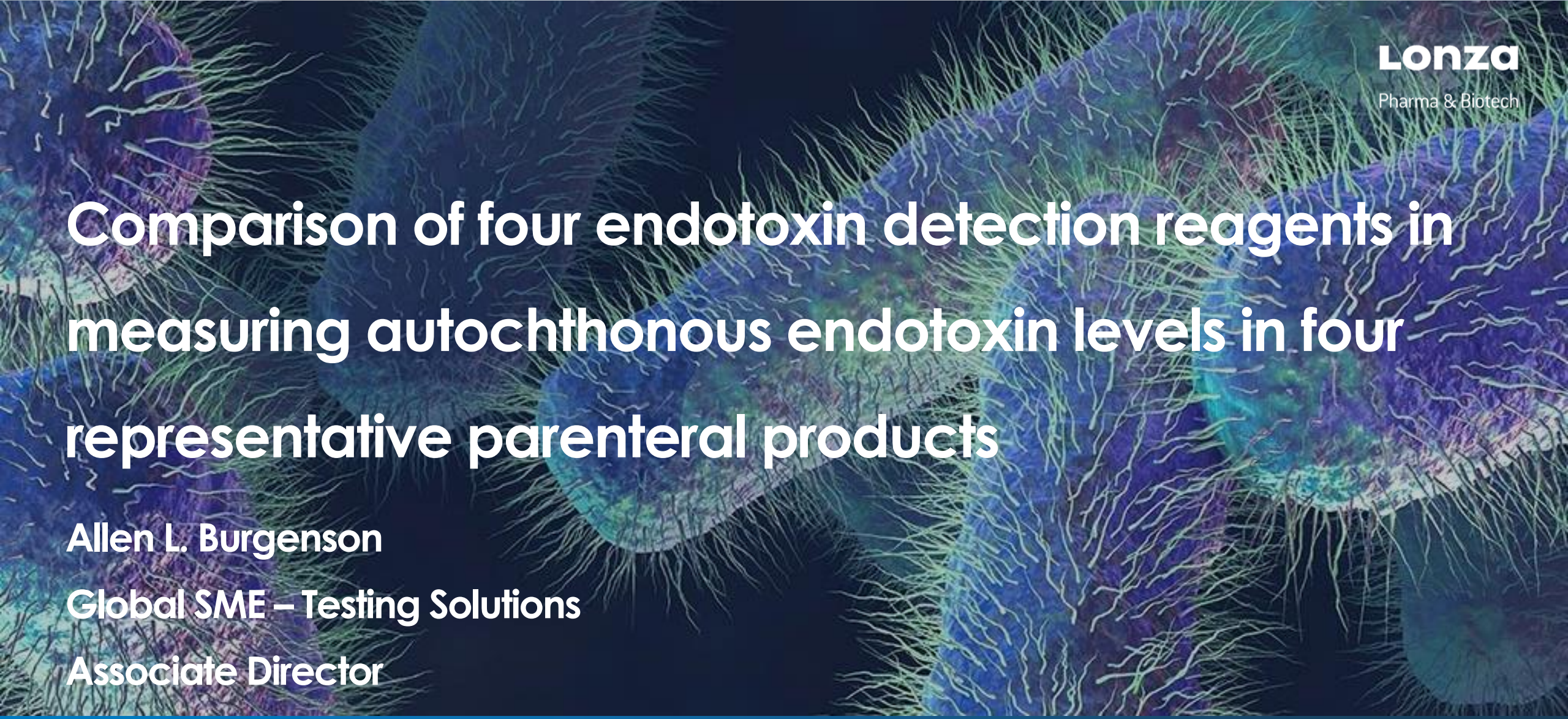

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Comparison of four endotoxin detection reagents in measuring autochthonous endotoxin levels in four representative parenteral products

Allen L. Burgenson
Global SME – Testing Solutions
Associate Director

Introduction

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- The following is a presentation of the data obtained by execution of a protocol containing the tasks requested by the United States Pharmacopeia (USP) as per USP <1085.1> USE OF RECOMBINANT REAGENTS IN THE BACTERIAL ENDOTOXINS TEST—PHOTOMETRIC AND FLUOROMETRIC METHODS USING RECOMBINANTLY DERIVED REAGENTS, to develop the data set required for inclusion of the recombinant Factor C (rFC) assay in that Compendia

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- Four products proposed by EDQM for inclusion in studies were contained in the protocol.
  EDQM ultimately did not carry out the proposed studies.

Background for the study

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- When water systems work as designed, they work very well

- However, when they break, they don't
  - Lack of appropriate maintenance and repairs
  - Wear and tear on critical components
  - Inadequate sanitization after repairs

- Hazard Analysis and Critical Control Points (HACCP)
  - A step at which control can be applied and is essential to prevent or eliminate a safety hazard or reduce it to an acceptable level

Background for study

Potential contamination of products due to contamination of the water system

Case studies

01 Leaking valve in production
Improper/incomplete repair of a valve causing contamination of the water system

02 Malfunctioning heat exchanger in WFI loop
Malfunction (pinholes) in a heat exchanger using mains water to cool WFI loop

03 Improper come-up from shutdown
Improper completion of maintenance operations after shutdown. Additional tasks performed outside of the scheduled maintenance tasks

Tim Sandle, PhD, *Bacterial endotoxin contamination of water systems*. (2019) *www.pharmamicroresources.com*

Background for study

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In each of the case studies cited by Sandle, it was *autochthonous* endotoxin from *autochthonous* microorganisms causing the endotoxin contamination of the water systems

autochthonous

adjective au·toch·tho·nous | \ ȯ-ˈtäk-thə-nəs \

Definition of *autochthonous*

01 INDIGENOUS, NATIVE an *autochthonous* people, *autochthonous* plants

02 formed or originating in the place where found *autochthonous* rock, an *autochthonous* infection

Such contamination is not uncommon in industry, and we must be able to detect it in our CCP samples

Relevant guidance

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- USP General Notices 6.30: The alternative method or procedure must be fully validated (see *Validation of Compendial Procedures* ⟨1225⟩) and must produce comparable results to the compendial method or procedure within allowable limits established on a case-by-case basis.

- FDA 2012 Guidance: Firms may use alternative methods and/or procedures if they provide advantages in terms of accuracy, sensitivity, precision, selectivity, or adaptability to automation or computerized data reduction, and in other special circumstances. Such alternative procedures and methods should be validated as described in the USP General Chapter and should be shown to achieve equivalent or better results.

- USP <1225>: Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for the intended analytical applications

Relevant guidance

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FDA Guidance for Industry: Analytical Procedures and Methods Validation for Drugs and Biologics.

- The new method coupled with any additional control measures is equivalent or superior to the original method for the intended purpose (comparability)

- The new analytical procedure is not more susceptible to matrix effects than the original procedure (suitability). Note: Products have specific, calculated endotoxin limits and associated MVDs. The alternative test may have different interference properties than the compendial test, but in any case, dilution may not exceed the MVD.

