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Original Research Article

Evaluation of the Suitability of Kinetic Chromogenic LAL Assay for Determination of Endotoxin Levels in Heparin Sodium Injection

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HIGHLIGHTS

- Bacterial endotoxins are important contaminants associated with injectable pharmaceuticals.
- Kinetic chromogenic LAL assay was used as the method to determine endotoxin levels in heparin injections.
- Selectivity, linearity and repeatability of the endotoxin chromogenic method was validated.

Keywords:
Endotoxin
Heparin injection
Inhibitory effect
Kinetic chromogenic
LAL assay
Validation

ABSTRACT

Determination of the levels of endotoxins in injectable products has always been one of the concerns of regulatory authorities and manufacturers. Since a number of pharmaceuticals interfere with the LAL test to some degree, overcoming the inhibition or enhancement properties of a product is required as part of the validation of the LAL test for use in the final release testing of parenteral products. In this study, interference profile of Heparin injection in quantitative chromogenic LAL test was evaluated and the method of overcoming was investigated and validated. The results indicate that dilution as the most widely used technique for overcoming interference could not eliminate LAL interference in the aforementioned medicinal product. The inhibitory nature of heparin occurs due to its anticoagulant properties and can be overcome by using divalent cations such as magnesium. Three concentrations of magnesium chloride were evaluated for elimination of heparin's inhibitory effect. All three concentrations studied (5, 10 and 25 mM) could effectively eliminate the inhibitory effects of heparin. Hence, one-way analysis of variance was used to determine statistically significant differences between these three concentrations. The results of ANOVA statistical method showed the optimal recovery of spiked endotoxin was at a concentration of 10 mM of magnesium chloride. In consequence, chromogenic LAL test using 10 mM of magnesium chloride as diluent could be a validated method of choice for heparin LAL assay.

Introduction

Bacterial endotoxins and pyrogens are one of the most important contaminants associated with injectable pharmaceuticals. Currently the most commonly used test for determination of endotoxin level is Limulus Amebocyte Lysate (LAL) assay (Chalupniak et al., 2014). The LAL method is based on an endotoxin-induced coagulation reaction which offers a compendially-approved sensitive and selective test to determine endotoxins (European Directorate for the Quality

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of Medicines, 2013; United States Pharmacopeial Convention, 2014). However, the main concern associated with this method is the interaction of the LAL method with some substances tested for endotoxins, including metals, amino acids, antibiotics and enzymes which inhibit or enhance the coagulation reaction of LAL (Yuu et al., 2011). LAL (Enzyme cascade) and endotoxin are the major causes of bacterial endotoxin testing (BET) interference. LAL interference could be a result of non-neutral pH, ionic strength, chelating of divalent cations, serine protease inhibitors, solvents and beta glucans. Endotoxin interference is related to aggregation and adsorption phenomena (Cooper et al., 1997).

Prior to LAL testing of any product, it has to be screened for interference (both inhibition and enhancement). Should interference exist then, as an integral part of the validation process for the use of LAL in the final release testing of injectable and medical devices, overcoming the inhibition or enhancement of the product is required by regulatory bodies (Food and Drug Administration, 1992).

Three types of LAL endotoxin testing methods namely the gel-clot, the chromogenic and the turbidimetric methods, have been approved by the United States Pharmacopeia and European Pharmacopoeia for the evaluation of injectable drugs, medical devices, and pharmaceutical raw materials (Joiner et al., 2002). Among these methods, the kinetic chromogenic assay is less affected by the inhibitory effect of those products which may interfere with the clotting mechanism in turbidimetric and gel clot assays (Lonza, 2012a).

The object of the present study was to investigate the suitability of the kinetic chromogenic LAL assay for the determination of endotoxin levels in heparin injections. Hence, the interference pattern of heparin was assessed and the way to eliminate the interference was evaluated and optimized. The method was then validated to optimize the quality control testing required for the final release testing of the drug products.

