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Validation of LAL test on some antibiotics: A study on the causes of different inhibitory effects

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ABSTRACT

Validation process is a program for ensuring products with proven acceptable and predetermined specification. The LAL test used for detection of endotoxin in the sterile parenteral products is one of the tests that require validation. During this study, validation of 12 parenteral antibiotics including penicillin and cephalosporin have been done using gel clot method and obtained results which prescribed relationship between molecular structure and inhibitory dilution. The inhibitory dilution for potassium penicillin G was 5 millions units, potassium penicillin G was 1 millions units and natrium penicillin G was 1:40. Ampicillin, penicillin G 6:3:3, 400,000 and 800,000 unit penicillin had inhibitory dilutions up to 1:20 . Cloxacillin inhibitory dilution was 1:80. and Cefotaxim, Cefepime, Ceftizoxime dilution results were 1:40, Cefuroxim, Ceftazidime were 1:20 and this result for Cefazolin was 1:80.The results were studied and presented their according to pharmaceutical group separately. None of evaluated antibiotics showed enhancement effect but inhibitory was observed which made a significant conclusion on the role of dilutions on this phenomenon.

Key words: LAL test, Gel clot, validation, parenteral antibiotics

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1. Introduction

Due to direct contact of parenteral products to blood and human body tissues, these products should be pyrogen free, therefore it should be proved by manufacturer, before approving products for use in pharmaceutical market (Loyd et al., 2008 and USP, 2008). Official method for detecting pyrogenes in US Pharmacopeia is Rabbit test (USP, 2008 and BP, 2009).This test significantly depends on the physiological parameters of tested animals like other biological tests. Also the results would be related to environmental factors including physical conditions, temperature, pressure, humidity and stressors during the experiment. In addition it costs more (Kevin et al., 2007) for detecting pyrogens. One of the most important of them is endotoxines derived from gram-negative bacteria. (kevin et al., 2007) LAL test (Limulus Amebocyte Lysate) can be used (USP, 2008).

LAL test is more sensitive, more accurate and faster than the rabbit test and costs less, but one of the major limitations of LAL test, is enhancement and inhibition of some compounds on it (Eli et al., 2007). To the inhibitory effect, some of the factors preventing the formation of gel and result in false negative and the enhancement effect are when gel formation is observed because of the nature of the antibiotic, not the presence of endotoxin.

Thus, for validation of LAL test and ensuring the accuracy of results on parenteral product, the inhibition and enhancement effects should be investigated first (Ludwig et al., 2007). Providing observation of these effects products should be diluted up to the Maximum Dilution Factor (MVD) (USP, 2008). Present study is investigated for achieving this purpose and find logical relationship between chemical molecular structure and the inhibitory effects on some parenteral antibiotics made by Jaber ibn Hayyan pharmaceutical company. In this study, the inhibitory-enhancement effect was tested on 12 antibiotics.

2. Methodology

2-1. Equipments

Pyrogen Free disposable syringes, Glassy 75 × 10 mm tubes (previously got Pyrogen Free.), sampler with multi test disposable pyrogen free tips made in United States companies Cambrex and Endosafe, Pyrogen free empty vials, Water bath 37 ºC: Rost Frei mode. Oven: MMM ECOCELL 222 and Lab oven Model made in Germany, Traceable Timer made in United states, Tube shaker : MS2 Minishakr model made in United states, Laminar air flow : Beast bsc 126 model made in India, Refrigerator Model: SAMSUNG Construction USA, freezer -20ºC:SAMSUNG model made in korea.

2-2. Materials

Single and multi-test LAL gel clot kits made in United States companies Cambrex and Endosafe, LAL reagent water (LRW), Sulphochromic solution for washing the tubes, Isopropyl alcohol 75° or Ethanol 75°

2-3. Methods

2- 3-1. Confirmation of lysate sensitivity

According to the characteristics declared by LAL kit manufacturer, accuracy and validity of the method must be checked so performance of confirming the desired precision kit was done (Walkersville, 2009) and inhibitory effect was performed on each product. After adding water to standard vial of Endotoxin due to potency of it dilution 1EU/ml was prepared and dilutions of 0.03, 0.06, 0.125, 0.25 and 0.5 EU/ml were prepared as are seen in Table1.