Getting the work done

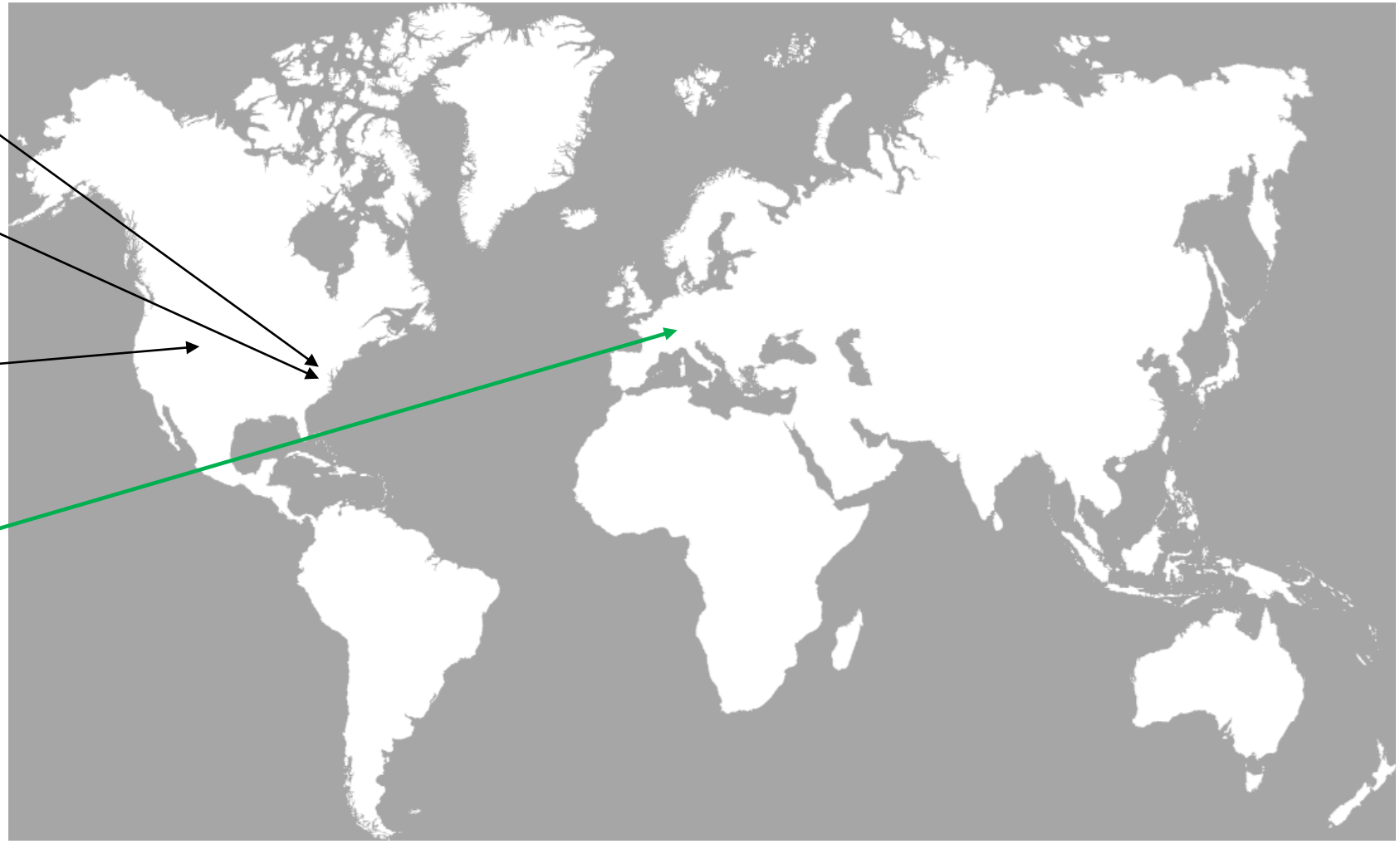
Finding a neutral 3rd party laboratory

Eurofins Lancaster Laboratories (Lancaster PA)

BioReliance (Rockville MD)

Nelson Laboratories (Salt Lake City UT)

Labor L+S (Bad Bocklet, DE)



The goals of the study

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- Is endotoxin from autochthonous bacteria detectable using LAL and recombinant methods?
- Are glucans present in the tested post carbon water sample?
- Can endotoxin be measured in final products using LAL and recombinant methods?
- Are there differences between the methods?

Products tested

As per the original EP ring trial proposal

- Acyclovir IV
- Gentamicin IV
- Insulin
- IV Saline

- Each product was tested by spiking a water sample containing autochthonous endotoxin (at approximately the product ERL) from the Walkersville water production system
- Products tested at approximately 1/2 ERL* (where possible) and 1/20 ERL* (where possible)
- Each product tested using each endotoxin detection reagent
- Each product tested with and without beta glucan blocker