Materials and Methods

Materials and Equipment

The following materials have been employed: Kinetic-QCL (Lonza, USA) with a sensitivity range of 0.005 EU/ml - 50.0 EU/ml; The Kinetic-QCL™ Kinetic Chromogenic LAL assay is the most sensitive of the LAL assays and is able to quantify the concentration of endotoxin over a wider range and includes Kinetic-QCL™ reagent, K50-643, Control Standard Endotoxin, *E. coli* O55:B5 endotoxin, E50-643, LAL Reagent Water, W50-640. Magnesium chloride hexahydrate (Merck, Germany), heparin injection 10000 IU/ml (Darou Pakhsh Pharmaceutical Company, Iran), Microplate Reader- SPECTROstar Nano and MARS data analysis software V3.01 R2 (BMG, Germany). Endotoxin-free glass dilution tubes (13 mm×100 mm) depyrogenated by heating at 250ºC for 30 min, Socorex micropipettes Acura825 with sterile, pyrogen-free individually wrapped tips, Eppendorf research plus multichannel, sterile, flat-bottom non-pyrogenic polystyrene 96-well microplates

Preparation of LAL reagent and CSE

The LAL reagent and Control Standard Endotoxin (CSE) were prepared by reconstituting the lyophilized vials according to the instructions contained in the Lonza manual. When conducting the test, five serial dilutions of the CSE, starting from 50 EU/ml, were prepared using water for BET as diluent to obtain final endotoxin concentrations of 0.005, 0.050, 0.5 and 5 EU/ml (European Directorate for the Quality of Medicines, 2013).

Sample preparation

Samples (sample ID HS-01, HS-02 and HS-03) were selected randomly from three batches of heparin 10000 IU/ml injection. Each sample was diluted with water BET to give a solution containing 1000 IU of heparin activity per ml (solution A). The endotoxin limit concentration of solution A should be less than 10 IU of endotoxin per ml. Then serial dilutions of solution A were prepared according to the maximum valid dilution (MVD) from 1:250 to 1:2000 by dilution factors of 2 (European Directorate for the Quality of Medicines, 2013).

Determination of the MVD

The greatest dilution at which endotoxin limit can be detected is the MVD (Dawson, 1995). The MVD was determined for solution A by dividing the endotoxin limit concentration of solution A (ERL) to the lowest endotoxin concentration used for plotting the standard curve (Lambda) (European Directorate for the Quality of Medicines, 2013):

MVD=ERL/Lambda
MVD=10/0.005
MVD=2000 (1: 2000)

Spiked sample preparation

Solution A's were fortified with standard endotoxin (CSE) to concentration of 10 EU/ml. Then serial dilution of spiked solution A's were prepared in a similar way to the sample dilution (European Directorate for the Quality of Medicines, 2013).

Diluents

LAL reagent water was used as diluent in trials.

Magnesium chloride solution (with concentration of 5, 10 and 25 mM) in LAL reagent water was used as diluent in the tests after finding the inhibitory effect. These diluents were used as blank in each relative test.

Principle of the method

In the kinetic chromogenic LAL assay, a sample is mixed with the LAL substrate reagent, placed in an incubating plate reader at 37±1ºC, and the absorbance at 405 nm of each well of the microplate is automatically monitored, using the initial absorbance reading of each well as its own blank. The reader determines the time required for the absorbance to appearance of yellow color and increases 0.200 absorbance units. This time is termed reaction time which is inversely proportional to the amount of endotoxin present. The concentration of endotoxin in unknown samples can be calculated from a standard curve (Lonza, 2012b; European Directorate for the Quality of Medicines, 2013).

Preliminary evaluation of the inhibitory effect of the heparin with LAL assay

To evaluate the potential interference of heparin injection with the LAL coagulation reaction, BET was performed using LAL reagent water as diluent to obtain the MVD. Three batches of heparin injection were tested in triplicate, at dilutions of 1:250, 1:500, 1:1000 and 1:2000, corresponding to the MVD, along with the negative control, in triplicate. To assess the inhibitory effect, the spiked sample solution containing 10 EU/ml of CSE was also tested in triplicate. The recovery of endotoxin added to spike the sample solution was calculated using the standard curve. The acceptable spike recovery must be between 50-200% (European Directorate for the Quality of Medicines, 2013; United States Pharmacopeial Convention, 2014; United States Pharmacopeial Convention, 2014). To increase the reactivity of the LAL reagents and overcome the inhibitory effect of the product, 5, 10 and 25 mM magnesium chloride solutions were used as diluents in triplicate in three different tests. The results of the preliminary evaluation allowed us to determine the optimal concentration of magnesium chloride in which LAL assay could work for heparin injection without any interference and increase the recovery of endotoxin (Lonza, 2012c).

Statistical tests

The effect of the three concentrations of magnesium chloride on elimination of the interference of heparin sodium was statistically investigated. Statistical tests were performed with GraphPad Prism 6.01 software (GraphPad software, La Jolla, USA). Data were analyzed using one-way ANOVA followed by Tukey's post hoc. Differences with P values of <0.05 were regarded as significant.

