

Determination of NID factor in cephalosporin antibiotics

Ramesh Pennamareddy¹, K. Prabakar¹ and J. Pandiyan²

¹P.G. Department of Zoology, Jamal Mohamed College (Autonomous), Tiruchirappalli;

²P.G. Research Dept. of Zoology & Wildlife Biology, AVC College (Autonomous), Mannampandal-609 305, TN, India
bacterialendotoxin@gmail.com

Abstract: Cephalosporin antibiotics are the most widely-used as life saving drugs. The drug is subjected to bacterial endotoxins test before releasing into market but due to interfering factors in most of the cases there is a probability of getting false positive results. Hence, non-interfering dilution (NID) to be selected before validating the product. Single product from 1st to 4th generation cephalosporins were selected in this study and the NID factor among those Cephalosporin antibiotics is found between \pm two fold.

Keywords: Cephalosporin antibiotics, endotoxin test, non-interfering dilution (NID).

Introduction

Bacterial endotoxin is one of the most potent activator of mammalian immune system. In general, as per the United State Pharmacopeias (USP,1999) the threshold pyrogenic dose is 5 EU/kg/hr for parenteral drugs and 0.2 EU/kg/hr for intrathecal drugs (Mc Closky *et al.*, 1943). 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 pyrogenicity, shock, coagulation and inflammation (Bang, 1956; Atherton & Furr, 2009). Gram negative bacterial outer membrane lipopolysacchride (LPS) induces a cascade of defense mechanism that is known as fever and inflammation (Good & Lane, 1977). So it is mandatory to check the presence of endotoxin level in parenteral drugs before releasing the product into market.

The LAL reaction (Michael *et al.*, 2000) 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 Cephalosporin 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 evaluate the NID factor in different Cephalosporin antibiotics (1st to 4th Generation) before validating the product to avoid false positive

results, which may cause severe complication in the patients as discussed in literature.

Materials and methods

Materials

Lyophilized Limulus Amoebocyte Lysate of 0.125sensitivity (LAL), control standard endotoxin (CSE) 5 Eu/ng, LAL reagent water (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, Cephalothin for Injection, Cefuroxime Sodium, Ceftriaxone Sodium and Cefpirome sulphate for Injection were used for determination of NID by gel clot technique. The sensitivity of the Lysate (labeled 0.125Eu/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 (Sullivan & Watson, 1975).

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 2 λ , λ , $\lambda/2$ & $\lambda/4$, where λ is the labeled claim sensitivity of Lysate.

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

$$MVD = (\text{Endotoxin limit} \times \text{Concentration of sample solution}) / (\lambda)$$
 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).

Cephalothin for injection sample preparation: Batch No: CFI-0109, Potency=50 mg/mL, E.L=0.13 Eu/mg, Lysate sensitivity is 0.125 Eu/mL and MVD = 52. The following test dilutions are prepared by 1:52 (0.96 mg/mL), 1:26 (1.92 mg/mL), 1:13 (3.84 mg/mL), 1:6.5 (7.69 mg/mL) & 1:3.2 (16.38 mg/mL).

Cefuroxime Sodium sample preparation: Batch No: CXN-0109, Potency=50 mg/mL, E.L=0.10 Eu/mg, Lysate sensitivity is 0.125 Eu/mL and MVD = 40. The following test dilutions are prepared by 1:40 (1.25 mg/mL), 1:20 (2.5 mg/mL), 1:10 (5 mg/mL), 1:5 (10 mg/mL) & 1:2.5 (20 mg/mL).

Ceftriaxone sodium sample preparation: Batch No: CTN-0109, Potency=50 mg/mL, E.L=0.20 Eu/mg, Lysate sensitivity is 0.125 Eu/mL and MVD = 80. The following test dilutions are prepared by 1:80 (0.62 mg/mL), 1:40 (1.25 mg/mL), 1:20 (2.5 mg/mL), 1:10 (5 mg/mL) & 1:5 (10 mg/mL).

Cefpirome sulphate for Injection sample preparation: Batch No: CSI-0109, Potency=50 mg/mL, E.L=0.20 Eu/mg, Lysate sensitivity is 0.125 Eu/mL and MVD = 80. The following test dilutions are prepared by 1:80 (0.62 mg/mL), 1:40 (1.25 mg/mL), 1:20 (2.5 mg/mL), 1:10 (5 mg/mL) & 1:5 (10 mg/mL).

Above mentioned Cephalosporin antibiotics are validated after adjusting their pH to 6-8, The NID factor was evaluated in the preliminary screening, variation among them is \pm two fold.