Endotoxin detection reagents, software, and hardware

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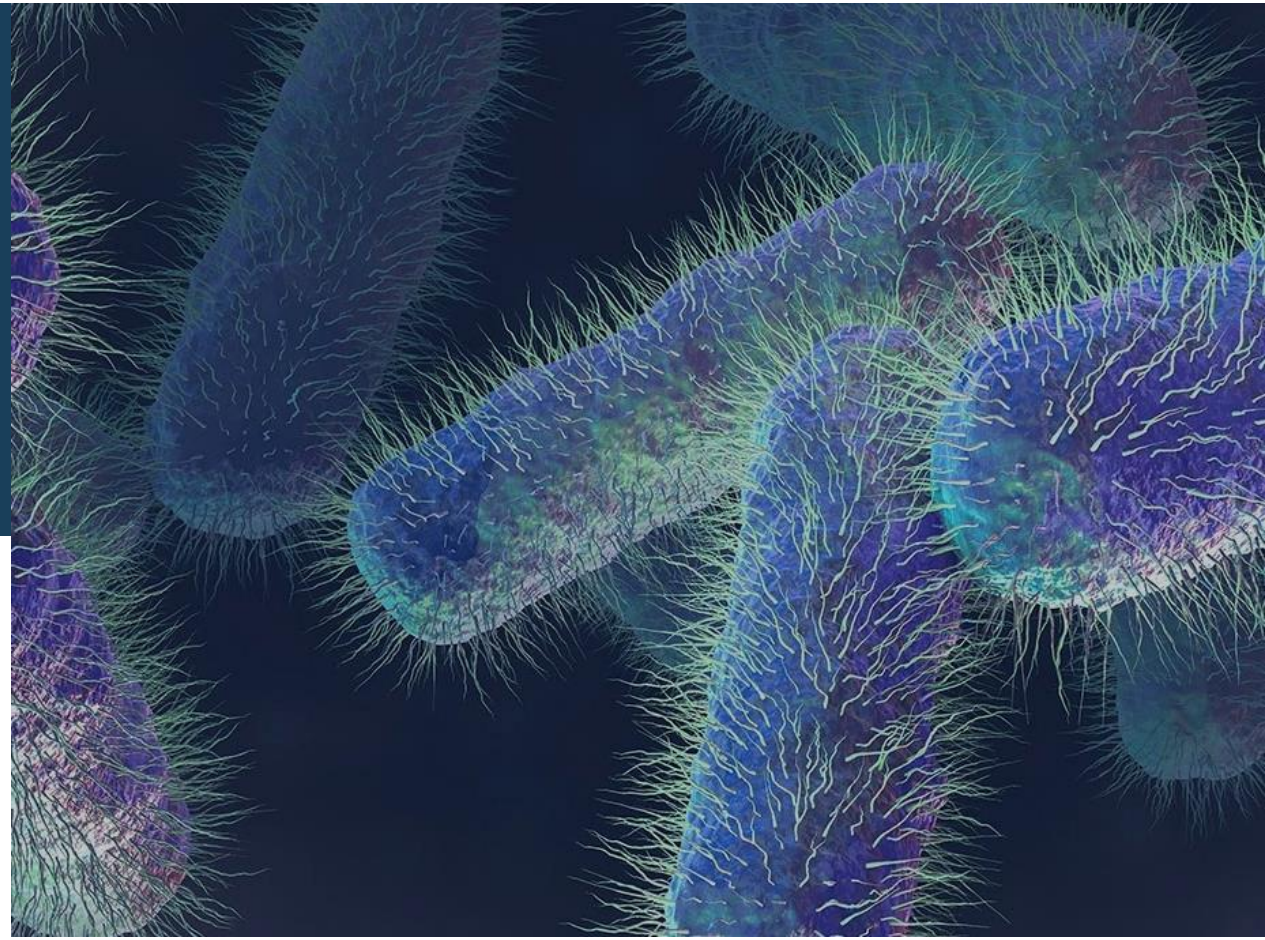
- Kinetic turbidimetric reagent
- Kinetic chromogenic reagent
- Recombinant Factor C reagent I
- Recombinant Factor C reagent II
- WinKQCL® Software on laptop
- PyroWave® Fluorescence Reader
- BioTek™ ELx808™ optical density reader



Establishing endotoxin limits

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Endotoxin limits were set based on the worst-case found in the manufacturer's package insert using the compendial formula ERL=K/M where

K = (pyrogenic threshold of 5 EU/kg)(patient weight in kg)

M = patient dose (in mL, mg, etc.) per hour

Some worst-case situations were pediatric use

Establishing endotoxin limits

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Product	Highest Dose	Dose Type	Weight Kg.	ERL	Units	Chrom MVD	rFC MVD	Turb MVD
Acyclovir	20 mg/kg IV q8hr	pediatric	40	12.5	EU/mL	2500	2500	1250
Gentamycin sulfate USP	10.5 mg/kg/day IV/IM divided q8hr	adult	70	1.42	EU/mL	284	284	142
IV Saline	0.25 EU/mL	adult	70	0.25	EU/mL	50	50	25
Insulin Regular Human - Injection	1.5 units/kg/day during puberty	pediatric	40	333.3	EU/mL	66667	66667	33333

Test protocol execution

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Post carbon bed water samples

- A water sample was taken after the activated carbon bed in the Walkersville WFI production system
  - Potential source of downstream contamination by autochthonous endotoxin or organisms
  - Simulates a breach of those downstream purification systems
  - Easy to obtain a high level of autochthonous endotoxin
- Water sample tested for endotoxin contamination levels in Walkersville
- Sample frozen for long term storage and shipment
- Sample received, thawed, and tested at contract testing lab for endotoxin levels

Test protocol execution

Post carbon bed water endotoxin content determination

- As the endotoxin and glucan values for the post carbon water sample were unknown, those levels were first determined at the contract test lab using several dilutions in a screening before use as the inoculant. The samples were tested in duplicate to determine a non-interfering dilution for further water testing within the study.

Water plate setup				Water samples (post carbon water)			
	Standard Curve			without Spike		with Spike (PPC)	
	A	B	C	D	E	F	G
1	50	50	50	undil	undil	undil	undil
2	5	5	5	1:5	1:5	1:5	1:5
3	0,5	0,5	0,5	1:10	1:10	1:10	1:10
4	0,05	0,05	0,05	1:100	1:100	1:100	1:100
5	0,005	0,005	0,005	1:1.000	1:1.000	1:1.000	1:1.000
6	Blank	Blank	Blank	1:10.000	1:10.000	1:10.000	1:10.000
7	Blank	Blank	Blank	1:100.000	1:100.000	1:100.000	1:100.000
8				1:1.000.000	1:1.000.000	1:1.000.000	1:1.000.000

Test protocol execution

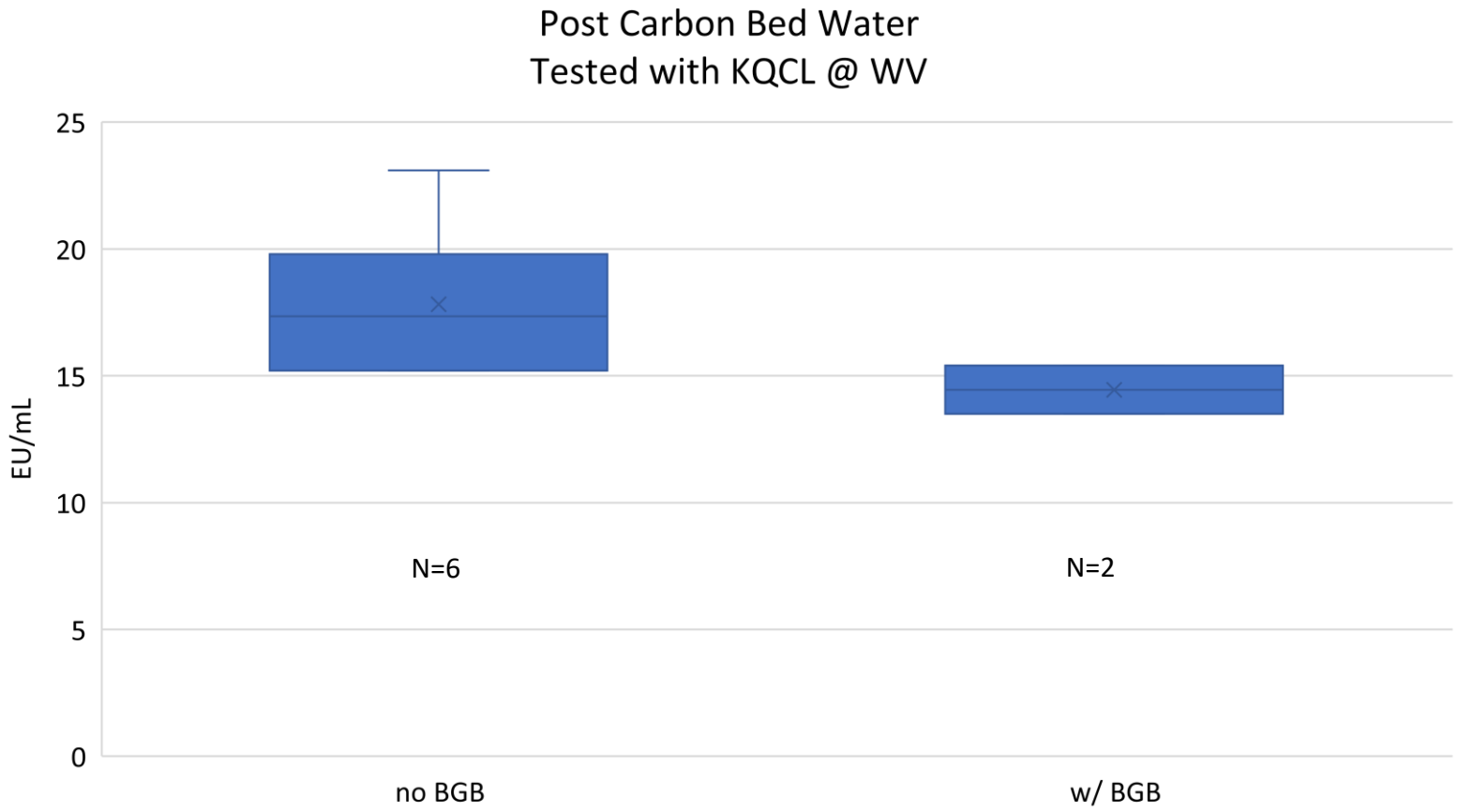
Post carbon bed water glucan content determination

