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Resolving Test Interference in Detection of Endotoxin's in 3rd Generation Cephalosporin Drug

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Abstract: The effect of varying the pH and ionic strength in the raw materials used in the finished product, 3rd generation Cephalosporin drug Cefepime for Injection was investigated. The interfering factors inhibiting the endotoxins in the raw material Cefepime hydrochloride and L-Arginine while quantifying with Limulus Amoebocyte Lysate are sorted out by neutralizing the ionic concentration in the raw materials using alkali/ acid and validated the final product along with its raw materials. This technique can be used for other cephalosporin group and parenteral drugs to overcome the interference.

Keywords: Interference, Limulus Amoebocyte Lysate (LAL), Control Standard Endotoxin (CSE).

Introduction

Bacterial endotoxin is one of the most potent activator of mammalian immune system. In general, as per the United State Pharmacopeias (USP) the threshold pyrogenic dose is 5 EU/kg/hr for parenteral drugs and 0.2 EU/kg/hr for intrathecal drugs (5). When endotoxin enters into human blood these toxins induces white blood cells (WBC) to release cytokines, such as tissue necrosis factor (TNF), interleukin-1 and interleukin-8, which mediate a complex biological response including pyrogensity, shock, coagulation and inflammation (5,4&7). Gram negative bacterial outer membrane Lipopolysacchride (LPS) induces a cascade of defense mechanism that is known as fever and inflammation (2). So it is mandatory to check the presence of endotoxin level in parenteral drugs before releasing the product into market.

The LAL reaction with endotoxin requires pH neutrality and optimum levels of Na+ and divalent cations. A uniform temperature of 37° C optimizes the rate of reaction. Most therapeutic drug products requires dilution with LAL reagent water (LRW) before testing to avoid interference, where inhibition is failure to recover the positive control, and enhancement is excess recovery. There are 3 principle causes of invalid or inhibitory results in gel clot testing are 1. Loss of purified Endotoxin used for product positive controls (PPC). 2. Adverse chemical conditions such as non-neutral pH or sub optimal levels of sodium ions and divalent cations (Mg++ and Ca++). 3. Inadequate controlled test parameters including testing accessories, reagents and analyst proficiency.

The aim of the study is to sort out the interfering factors which lead to the diverse results in finished and raw materials of Cefepime for injection. The false positive results may cause severe complication in the patients as discussed in literature.

Materials and Methods

Lyophilized Limulus Amoebocyte Lysate of 0.125 sensitivity Lot# A4121X (LAL), control standard endotoxin 5 Eu/ng Lot# EX83372 (CSE), LAL reagent water Lot# 99732080 (LRW) of Endosafe US, Depyrogenated (250°C for 30 min)10 X 75 mm assay tubes, 16X100 mm dilution tubes ,pyrogen free Micropipette tips, vortex mixture, 1N NaoH, 1N HCL, Cefepime HCL (Sterile), L-Arginine (Sterile) are Cefepime for Injection were used for determination of Endotoxin content by the gel clot technique.

The sensitivity of the Lysate (labeled 0.125 Eu/mL) was determined by using known amount of E.coli control standard endotoxin.

In the gel-clot techniques, the reaction end point is determined from dilutions of the material under test in

direct comparison with parallel dilutions or a reference endotoxin, and quantities of endotoxins are expressed in Endotoxin units.

1. Preparation of Standard stock solution and standard solutions: The CSE having a defined potency of 50 EU/Vial was reconstituted with 5ml of LRW and mixed intermittently for 30 minutes using a vortex mixture and this concentrate was used to prepare 2λ, λ, λ/2 & λ/4, where λ is the labeled claim sensitivity of Lysate.

2. Preparation of sample solution: Test samples were diluted to the required concentrations based on the formulae MVD. MVD is the maximum valid dilution, which is allowable dilution of the specimen at which the endotoxin limit can be determined. The general equation to determine MVD is

MVD = (Endotoxin limit X Concentration of sample solution)/ (λ). Where E.L is the endotoxin limit of the test sample, which is specified in the individual monograph in terms of volume or units of active drug (in EU/mg).