Table1. *How to prepare standard dilutions.*

Vial	Endotoxin + LRW	Final dilution of Endotoxin(EU/ml)
1	1IU/ml + 1ml	0.5
2	1ml of vial 1 + 1ml	IU/ml 0.25
3	1ml of vial 1 + 1ml	0.125
4	1ml of vial 1 + 1ml	0.06
5	1ml of vial 1 + 1ml	0.03

After preparing different concentrations of endotoxin vials, Lysate vials prepared and 0.1ml Lysate added to each 0.1ml of sample. Examples included product, positive control and negative control. Pyrogen Free Water was used as negative control. After adding different concentrations of endotoxin to Lystae vials according to Table 2 (in this study Lystae sensitivity was equivalent to 0.06 EU / ml):

Table 2. *Gel clot formation in the presence of different dilutions of endotoxin.*

Repeat	0.5	0.12	0.06	0.03	water	Endpoint
1	+	+	+	–	–	0.06
2	+	+	+	–	–	0.06
3	+	+	+	+	–	0.03
4	+	+	+	–	–	0.06

The results of the endpoints were investigated after one hour incubation in 37ºC water bath. of logarithms of endpoints were calculated corresponding in Table 3, after calculating the mean, anti-logarithm of mean was:

Table 3. *Logarithmic calculated endpoint.*

Average= -1.297

Endpoint IU/ml	$\log10_{Endpoint}$
0.06	-1.222
0.06	-1.222
0.03	-1.522
0.06	-1.222

According to the acceptable range that is 0.5-2λ this result due to λ=0.06 is actually acceptable.

2-3-2. Determination the inhibitory-enhancement effect on products

After confirming sensitivity of Lysate the next step was validation of LAL test on 12 different antibiotics. For this aim four different dilution of the product including the contaminated product with 2λ endotoxin, 2 negative controls (LRW) and 2 positive controls that are 2λ standard endotoxin; were developed. Diluting products is permitted up to maximum valid dilution (MVD). If the not repose in the range, more sensitive Lysate kit should be used.

2-3-3. Gel clot test on products

Different dilutions of desired antibiotic vials were prepared. Then 100μl of each dilution added to the pyrogen free 75 × 10 mm tubes, negative control tubes containing water (LRW) and two positive control tubes containing standard endotoxin were considered. After Lystae preparation in aseptic condition, 100 μl of Lysate added to negative control tubes, sample tubes and positive control tubes respectively. After mixing them slowly, all of tubes were placed at 37 ºC water baths. Positive samples form a stable gel that with the 180° inversion of tubes gel clot remains stable. This is observed in endotoxin-contaminated samples and positive controls.Negative samples do not have formed gel, which is observed in negative control samples and in some dilutions that samples show inhibition. It was observed increase opacity because of viscosity considered negative. Negative control confirms the accuracy of used method. In this study some parentral antibiotics of Jaber ebn Hayyan company of groups of penicillins and cephalosporins were investigated. potassium penicillin G 1000000 unit, penicillin G 6.3.3, sodium penicillin G 5,000,000 units, Procaine Penicillin800000 units, Ampicillin, penicillin 1g, cloxacillin 500 mg were selected antibiotics of penicillins (Williams, 2002; Graham, 2001) and cefazolin 1g, ceftizoxime 1g, cefuroxim 1500 mg, ceftazidime 1g, cefotaxim 1g and cefepime 2g were selected antibiotics of cephalosporins. (Williams, 2002; Graham, 2001)

3. Results

For validation enhancement and inhibition were studied. Results indicated that the inhibitory effect is seen in different dilutions for each antibiotic, although none of the products show inhibition. The results of inhibitory effect are 3 in the tables 1-3 to 3-12.
No gel formation marked in the tables with negative sign and gel formation marked with positive sign. Repetitions of each test determined by number in paranthesis beside every signs stated. Meaning of sample is mentioned to vial of desired antibiotic and also Sample (-) is contaminated samples with 2λ standard endotoxin, Control (+) is only 2λ standard endotoxin and Control (-) is Water (LRW).