Dilution factor	Detection limit [pg/ml]	Beta-Glucan [pg/ml]	PPC Recovery [%]
1:10	100	< 100	179
1:100	1.000	< 1.000	137
1:1.000	10.000	< 10.000	119
1:10.000	100.000	< 100.000	142
1:100.000	1.000.001	< 1.000.001	90

- Result: Post carbon water sample was free of detectable beta glucans

Post carbon water – pre-freezing endotoxin content

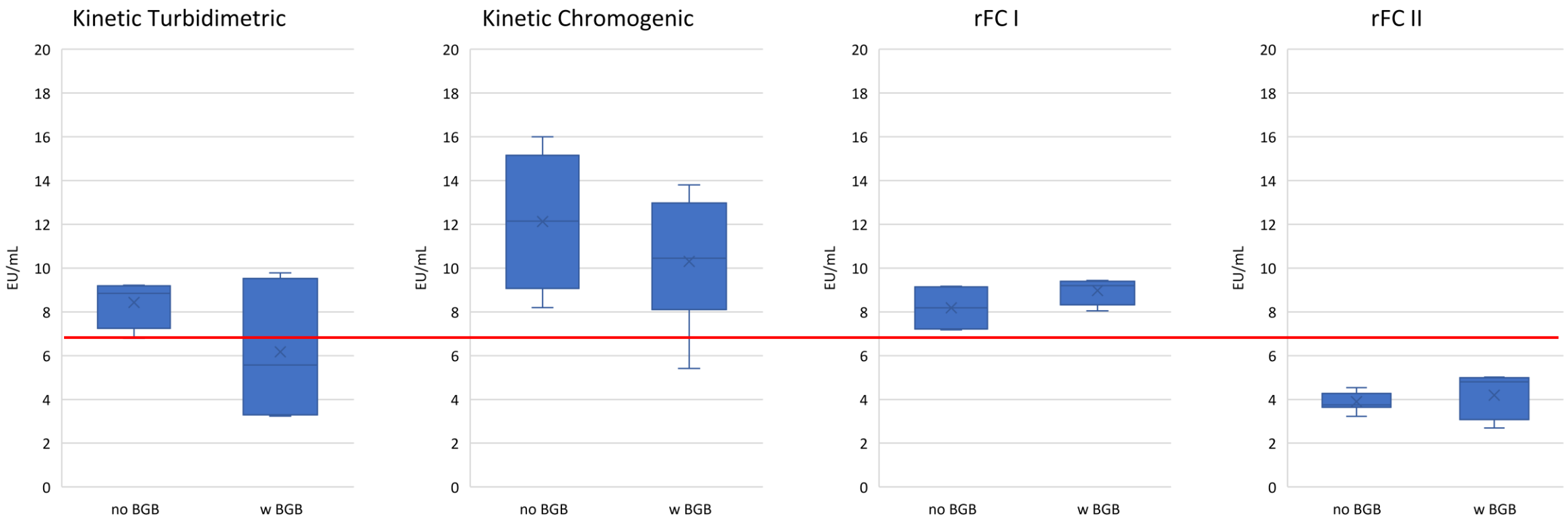
Test results, two analysts, with and without BGB PPC=5 EU/mL



Mean w/o BGB	17.8
Mean w/ BGB	14.45
Difference	3.4
Reduction	19%

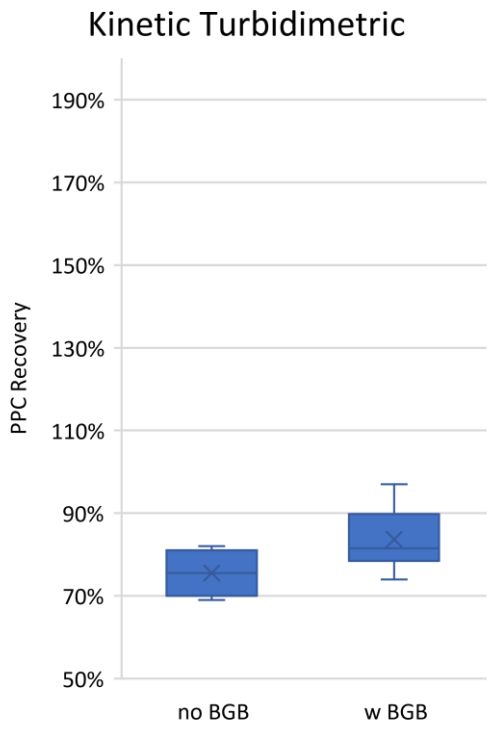
Post carbon water – post-freezing endotoxin content

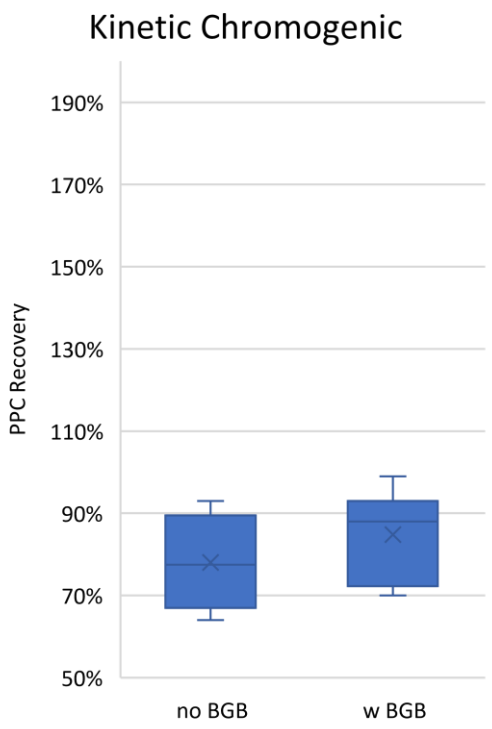
Test results, two analysts, with and without BGB PPC=0.5 EU/mL