Method Validation Study

Following finding the optimum concentration of magnesium chloride solution, which minimized heparin sodium interference in the LAL assay, the aforementioned parameters were evaluated to validate the method.

Initial Qualification assay

The initial qualification assay was performed as a log/log linear correlation of the individual reaction time values for each replicate of standard endotoxins. The standard curve was prepared by plotting the log of the individual reaction time value (in second) for each replicate against the log of endotoxin concentration. Five concentrations of CSE in triplicate (0.005, 0.05, 0.5, 5.0 and 50 EU/mL) were applied to determine the slope, y-intercept and correlation coefficient (Fernando et al., 2013).

Linearity

The linearity of three batches of heparin injection at dilution of 1:500 and LAL reagent water as blank were tested in triplicate for evaluation of linearity. The acceptable value of the correlation coefficient (r) must be ≥ 0.980 (Fernando et al., 2013).

Selectivity

Selectivity analysis was employed to demonstrate the ability of the bio-analytical method to quantify and differentiate the endotoxin from the components that might be present in the product, including metabolites, impurities, degradation compounds and matrix components (Fernando et al., 2013).

Three batches of heparin injection at dilution of 1:500 were tested in triplicate for evaluation of selectivity. The spiked recovery sample was tested in comparison with the non-contaminated product using the appropriate concentration of magnesium chloride solution obtained from section 2.8. . The acceptable spiked recovery must be between 50-200%.

Repeatability

The LAL test will meet the acceptance criteria for repeatability when the spike recovery value for three batches of heparin injection in 1:500 dilution and in triplicate is between 5 EU/ml and 20 EU/ml with CV of less than 10 percent (Rustichelli et al., 2013).

Table 1. Determination of endotoxin in heparin injection using LAL reagent water as diluent.

Sample	Sample ID	Dilution	Endotoxin concentration (EU/ml)
Water for BET		Direct	N.D
< 0.005 EU/ml	HS-01	1/250	N.D
		1/500	
		1/1000	
		1/2000	
Heparin injection	HS-02	1/250	N.D
		1/500	
		1/1000	
		1/2000	
< 10 EU/1000 IU heparin	HS-03	1/250	N.D
		1/500	
		1/1000	
		1/2000	

N.D: Not detected

Y-Intercept: 3.11, Slope: -0.26,

Correlation coefficient: 0.995, Coefficient of variation: <10%

Results and Discussion

Preliminary evaluation of the inhibitory effect of heparin with LAL assay:

The results of preliminary investigation of the inhibitory effect of heparin, using water as diluent, were evaluated and are shown in Tables 1 and 2. As seen in Tables 1 and 2, the LAL assay was performed on heparin injection and heparin injection spiked with 10 EU/mL of CSE in triplicate, at dilutions of 1:250, 1:500, 1:1000 and 1:2000, corresponding to the MVD, along with the negative control, in triplicate using LAL reagent water as diluent to evaluate the recovery of endotoxin. The obtained results indicate an inhibitory effect of heparin injection on the

Table 2. Recovery of endotoxin in spiked heparin injection using LAL reagent water as diluent.

Sample	Sample ID	Dilution	Coefficient of variation (%)	Recovery of endotoxin (EU/ml)	Recovery (%)
Heparin injection and 10 EU/ml of endotoxin	HS-01	1/250	1.41	3.4	34%
		1/500	4.32	4.3	43%
		1/1000	4.97	2.1	21%
		1/2000	6.38	3.2	32%
Acceptance criteria 5.0-20.0 EU/ml (50-200%)	HS-02	1/250	2.94	3.8	38%
		1/500	3.74	4.3	43%
		1/1000	3.74	2.5	25%
		1/2000	2.82	2.7	27%
	HS-03	1/250	2.16	3.0	30%
		1/500	4.55	4.3	43%
		1/1000	6.16	1.7	17%
		1/2000	6.48	3.0	30%

Y-Intercept: 3.11, Slope: -0.26,

Correlation coefficient: 0.995, Coefficient of variation: <10%

Table 3. Determination of endotoxin in heparin injection using 5 mM $MgCl_2$ solution as diluent.