Table 3-1. *LAL test results for potassium penicillin G.*

dilution / Samples	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
Sample(+)	4(-)	4(-)	4(-)	4(+)	4(+)	4(+)	4(+)	4(+)
Sample	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)
Control(+)	2(+)							
Control(-)	2(-)							

Pen G K1000000 U/vial
MVD: 1666times
Endotoxin limit in drug monograph: 0.01 Eu/100U

Table 3-2. *LAL test results for penicillin G 6.3.3.*

dilution / Samples	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
Sample(+)	4(-)	4(-)	4(+)	4(+)	4(+)	4(+)	4(+)	4(+)
Sample	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)
Control(+)	2(+)							
Control(-)	2(-)							

Pen 6-3-3
MVD: 2000times
Endotoxin limit in drug monograph: 0.01 Eu/100U

Table 3-3. *LAL test results for penicillin Na.*

dilution / Samples	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
Sample(+)	4(-)	4(-)	4(-)	4(-)	4(+)	4(+)	4(+)	4(+)
Sample	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)
Control(+)	2(+)							
Control(-)	2(-)							

Pen Na 5000000 U/vial
MVD: 8333times
Endotoxin limit in drug monograph: 0.01 Eu/100U

Table 3-4. *LAL test results for penicillin procaine.*

dilution / Samples	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
Sample(+)	4(-)	4(-)	4(-)	4(-)	4(+)	4(+)	4(+)	4(+)
Sample	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)
Control(+)	2(+)							
Control(-)	2(-)							

Pen Procaine 800000 U/vial
MVD: 1333times
Endotoxin limit in drug monograph: 0.01 Eu/100U

Table 3-5. *LAL test results for ampicillin.*

Dilution / Samples	1/10	1/20	1/40	1/80	1/160	1/320
Sample(+)	4(-)	4(-)	4(+)	4(+)	4(+)	4(+)
Sample	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)
Control(+)	2(+)					
Control(-)	2(-)					

Ampicillin 1g/vial
MVD: 2500times
Endotoxin limit in drug monograph: 0.15Eu/mg

Table 3-6. *LAL test results for cloxacillin.*

Dilution / Samples	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
Sample(+)	4(-)	4(-)	4(-)	4(-)	4(+)	4(+)	4(+)	4(+)
Sample	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)
Control(+)	2(+)							
Control(-)	2(-)							

Cloxacillin 500mg/vial
MVD: 3333times
Endotoxin limit in drug monograph: 0.04Eu/mg

Table 3-7. *LAL test results for cefazolin.*

dilution / Samples	1/10	1/20	1/40	1/80	1/160	1/320	1/640
Sample(+)	4(-)	4(-)	4(-)	4(-)	4(+)	4(+)	4(+)
Sample	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)
Control(+)	2(+)						
Control(-)	2(-)						

Cefazolin 1g/vial
MVD: 2500times
Endotoxin limit in drug monograph: 0.15Eu/mg

Table 3-8. *LAL test results for ceftizoxim.*

dilution / Samples	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
Sample(+)	4(-)	4(-)	4(-)	4(+)	4(+)	4(+)	4(+)	4(+)
Sample	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)
Control(+)	(+)2							
Control(-)	(-)2							

Ceftizoxim 1g/vial
MVD: 1666times
Endotoxin limit in drug monograph: 0.01 Eu/mg

Table 3-9. *LAL test results for cefuroxime.*

Dilution / Samples	1/10	1/20	1/40	1/80	1/160	1/320
Sample(+)	4(-)	4(-)	4(+)	4(+)	4(+)	4(+)
Sample	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)
Control(+)	2(+)					
Control(-)	2(-)					

Cefuroxime 1500mg/vial
MVD: 2500times
Endotoxin limit in drug monograph: 0.1 Eu/mg

Table 3-10. *LAL test results for ceftazidim.*

Dilution / Samples	1/10	1/20	1/40	1/80	1/160	1/320	1/640
Sample(+)	4(-)	4(-)	(+)4	(+)4	(+)4	(+)4	(+)4
Sample	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)
Control(+)	2(+)						
Control(-)	2(-)						

Ceftazidim 1g/vial
MVD: 1666times
Endotoxin limit in drug monograph: 0.01 Eu/mg

Table 3-11. *LAL test results for cefotaxim.*

Dilution / Samples	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
Sample(+)	4(-)	4(-)	4(-)	4(+)	4(+)	4(+)	4(+)	4(+)
Sample	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)
Control(+)	2(+)							
Control(-)	2(-)							

MVD: 3333times
Endotoxin limit in drug monograph: 0.02 Eu/mg

Table 3-12. *LAL test results for Cefepime.*

Dilution / Samples	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
Sample(+)	4(-)	4(-)	4(-)	4(+)	4(+)	4(+)	4(+)	4(+)
Sample	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)	4(-)
Control(+)	2(+)							
Control(-)	2(-)							