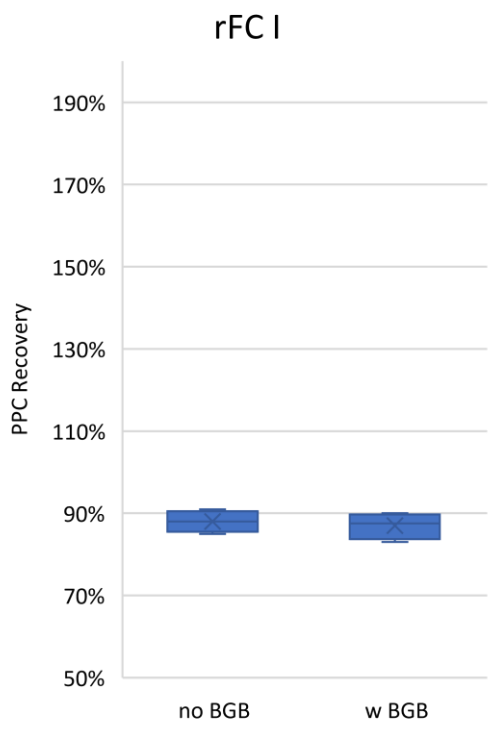


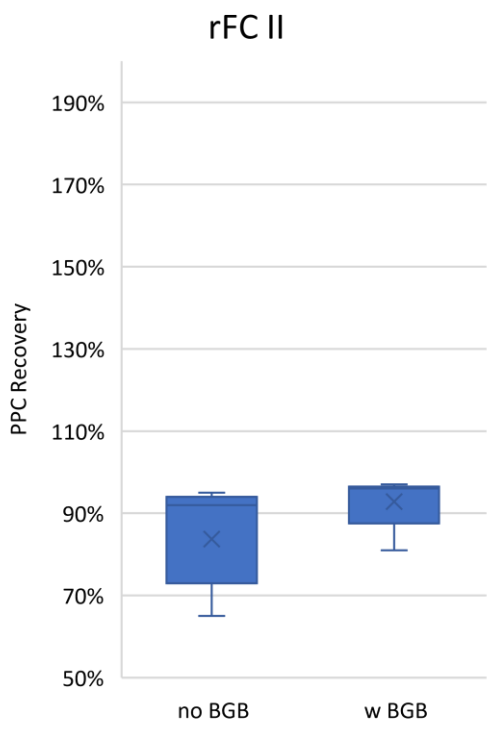
Post carbon water – post-freezing PPC recovery

Test results, two analysts, with and without BGB PPC= 0.5 EU/mL









Test protocol execution

Inoculum preparation

- Based on the arithmetic mean of the triplicate analysis, dilutions were prepared for spiking the different products on the day of contamination

- Post-carbon water was diluted with LALRW to approximately ERL and 1/10 ERL for each specific product to be tested. Each product was inoculated the day of the test. For some samples the measured endotoxin level of the post-carbon water sample was too low to prepare the spike levels (see tables below), the products were spiked at the highest level achievable.

Product	ID #	Product ERL	Proposed Spike	Actual Spike
Acyclovir	#1	12.5	6.25	3.5
Acyclovir	#1a	12.5	0.625	.35
Gentamycin	#2	1.42	0.71	0.71
Gentamycin	#2a	1.42	0.071	0.071
IV Saline	#3	0.25	0.125	0.125
IV Saline	#3a	0.25	0.0125	0.0125
Insulin	#4	333	166.6	3.5
Insulin	#4a	333	16.6	0.35

Protocol execution

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- Each product was tested using multiple dilutions from both the “ERL*” and “1/10 ERL*”
- Concentrations in duplicate, plus duplicate PPCs at that concentration
- The tests were then repeated using beta glucan blocker

Depending on dilution value from section 5.3, if dilution factor ≤1:100

	Plate setup per product (KQCL, PG, EZ II)			Product spike at ERL				Product spike at 1/10 ERL				Post Carbon
	Standard Curve			Product		Product + PPC		Product		Product + PPC		Water
	A	B	C	D	E	F	G	H	I	J	K	L
1	50	50	50	undil	undil	undil	undil	undil	undil	undil	undil	undil (U)
2	5	5	5	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	undil (U)
3	0,5	0,5	0,5	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5	U+PPC
4	0,05	0,05	0,05	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10	U+PPC
5	0,005	0,005	0,005	1:50	1:50	1:50	1:50	1:50	1:50	1:50	1:50	*dil.5.3**
6	Blank	Blank	Blank	1:100	1:100	1:100	1:100	1:100	1:100	1:100	1:100	*dil.5.3**
7	Blank	Blank	Blank	1:1.000	1:1.000	1:1.000	1:1.000	1:1.000	1:1.000	1:1.000	1:1.000	*dil.5.3*+PPC*
8	Blank	Blank	Blank	1:10.000	1:10.000	1:10.000	1:10.000	1:10.000	1:10.000	1:10.000	1:10.000	*dil.5.3*+PPC*