Sample	Sample ID	Dilution	Endotoxin concentration (EU/ml)
5 mM $MgCl_2$ < 0.005 EU/ml	-	Direct	0.001
Heparin Injection < 10 EU/1000 IU Heparin	HS-01	1/250	0.17
		1/500	0.22
		1/1000	0.20
		1/2000	0.14
	HS-02	1/250	0.18
		1/500	0.25
		1/1000	0.32
		1/2000	0.10
	HS-03	1/250	0.15
		1/500	0.25
		1/1000	0.30
		1/2000	0.12

Y-Intercept: 3.09, Slope: -0.25

Correlation coefficient: 0.991, Coefficient of variation: <10%

LAL assay since the recovery of spiked endotoxin was less than 50 percent in all concentrations which is not in compliance with the acceptance criteria (50-200%) recommended by the regulatory guidelines.

In order to overcome this issue, magnesium chloride solution at concentrations of 5, 10 and 25 mM was used as diluent to provide divalent cations required by the LAL reaction. The results of adding magnesium chloride are summarized in Tables 3 to 8. They confirmed the suitability of magnesium chloride for reduction of inhibitory effect of heparin in the LAL test because endotoxin recovery was increased to acceptable levels (>50%) at 5, 10, 25

Table 4. Recovery of endotoxin in spiked heparin injection using 5 mM $MgCl_2$ solution as diluent.

Sample	Sample ID	Dilution	Coefficient of variation (%)	Recovery of endotoxin (EU/ml)	Recovery (%)
Heparin and 10 EU/ml of endotoxin	HS-01	1/250	0.82	5.0	50%
		1/500	2.16	5.0	50%
		1/1000	3.74	5.55	55%
		1/2000	4.32	5.58	56%
Acceptance criteria 5.0-20.0 EU/ml (50-200%)	HS-02	1/250	1.41	5.37	54%
		1/500	2.94	5.14	51%
		1/1000	3.27	5.68	57%
		1/2000	4.97	6.23	62%
	HS-03	1/250	1.41	5.21	52%
		1/500	2.16	5.08	51%
		1/1000	3.74	5.57	56%
		1/2000	3.56	5.90	59%

Y-Intercept: 3.09, Slope: -0.25

Correlation coefficient: 0.991, Coefficient of variation: <10%

Table 5. Determination of endotoxin in heparin injection using 10 mM $MgCl_2$ solution as diluent.

Sample	Sample ID	Dilution	Endotoxin concentration (EU/ml)
10 mM $MgCl_2$ < 0.005 EU/ml	-	Direct	0.001
Heparin injection <10 EU/1000 IU heparin	HS-01	1/250	0.21
		1/500	0.26
		1/1000	0.22
		1/2000	0.20
	HS-02	1/250	0.22
		1/500	0.30
		1/1000	0.27
		1/2000	0.26
	HS-03	1/250	0.26
		1/500	0.31
		1/1000	0.34
		1/2000	0.30

Y-Intercept: 3.13 Slope: -0.27

Correlation coefficient: 0.993, Coefficient of variation: <10%

mM magnesium chloride. In order to find the optimal concentration of magnesium chloride the obtained values of endotoxin recovery were compared using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc*. The statistical analysis showed that the maximum recovery of endotoxin was obtained with magnesium chloride concentration of 10 mM. So, 10 mM magnesium chloride was considered as optimal concentration for LAL chromogenic test method validation.

Validation study

Initial qualification assay

The results of the initial qualification assay are presented

Table 6. Recovery of endotoxin in spiked heparin injection using 10 mM $MgCl_2$ as diluent.

Sample	Sample ID	Dilution	Coefficient of variation (%)	Recovery of endotoxin (EU/ml)	Recovery (%)
Heparin injection and 10 EU/ml of endotoxin	HS-01	1/250	0.82	6.3	63%
		1/500	1.41	6.9	69%
		1/1000	2.94	6.0	60%
		1/2000	4.24	5.9	59%
Acceptance criteria 5.0-20.0 EU/ml (50-200%)	HS-02	1/250	1.41	5.9	59%
		1/500	1.41	6.7	67%
		1/1000	1.63	6.2	62%
		1/2000	4.32	6.0	60%
	HS-03	1/250	0.82	6.0	60%
		1/500	2.16	6.8	68%
		1/1000	2.16	6.1	61%
		1/2000	4.08	6.1	61%

Y-Intercept: 3.13 Slope: -0.27

Correlation coefficient: 0.993, Coefficient of variation: <10%

Table 7. Determination of endotoxin in heparin injection using 25 mM MgCl2 solution as diluent.