3. Cefepime Hcl sterile sample preparation: Batch No: CHS-0101, Potency=100mg/mL , E.L=0.04 Eu/mg , Lysate sensitivity is 0.125 Eu/mL and MVD = 32.The following test dilutions are prepared by 1:32 (3.125 mg/mL), 1:16 (6.25 mg/mL), 1:8 (12.5 mg/mL), 1:4 (25 mg/mL) & 1:2 (50 mg/mL).

4. L-Arginine sterile sample preparation: Batch No:LAS-0102, Potency=100mg/mL , E.L=0.03 Eu/mg , Lysate sensitivity is 0.125 Eu/mL and MVD = 24.The following test dilutions are prepared by 1:24 (4.16 mg/mL), 1:12 (8.33 mg/mL), 1:6 (16.66 mg/mL), 1:3 (33.33 mg/mL) & 1:1.5 (66.66 mg/mL)

5. Cefepime for Injection sample preparation: Batch CPI -0103, Potency=100mg/mL , E.L=0.06 Eu/mg , Lysate sensitivity is 0.125 Eu/mL and MVD = 48. The following test dilutions are prepared by 1:48 (2.08 mg/mL), 1:24 (4.16 mg/mL), 1:12 (8.33 mg/mL), 1:6 (16.66 mg/mL) & 1:3 (33.33 mg/mL.

Cefepime for injection will be prepared by blending Cefepime hydrochloride with L-Arginine. Cefepime hydrochloride is acidic and L-Arginine is basic in pH, while the finished product Cefepime for Injection pH is 4-6.

Method: Equal volume of test sample and LAL reagent is added in a depyrogenated test tube of 10 X 75 mm and incubate this mixture at 37± 1°C for 60±2 min. Then invert the tube by 180° and look for gel formation. If a gel inside the test tube is able to maintain its integrity after inverting the tube to 180° then it is a positive reaction which indicates presence of Endotoxin in the sample greater than the limit. Other than this any condition is considered as negative which indicates absence of endotoxin in the sample (lesser than the lysate sensitivity).

Product Testing: For testing products equal volume of drug (sample) and LAL reagent is taken and following tubes are prepared (6)

Negative Product Control (NPC) - Sample + LAL

Positive Product Control (PPC) - Sample + CSE (2λ) + LAL

Negative Water Control (NWC) - LRW + LAL

Positive Water Control (PWC) - LRW + CSE (2λ) + LAL

Majority of times it has been a common observation that if a product is tested directly it inhibits the LAL test and thus shows interference (1 & 3).

Interference: Interference is defined as a significant difference between the end points of positive water control and positive product control using standard endotoxin.

This interference could be either inhibition wherein the recovery of endotoxin is below than the expected or enhancement wherein the recovery of endotoxin is higher than expected

Product Validation: Product needs to be validated before start for routine testing. Validation is a test condition where an endotoxin standard is detected with the same efficiency in a test sample as it is in LRW. This validation study consists of two different phases wherein in Phase I (Preliminary screening) involve interference testing and Phase II consists of validation of product.

Significance of product validation is that it gives information on whether there are any interfering factors in the drug product to the LAL test and also it gives an idea of the approximate levels of endotoxin content in the drug product. It also covers manufacturing of product and formulation of the product.

It is always advisable to carry out revalidation if product formulation is changed and which is likely to affect the interference pattern of the product for LAL test. Also revalidation is to be conducted for any product if there is any change in manufacturing procedures or in vendor.

Phase I: Preliminary Screening / interference Study (9) : In this two identical series of product dilutions (two-fold dilutions), one spiked with 2λ, and one left unspiked. The result of Phase I will tell you the non-interfering dilution (NID) of the product, which is used for the actual validation (Phase II). The non-interfering dilution (NID) is the first set of PPC that shows a gel.