*dilution determined in section 5.3

- *actually 1/2 and 1/20 ERL

Criteria for inclusion of results

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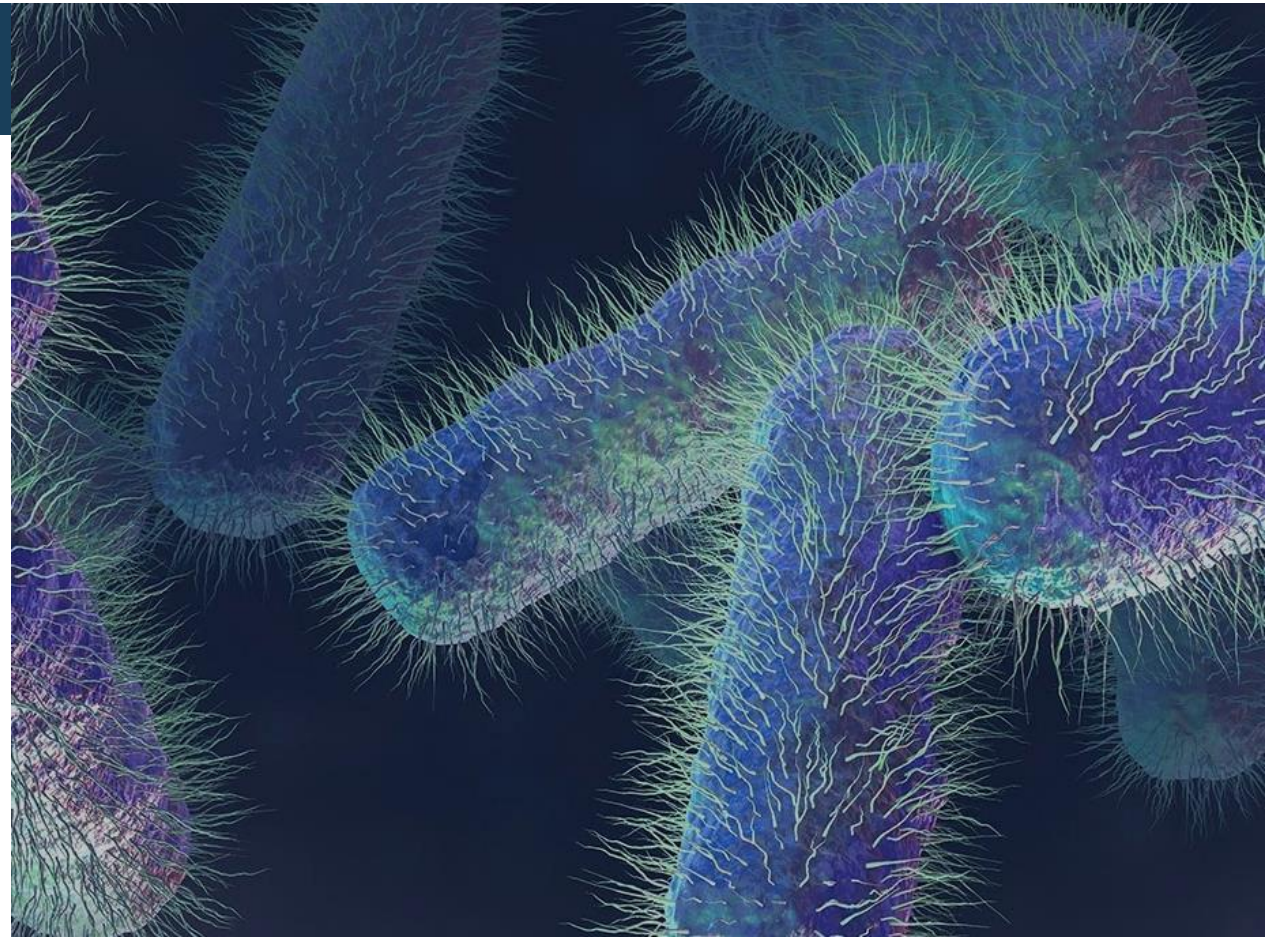
All included samples must have:

- a PPC recovery between 50 and 200%
- a measurable endotoxin level at that dilution

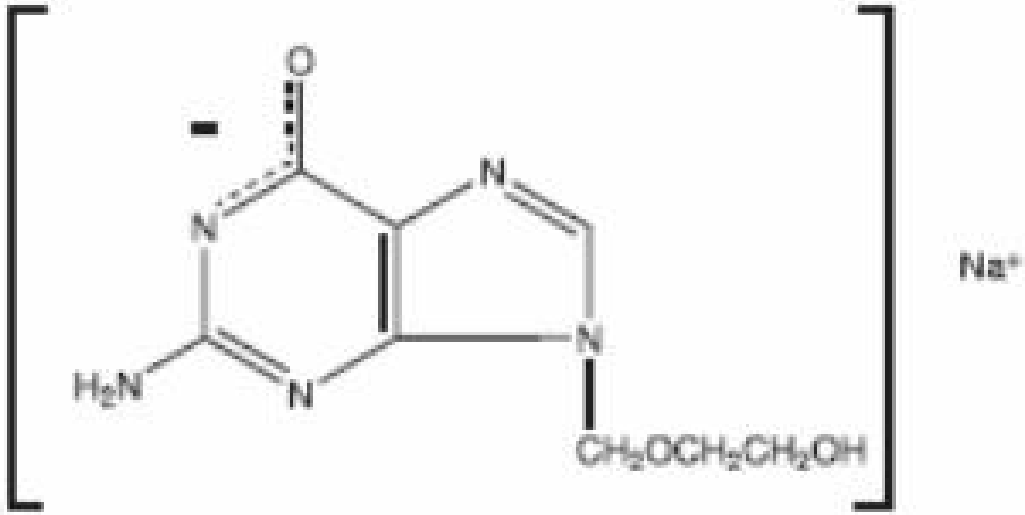


Acyclovir

ERL = 12.5 EU/mL

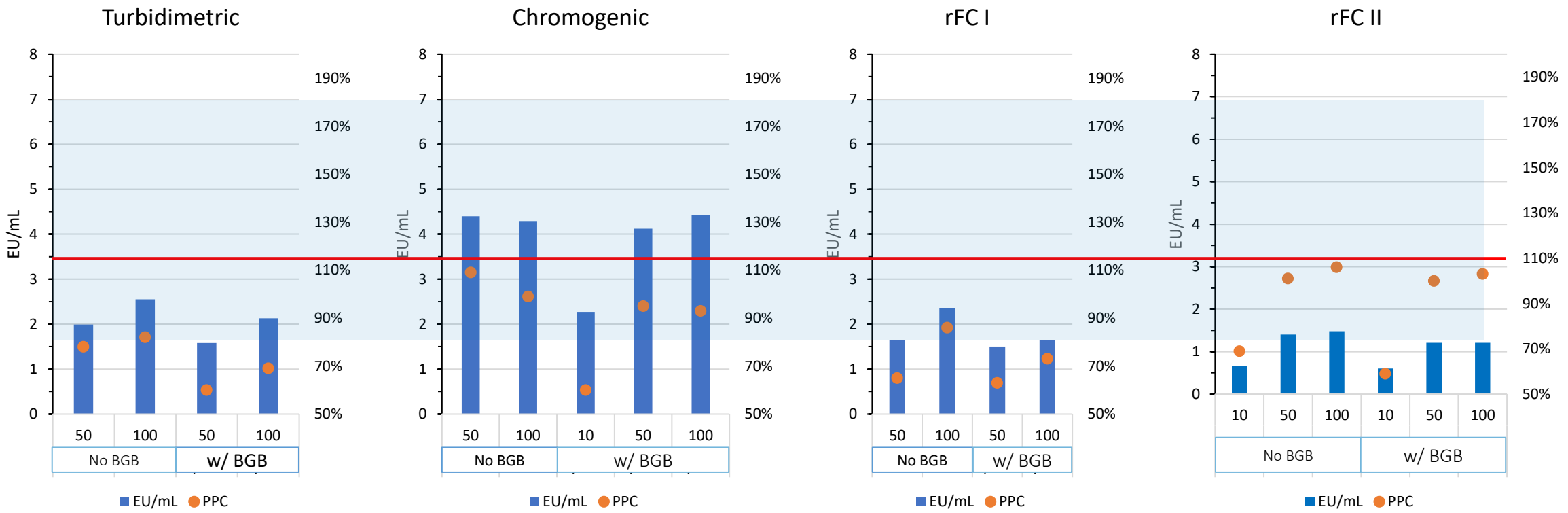
- Acyclovir is an antiviral drug active against herpes viruses. Acyclovir Injection is a formulation for intravenous administration. Acyclovir Injection is a sterile solution containing acyclovir 25 mg/mL. Acyclovir Injection is available in 20 mL and 40 mL vials, with each mL containing acyclovir sodium equivalent to 25 mg acyclovir.