Sample	Sample ID	Dilution	Endotoxin concentration (EU/ml)
10 mM $MgCl_2$ < 0.005 EU/ml	-	Direct	0.001
Heparin injection <10 EU/1000 IU heparin	HS-01	1/250	0.19
		1/500	0.25
		1/1000	0.19
		1/2000	0.18
	HS-02	1/250	0.21
		1/500	0.30
		1/1000	0.31
		1/2000	0.33
	HS-03	1/250	0.20
		1/500	0.28
		1/1000	0.30
		1/2000	0.30

Y-Intercept: 3.1, Slope: -0.24

Correlation coefficient: 0.998, Coefficient of variation: <10%

in Table 9. As can be seen, the coefficient of variation of the reaction times for the replicates was less than 10%. Meanwhile the slope and Y-intercept and correlation coefficient were -0.24, 3.08 and 0.997, respectively, which meet the relative acceptance criteria in FDA guideline on validation of the LAL test (Fernando et al., 2013).

Linearity

Table 10 shows the results of the test for linearity which were obtained by triplicate testing of three batches of heparin injection spiked with 10 IU/ml of endotoxin. The obtained values are in compliance with the acceptable criteria for Coefficient of variation and percentage of

Table 8. Recovery of endotoxin in spiked heparin injection using 25 mM $MgCl_2$ solution as diluent.

Sample	Sample ID	Dilution	Coefficient of variation (%)	Recovery of endotoxin (EU/ml)	Recovery (%)
Heparin injection and 10 EU/ml of endotoxin	HS-01	1/250	0.82	5.1	51%
		1/500	2.16	6.5	65%
		1/1000	2.94	6.2	62%
		1/2000	3.27	5.9	59%
Acceptance criteria 5.0-20.0 EU/ml (50-200%)	HS-02	1/250	0.82	5.3	53%
		1/500	1.63	6.8	68%
		1/1000	5.72	6.3	63%
		1/2000	3.56	6.0	60%
	HS-03	1/250	1.63	5.1	51%
		1/500	2.94	6.6	66%
		1/1000	2.94	6.4	64%
		1/2000	6.68	6.2	62%

Y-Intercept: 3.1, Slope: -0.24

Correlation coefficient: 0.998, Coefficient of variation: <10%

Table 9. Validation study of the kinetic-QCL LAL assay for heparin injection: Initial qualification assay.

Standard endotoxin (EU/ml)	Coefficient of variation (%)	Correlation coefficient	Slope	Y-Intercept
50	0.47%	0.9970	-0.24	3.08
5	4.92%			
0.5	5.71%			
0.05	2.94%			
0.005	3.55%			

Y-Intercept: (2.500 to 3.500), Slope: (-0.40 to -0.10),
Correlation coefficient: $r \geq 0.98$, Coefficient of variation: <10%

endotoxin recovery suggested in the regulatory guidelines (Rustichelli et al., 2013).

Test of selectivity

The results of the test for selectivity analysis in three batches of heparin injection are summarized in Table 11. According to the obtained results, there was no reaction between blanks and LAL reagent, confirming that the kinetic chromogenic assay detects only endotoxin.The spiked recovery sample was tested in comparison with the non-contaminated product using the appropriate concentration of magnesium chloride solution obtained from section 2.8.

Repeatability

As shown in Table 12, the CV% of the reaction times obtained for the triplicate tests of three batches of heparin injection were <10% which is in compliance with the regulatory requirements (Rustichelli et al., 2013). In comparison with the pyrogen test using rabbits, BET is a highly sensitive and simple method for detection of endotoxins. BET is a regulatory requirement for quality control of injections and medical devices (European Directorate for the Quality of Medicines, 2013). All assays, independent of methodology are standardized using endotoxin in water. Therefore unless the sample is water, some substances or components of the solution may interfere with the LAL test even when sample solutions are diluted up to the MVD (Dawson, 1995; Yuu et al., 2011). The major interference mechanisms to be expected when testing various parenteral drugs for BET using the LAL test include: suboptimal pH conditions, aggregation or adsorption of control endotoxin spikes, unsuitable cation concentrations, enzyme or protein modification, nonspecific LAL activation and endotoxin masking (Williams, 2007). Materials such as heparin can bind divalent cations and reduce the aggregation state of endotoxin which may lead to interfere with LAL assay (Lonza, 2012c). In the present study we investigated the inhibitory effects of heparin by analyzing three batches of heparin injection available on the Iranian market. Furthermore, the suitability of kinetic chromogenic LAL assay for determination of endotoxin levels was evaluated. Since the Kinetic-QCL assay is less affected by inhibitory products which may interfere with the clotting mechanism of turbidimetric and gel clot assays, so, in the case of heparin injection, Kinetic-QCL assay could be the method of choice (Lonza, 2012b).