Results and discussions

Cefepime Hydrochloride:

Sample Dilution	1:2	1:4	1:8	1:16	1:32
Unspiked	--	--	--	--	--
Spiked	--	--	--	--	--

Table: 1

L-Arginine

Sample Dilution	1:1.5	1:3	1:6	1:12	1:24
Unspiked	--	--	--	--	--
Spiked	--	--	--	--	--

Table: 2

This assay shows that there is inhibition up to 1:32 (MVD) in Cefepime Hydrochloride and 1:24 (MVD) in L-Arginine. Due to Inhibition LAL is unable to detect the endotoxins even in spiked sample After analyzing the sample using different procedures, finally In order to sort out this inhibition problem the acidic pH of the Cefepime Hydrochloride (1.5 – 2.7) is adjusted to 6-8 with 1N NaoH and the basic pH of L-Arginine (11 – 10.2) is adjusted to 6-8 with 1 N Hcl and both the samples.

This assay shows no inhibition upto 1:2 dilution in Cefepime Hydrochloride, 1:1.5 in L-Arginine and the spike recovery at 1:2 and 1:1.5 dilutions onwards. Therefore the NID is 1: 4 (Cefepime hydrochloride), 1:3 (L-Arginine) and 1:6 (Cefepime for Injection). It is advisable to validate the product at not less than MVD/4 to take care of any batch to batch variation during regular production. So MVD/4 dilution is chosen for product validation.

Phase II: Validation of Product

For validation, test and compare two identical series of endotoxin dilutions bracketingλ; One prepared in LRW and another prepared in product diluted to the proposed test dilution. Here dilution selected for validation is 1:4. (Hot spike method).

Cefepime Hydrochloride: (Results after adjusting the Acidic pH to the range of 6-8 with 1 N NaoH).

Sample Dilution	1:2	1:4	1:8	1:16	1:32
Unspiked	++	++	--	--	--
Spiked	++	++	++	++	++

Table: 3

L-Arginine (Results after adjusting the Basic pH to the range of 6-8 with 1 N Hcl)

Sample Dilution	1:1.5	1:3	1:6	1:12	1:24
Unspiked	++	++	--	--	--
Spiked	++	++	++	++	++

Table: 4

Cefepime for Injection:

Sample Dilution	1:3	1:6	1:12	1:24	1:48
Unspiked	--	++	--	--	--
Spiked	++	++	++	++	++

Table: 5

Example of results:

Endotoxin/product												
Replicates	**Cefepime Hydrochloride**				**L-Arginine**				**Cefepime for Injection**			
	0.25 Eu/mL	0.125 Eu/mL	0.0625 Eu/mL	0.0312 Eu/mL	0.25 Eu/mL	0.125 Eu/mL	0.0625 Eu/mL	0.0312 Eu/mL	0.25 Eu/mL	0.125 Eu/mL	0.0625 Eu/mL	0.0312 Eu/mL
1	+	+	-	-	+	+	-	-	+	+	-	-
2	+	+	-	-	+	+	-	-	+	+	-	-
3	+	+	-	-	+	+	-	-	+	+	-	-
4	+	+	-	-	+	+	-	-	+	+	-	-

Table: 6

Negative product control: --; Geometric Mean = 0.125 EU/ml

Endotoxin/ LRW				
Replicates	0.125 Eu/mL	0.0625 Eu/mL	0.0312 Eu/mL	0.0156 Eu/mL
1	+	+	-	-
2	+	+	-	-
3	+	+	-	-
4	+	+	-	-

Table: 7

Blank: --; Geometric Mean = 0.125 EU/ml

Successful validation requires that both series confirm label claim (Geometric mean) within +/- one two-fold dilution. Validation is conducted at this dilution on three batches of product.

BET Applications:

Large Volume Parenterals (LVPs), Multiple - ingredient drugs, Small Volume Parenterals (SVPs), Radiopharmaceuticals, Biologicals, Water system validation, Validation of Dry heat Sterilizer and Medical devices(8).

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