- The chemical name of acyclovir is 9-[(2-Hydroxyethoxy)methyl]guanine sodium. The molecular formula of acyclovir is C8H10N5O3·Na and it has the following structural formula:



Acyclovir

ERL = 12.5 EU/mL Spiked at 3.5 EU/mL MVD=1:2,500

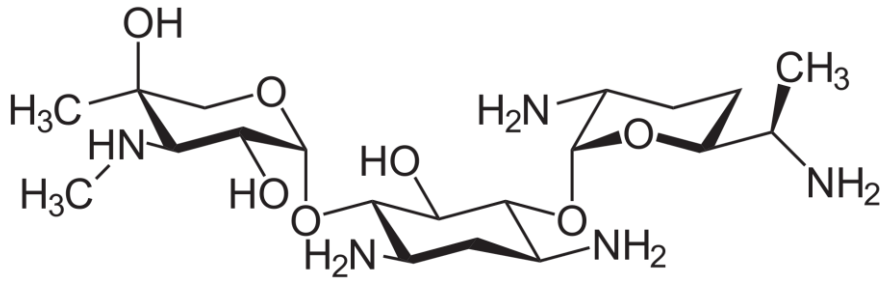


Gentamicin

ERL = 1.42 EU/mL

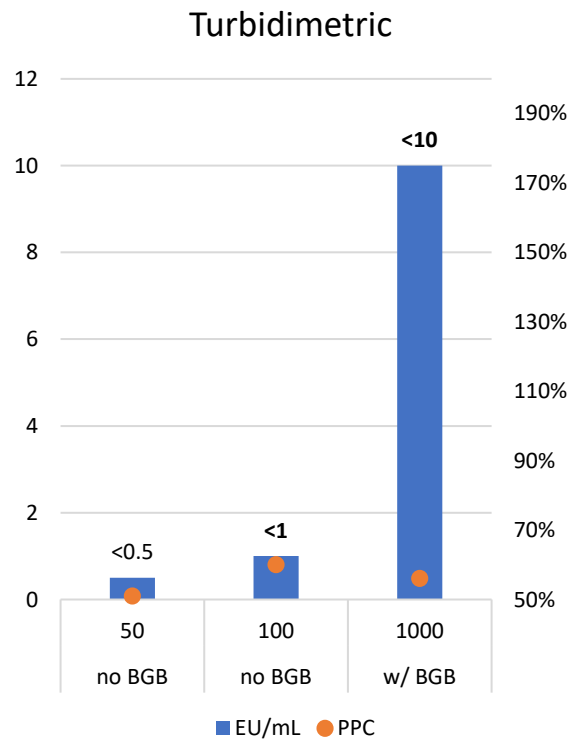
- Gentamicin injection is used to treat certain serious infections that are caused by bacteria such as meningitis (infection of the membranes that surround the brain and spinal cord) and infections of the blood, abdomen (stomach area), lungs, skin, bones, joints, and urinary tract. Gentamicin injection is in a class of medications called aminoglycoside antibiotics. It works by killing bacteria.

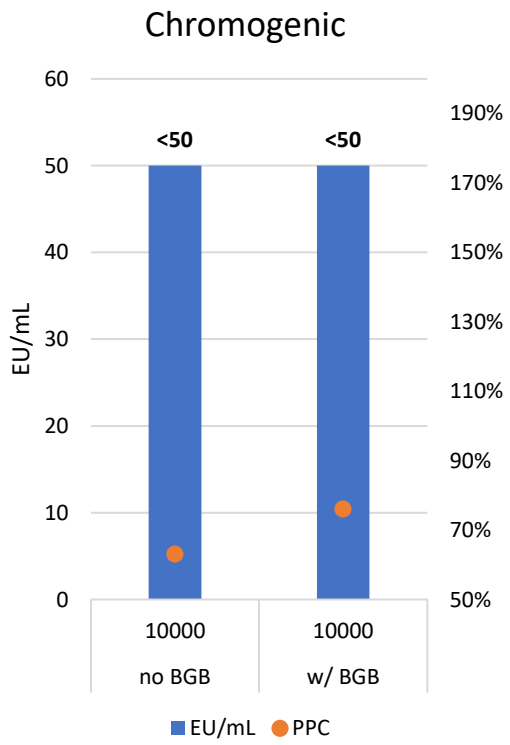
- Gentamicin has the following structural formula:

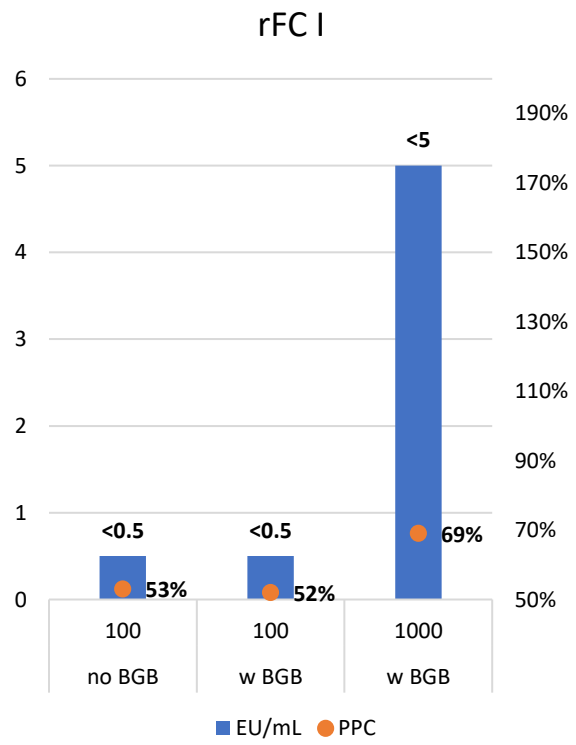


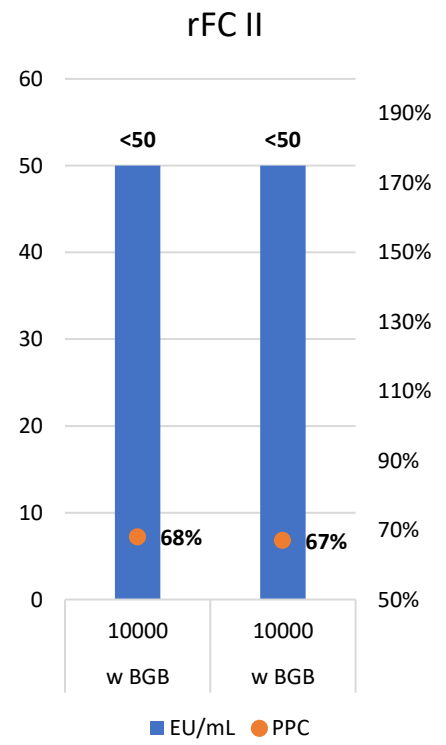
Gentamicin

ERL = 1.42 EU/mL Spiked at 0.7 EU/mL MVD=1:284 (1:142)