Cefepime 1g/vial
MVD: 666times
Endotoxin limit in drug monograph: 0.04Eu/mg

4. Discussion

Implementing quality assurance in a pharmaceutical company is not possible without validation of all processes affecting product quality, including laboratory tests (Ludwig, 2007). In this study, validation of LAL test was done on 12 parentral antibiotic and the dilution of inhibition was determined. According to importance of validation performance on all laboratory tests, it is necessary to validate LAL test (Ludwig, 2007). The result of the presence of logical relationship between

chemical structure and inhibitory dilution of antibiotics studied. This behavior is justified with regard to antibiotic groups. It's facilitated to consider two important groups of penicillin and cephalosporins and the results are as follow:

4 -1. Penicillins

Penicillins derivatives containing β-lactam structure that contain sulfur groups which is located in a ring (Williams, 2002)
Based on the results can understand:

Group 1: potassium penicillin G 5 million units, potassium penicillin G 1 million units, sodium penicillin G by dilution of 1:40
Group 2: Ampicillin, Penicillin G 6:3:3, Penicillin G 400,000 and 800,000 units by dilution of 1:20
Group3:cloxacillin by dilution of 1:80

All of these compounds have similar penicillin structure and vary because of different R group.
As can be seen in group 1 compounds, 1:40 dilution is resulted. In this group, sodium and potassium penicillin G have similar structure and only differ in their Na and K, but compared with the second group that have gel formation up to 1:20, the only difference between groups 1 and 2, is their salt. sodium or potassium penicillin G are pure but that of pure while penicillin G 6:3:3 is mixture of three materials and penicillin g 400000 and 800000units is a mixture of two materials, so the presence Other salts besides penicillin G act as impurity and cause changes in inhibition behavior and results Gel clot formation in other dilution.
Comparison of potassium or sodium penicillin G in group 1 with ampicillin, shows they have different R groups. R in group 1 is a benzene ring can have van der Waals binding but R group in ampicillin is  , which in addition having the ring of benzene to create van der Waals bindings can have bipolar – bipolar and hydrogen binding because of NH2 group (Cordier, 1999) so the difference in probable bindings with endotoxin caused differences in the observed dilution.
In group 2, comparison of ampicillin, penicillin G 6:3:3 and penicillin 400000 and 800000units declare ampicillin can have hydrogen binding and van der waals bounds because of 
That this group is seen in procaine part that is available in penicillin 6.3.3, 400000&800000 that cause all of these have inhibition up to 1:20.
In group 3 gel formation is up to 1:80 that's because of  that can have stronger bipolar-bipolar bounds.presence of chlorin atoms in R group also because different behavior in inhibition.it has 3pairs of unbound electrons that can have conju gation.

4-2.Cephalosporins

cephalosporins also have structure which the sulfur atom is in a hexagonal ring that causes differentiation of this group in comparison with penicillins. cephalosporins have similar structure and only difference is between R and R ' groups :

Group 1: Cefotaxime, Cefepime, Ceftizoxime by dilution of 1:40

Group 2: Cefuroxime, Ceftazidim by dilution of 1:20

Group3:Cefazolin by dilution of 1:80

The study on molecular structure of group 1 compounds shows they are similar to each other and the only difference is in R group which there is not in ceftizoxime there and is Co2H in cefepime and cefotaxime.These R groups are a weak acidic group that's because of positive charge on nitrogen so similarity in this group causes formation of gel clots up to 1:40. Also it should be considered that these R groups have hydrogen bond.In group 2, the difference between ceftazidime and cefuroxime is R and R' groups, and despite the different groups they have hydrogen bonds due to nitrogen and oxygen so compound in group 1 have acidic properties, and antibiotics of group 2 has basic properties.
Comparison of Group 2 and 3 shows that except of differences in functional groups, cefazolin in group 3 have a large group containing Nitrogen although antibiotics of group have basic properties but this is seen more and cause formation of gel clot in 1:80.
Validation LAL test was investigated on 12 antibiotics and inhibitory dilution of every antibiotic was determined. Inhibitory dilution was obtained between 1:20 to 1:80 for all of them. The possible relationship between inhibitory dilution and their molecular structure was studied. These studies indicate the existence of logical relationship between inhibition and the observed results. This relationship was because of same functional group that led to same inhibitory dilution.

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