Methods

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 $37 \pm 1^\circ\text{C}$ 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 (USP, 2009)

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 (van Noordwijk & DeJong, 1997).

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.

Table 1. Results of NID factor in 1st to 4th generation cephalosporins; -- no spike recovery

Cephalothin for Injection: (1 st Generation Cephalosporin) NID-1:6.5 (1:13 selected for validation)					
Sample Dilution	1:3.2	1:6.5	1:13	1:26	1:52
Unspiked	--	++	--	--	--
Spiked	--	++	++	++	++
Cefuroxime Sodium: (2 nd Generation Cephalosporin) NID-1:2.5 (1:10 selected for validation)					
Sample Dilution	1:2.5	1:5	1:10	1:20	1:40
Unspiked	++	++	--	--	--
Spiked	++	++	++	++	++
Ceftriaxone Sodium: (3 rd Generation Cephalosporin) NID-1:5 (1:20 selected for validation)					
Sample Dilution	1:5	1:10	1:20	1:40	1:80
Unspiked	++	++	--	--	--
Spiked	++	++	++	++	++
Cefpirome sulphate for Injection: (4 th Generation Cephalosporin) NID-1:10(1:40 selected for validation)					
Sample Dilution	1:5	1:10	1:20	1:40	1:80
Unspiked	--	++	++	--	--
Spiked	--	++	++	++	++

Results and discussions

Phase I: Preliminary Screening / interference study (Cooper, 1990).

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 reveal the non-interfering dilution (NID) of the product, which is used for the actual validation in Phase II. The non-interfering dilution (NID) is the first set of PPC that shows a gel.

Assay results after preliminary screening with pH adjusting to 6-8 are presented in Table 1.

This assay shows no inhibition from 1:6.5 dilution onwards in Cephalothin for Injection, 1:2.5 in Cefuroxime Sodium, 1:5 in Ceftriaxone Sodium and 1:10 in Cefpirome sulphate for Injection and the spike recovery from the NID onwards. It is advisable to validate the product next to MVD to take care of any batch to batch variation during regular production in the pharmaceutical industries.

Graphical representation of NID for four drug molecules selected for study are shown in Fig. 1 to 4.

Phase II: validation of product

For validation, test and compare two identical series of endotoxin dilutions bracketing λ ; One prepared in LRW and another prepared in product diluted to the proposed

test dilution. Here dilution selected for validation is provided in Table 1 (Hot spike method).