Insulin

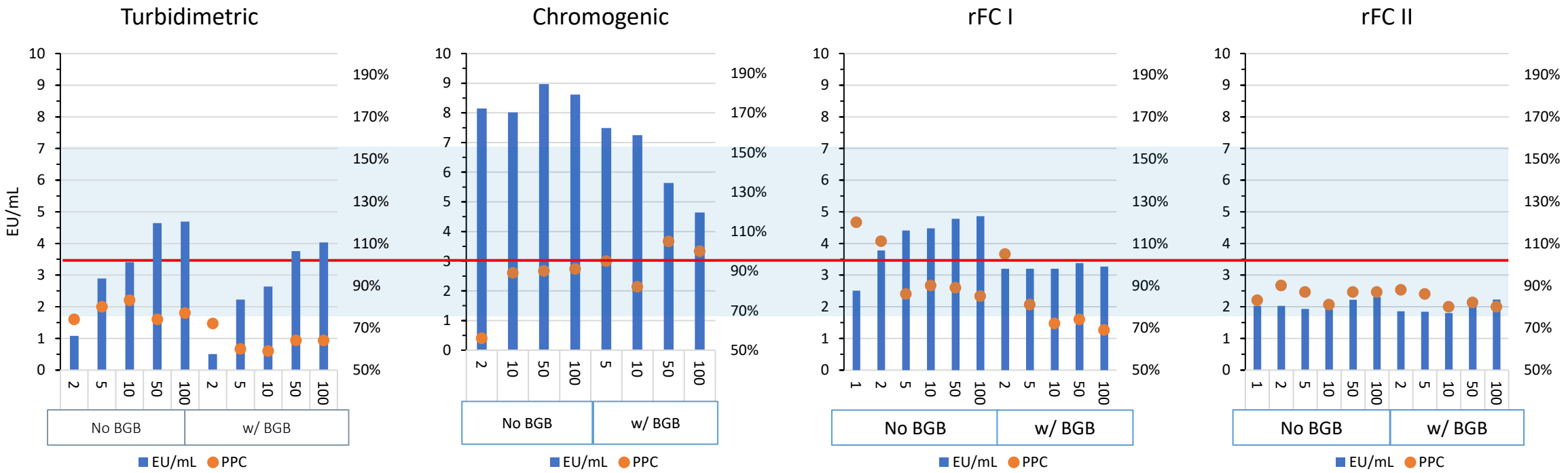
ERL = 330 EU/mL

- Human insulin is used to control blood sugar in people who have type 1 diabetes (condition in which the body does not make insulin and therefore cannot control the amount of sugar in the blood) or in people who have type 2 diabetes (condition in which the blood sugar is too high because the body does not produce or use insulin normally) that cannot be controlled with oral medications alone.

- Human insulin is in a class of medications called hormones.

Insulin

ERL = 330 EU/mL Spiked at 3.5 EU/mL MVD=1:66,000 (1:33,000)



IV Saline

ERL = 0.5 EU/mL

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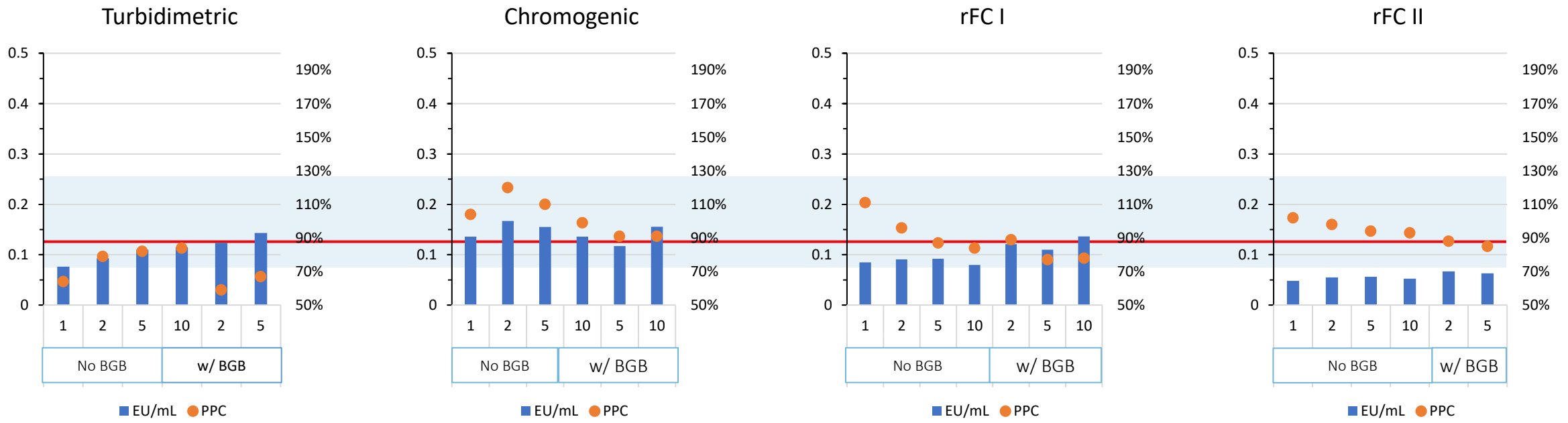
- Normal saline is a cornerstone of intravenous solutions commonly used in the clinical setting. It is a crystalloid fluid administered via an intravenous solution. Its indications include both adult and pediatric populations as sources of hydration and electrolyte disturbances. It can come in various concentrations; the two specifically addressed are 0.9% and 0.45%.

- 0.9% Normal Saline

- An isotonic concentration of sodium chloride is best suited for parenteral replacement of chloride losses that exceed or equal the sodium loss. Within each 100 mL of 0.9% sodium chloride Injection USP, there is 15.4 mEq of sodium ions and 15.4 mEq of chloride ions. Additionally, the osmolarity is 308 mOsmol/liter, and it has a pH range of 4.5 to 7.[1]

IV Saline

ERL = 0.5 EU/mL Spiked at 0.125 EU/mL MVD=1:100 (1:50)



Summary

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Endotoxin from autochthonous bacteria in post carbon water

- The analyzed post carbon bed water contains low levels of endotoxin (~7 EU/mL)
- The analyzed post carbon bed water contains levels of beta glucans below LOD (<100 pg/mL)
- Endotoxins from autochthonous organisms could be detected with all methods (LAL and rFC) in post carbon water

Endotoxin from autochthonous bacteria in tested products

- Products contaminated with endotoxins from autochthonous bacteria could be detected with all methods
- “The choice of reagent depends on the product!”

Acknowledgements

- Candice Stumbaugh
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Thank you

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