Table 10. Validation study of the kinetic-QCL LAL assay for heparin injection: Linearity.

Sample	Dilution	Recovered endotoxin (%)	Endotoxin concentration (EU/ml)	Coefficient of variation (%)
HS-01+ 10 EU/ml endotoxin	1:500	69%	7.16	1.41
HS-02+ 10 EU/ml endotoxin		67%	7.0	1.41
HS-03+ 10 EU/ml endotoxin		68%	7.11	2.16
Blank (10 mM $MgCl_2$)	Direct	-	0.001	0.82

Recovered endotoxin (50-200%),
Coefficient of variation: (<10%)

Table 11. Validation study of the kinetic-QCL LAL assay for heparin injection: Selectivity.

Sample	Dilution	Recovered endotoxin (%)	Endotoxin concentration (EU/ml)	Coefficient of variation (%)
HS-01	1:500	-	0.26	5.72
HS-02		-	0.3	5.73
HS-03		-	0.31	6.70
HS-01+ 10 EU/ml endotoxin		69%	7.16	1.41
HS-02+ 10 EU/ml endotoxin		67%	7.0	1.41
HS-03+ 10 EU/ml endotoxin		68%	7.11	2.16
Blank	Direct	-	0.001	0.82

Coefficient of variation: <10%

The results obtained confirmed the inhibitory effect of heparin injection with routine LAL assay because the recovery of spiked endotoxin was less than 50 percent. On the other hand, simple dilution, even up to the MVD could not overcome the interference properties of heparin. Since non-inhibitory dilution was not found even at MVD, to determine suitable level of a divalent cation that would inhibit heparin interference, magnesium chloride solution at concentrations of 5, 10 and 25 mM was used as a diluent. The acceptable range of recovery (>50%) was observed with all concentrations of magnesium chloride. Hence, to find the optimal concentration of magnesium chloride solution, the effect of the aforementioned magnesium chloride concentrations on the elimination of interference was compared using ANOVA statistical method.

The results of ANOVA revealed that for the dilution of 1:250, 1:500 and 1:1000 of heparin 1000 IU/ml injection, magnesium chloride at concentration of 10 mM showed significantly better effects on overcoming LAL interference.

Therefore, for the above dilutions of heparin, 10 mM magnesium chloride was found as the optimal concentration for providing suitable level of divalent cations required for the LAL assay. As heparin is a chelating agent it can reduce the aggregation state of endotoxins due to binding of divalent cations. This results in increased reactivity, which is observed as enhancement. In contrast, sequestration of cations makes them unavailable for optimum enzyme activity of LAL cascade resulting inhibition (Dawson, 2005). According to the ANOVA results for dilution of 1:2000, 5 mM magnesium chloride showed significant effects on endotoxin recovery. Decreased concentration of heparin at 1:2000 dilution is led to less binding of divalent cations. So, lower concentration of magnesium chloride (5 mM) was needed to eliminate the inhibitory effects.

The obtained results show that providing adequate quantities of the divalent cations such as magnesium salts can decrease the inhibitory effect of heparin in the LAL assay.

Table 12. Validation study of the kinetic-QCL LAL assay for heparin injection: Repeatability.

Sample	Dilution		Coefficient of variation (<10%)
HS-01+ 10 EU/ml Endotoxin	1:500	6.95	0.11
		7.00	
		6.75	
HS-02+ 10 EU/ml Endotoxin		6.85	0.11
		6.65	
		6.60	
HS-03+ 10 EU/ml Endotoxin		6.72	0.16
		6.66	
		7.02	

Conclusion

It was found that a 10 mM magnesium chloride solution, as diluent, produced the optimum recovery of endotoxins from Heparin injection. Based on this finding, a method validation of the Kinetic-QCL assay for determination of endotoxin level of heparin injection was performed. By evaluating method qualification, selectivity, linearity and repeatability, the endotoxin chromogenic method was validated in accordance with European/US Pharmacopeia. Therefore, this method is suitable as a release test for determination of bacterial endotoxin levels in heparin injection (Food and Drug Administration, 1992; European Directorate for the Quality of Medicines, 2013; United States Pharmacopeial Convention, 2014).

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