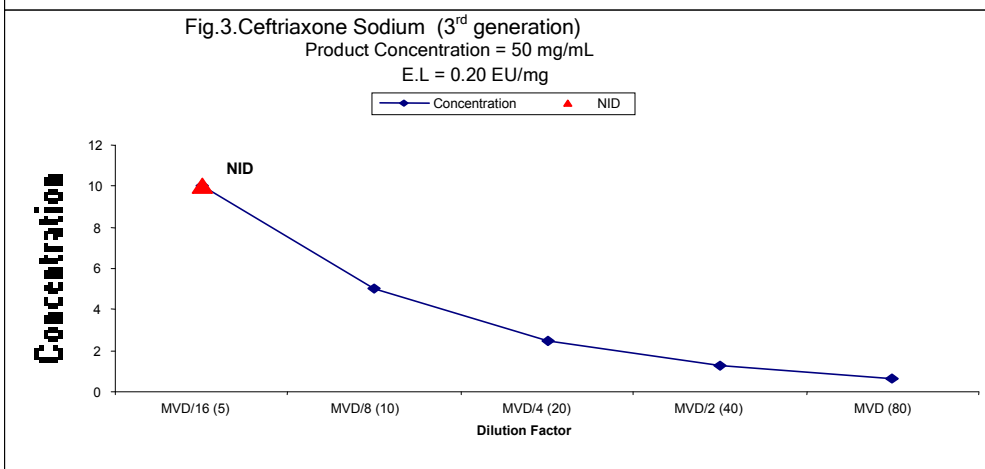
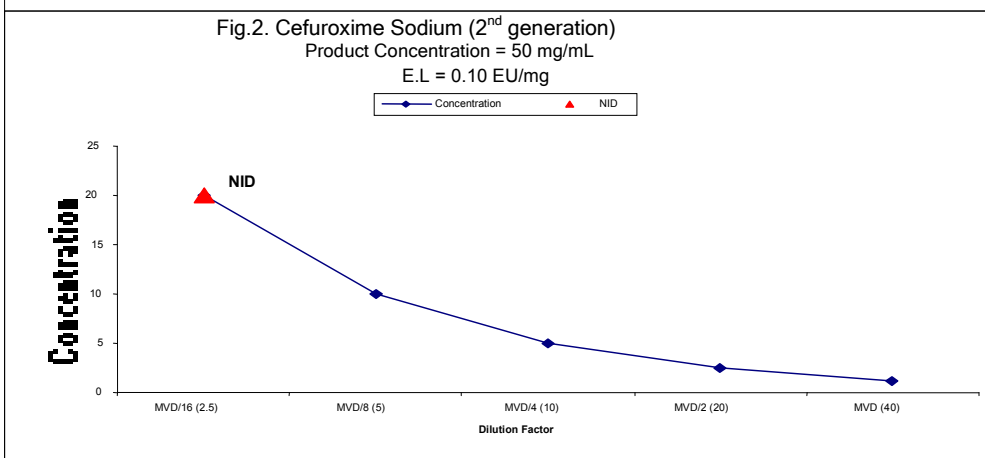
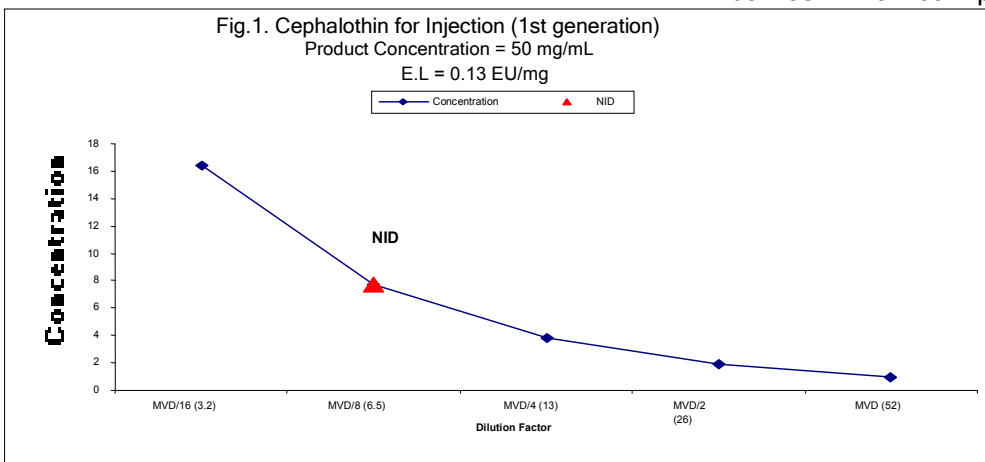
Analytical results of the cephalosporin group from Table 1 after pH adjustment reveal that NID factor between the four products is between MVD/8 and

MVD/16. In Table 2 results shows 100% recovery of the spiked endotoxins into the product. It means there is no inhibition or enhancement in these drug products during quantification of endotoxins, so there is no possibility of false positive results and we can apply the same methodology to understand the behavior of various cephalosporin group drug products before proceeding for actual testing. It was concluded that NID among four generations of Cephalosporin antibiotics is mostly \pm two fold.

BET applications include large volume parenterals (LVPs), multiple- ingredient drugs, small volume parenterals (SVPs), radiopharmaceuticals, biologicals, water system validation, validation of dry heat sterilizer and medical devices (Pearson, 1991).

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Phase II results

Assay results of the products after spiking with known concentration of endotoxin are presented in Table 2. Assay results of label claim sensitivity of the lysate are presented in Table 3. Successful validation requires that both series confirm label claim (Geometric mean) within \pm one two-fold dilution. Validation is conducted at this dilution on three batches of product.

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Table 2. Endotoxin/product; Negative product control: --; Geometric Mean = 0.125 EU/ml.

Replicates	Cephalothin for Injection			Cefuroxime Sodium				
	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	+	+	-	-	+	+	-	-
Replicates	Ceftriaxone Sodium			Cefpirome sulphate 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
1	+	+	-	-	+	+	-	-
2	+	+	-	-	+	+	-	-
3	+	+	-	-	+	+	-	-
4	+	+	-	-	+	+	-	-

Table 3. Endotoxin/LRW; Negative product control: --; Geometric Mean = 0.125 EU/ml

Replicates	0.25 Eu/mL	0.125 Eu/mL	0.0625 Eu/mL	0.0312 Eu/mL
1	+	+	-	-
2	+	+	-	-
3	+	+	-	-
4	+	+	-	-

