Research Article

Development of an *in vitro* Endotoxin Test for Monoolein–Water Liquid Crystalline Gel for Use as an Implant

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Abstract

Purpose: *Drugs that are administered by parenteral route must be apyrogenic. The aim of this study was to develop an in vitro endotoxin test for liquid crystalline gels for use as implants, using a monoolein–water liquid crystalline gel as a model.*

Methods: *The gel-clot technique was used. The gel was dissolved first in isopropyl myristate, and the endotoxins were extracted with water for bacterial endotoxin test. Tests for the labelled lysate sensitivity and interfering factors were performed to validate the developed method. The limit of detection of endotoxin in the gel was also determined.*

Results: *The labelled lysate sensitivity was confirmed. It was not influenced by the presence of extracts from the gels. Endotoxins in the contaminated test gels were completely extracted. Endotoxin concentration in the tested gels was below the calculated threshold endotoxin level.*

Conclusion: *A method to perform in vitro endotoxins test of liquid crystalline gels was successfully developed and validated. Application of the technique to gels currently being developed in our laboratories indicate that the gels were apyrogenic.*

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INTRODUCTION

Drugs that are administered by the parenteral route must be apyrogen [1-3]. Consequently, it is necessary to detect and/or quantify endotoxins in parenteral products such as implants. Bacterial endotoxins can provoke in humans, fever, shock, or even death [4]. To verify if parenteral drugs are apyrogenic, tests performed in rabbits are broadly accepted. Nevertheless, both ethical and economic reasons have led researchers to develop alternative methods. Among these alternative techniques, the most used is the *Limulus* amoebocyte lysate (LAL) test [5]. It is also known as bacterial endotoxin test. LAL test can detect or quantify bacterial endotoxins [6].

There are three types of bacterial endotoxin tests: gel-clot technique, which is based on gelation; turbidimetric technique, based on the development of turbidity after cleavage of an endogenous substrate; and chromogenic technique, based on the development of colour after cleavage of a synthetic peptide-chromogen [7]. The first type has this advantage: the decision to pass or fail the product under examination is based on the presence or absence of a gel-clot that is visible to the naked eye. For bacterial endotoxin tests, pharmacopoeias [3,7,8] stipulate that the test product must be soluble in or dilutable with water and it is the solution of the test product that is mixed with the LAL reagent. However, some products such as monoolein–water liquid crystalline gels are not soluble in water, and worse, they solidify in contact with water [9,10]. To the best of our knowledge, no pharmacopoeia or other literature has stipulated any protocol for *in vitro* endotoxin test (LAL test) for water-insoluble gel products.

Since our research group is currently developing monoolein–water liquid crystalline gels of gentamycin for use as bioresorbable implants for the treatment of chronic osteomyelitis [10-12], we set out to develop an *in vitro* test method for endotoxin in this particular product.

EXPERIMENTAL

Test product

The test product was a monoolein-water gel containing gentamicin (5 %w/w). It was prepared in our laboratory as follows: 10 g of gentamycin sulfate (Id Indis, Aartselar, Belgium) and 160 g of monoolein (Danisco Pharma, Brabrand, Denmark) were separately dissolved in 50 mL of deionized water and 50 mL of ethyl alcohol/ethyl ether 97.1/2.9 (Stella, Liège, Belgium), respectively. The solutions were sterilised by filtration and placed together in a 500 ml sterile glass flask that was then mounted on a rotary evaporator (model R-205, Büchi, Switzerland). The solvent mixture was evaporated at 50 ^{o}C, at rotating speeds varying from 150 to 235 revolutions per minute (rpm) and pressures ranging from – 0.70 to –0.92 bar. The final product was a liquid crystalline gel, which became very viscous in contact with aqueous fluids. The gel was used as a sterile sustained-release implant [10].

Apparatus and reagents

The *in vitro* endotoxins test (LAL test) was performed on samples of three batches of the gels (implants). The following apparatus and reagents were used: heat-stable apparatus (Thermolyne, USA); sterile and apyrogen pipettes; sterile and apyrogen tubes; vial containing 100 IU of standard endotoxins; *Limulus* amoebocyte lysate (LAL) reagent (Charles River Laboratories, USA) whose labelled lysate sensitivity (λ) was 0.015 IU/ml; apyrogen isopropyle myristate; and water for bacterial endotoxin test (Charles River Laboratories, USA). Solutions of standard endotoxins and *Limulus* amoebocyte lysate were rehydrated with water for bacterial endotoxin test (Charles River Laboratories, USA).

Test for confirmation of labelled lysate sensitivity

The labelled sensitivity of the LAL reagent was verified according to three pharmacopoeias [3,7,8] whose methods are slightly different. In the procedures, series of two-fold dilutions of the standard endotoxins were prepared to give concentrations of 2λ, 1λ, 0.5λ and 0.25λ where λ is the labelled sensitivity of the LAL reagent in endotoxin units per ml. Dilution of the standard endotoxins was carried out with water for bacterial endotoxin test (BET). The test was performed at four standard concentrations in quadruplicate and it included negative controls. A volume (100 µl) of LAL reagent was mixed with an equal volume of the standard solutions in each tube. The mixture was incubated in the heat-stable apparatus at 37 ± 1 °C for 60 ± 2 min, avoiding vibration. A positive reaction was characterised by the formation of a firm gel that remained when inverted through 180° in one smooth motion. Such a result was recorded as positive (+). A negative result (-) was indicated by the absence of such a gel or by the formation of a viscous gel that did not maintain its integrity. The test was not valid if any negative control was positive. The end-point was the last positive result in a series of decreasing concentration of endotoxin. The mean value of the logarithm of the end-point concentration was calculated and then the antilogarithm of the mean value using Eq 1.

Geometric mean end-point = antilog [(Σe)/f] … (1)

where *Σe* = sum of the log end-point concentrations of the dilution series used and *f* = number of replicates.

The geometric mean end-point concentration is the measured sensitivity of the LAL reagent (IU/ml). If this was not less than 0.5λ and not more than 2λ, the labelled sensitivity was confirmed and was used in the subsequent tests performed with this LAL reagent.

Extraction of endotoxin from the test product

As the monoolein–water liquid crystalline gel of gentamicin was insoluble (in water) and became solid in contact with water, we used a technique to extract endotoxin from it as follows. An amount (100 mg) of the gel was placed in a tube and melted at 40 °C in the heat-stage apparatus. The molten gel was dissolved first in 6 ml of isopropyl myristate in the tube, and then 5 ml of water for BET was added. It was mixed for 10 min using a vortex mixer and then left to settle for 20 min. The lipidic phase (the supernatant) was removed and the aqueous phase (extract) used as the test solution. Test for interfering factors was performed to validate this technique of extraction, and to verify the absence of interference of the extract during LAL test.

Test for interfering factors

Solutions A, B, C and D were prepared as shown in Table 1. The extract was the aqueous solution that stemmed from the extraction process. The test was performed as described for confirmation of labelled lysate sensitivity. Different concentrations of solution B were obtained by adding standard endotoxin to the gel so as to give theoretic concentrations of 2λ, 1λ, 0.5λ, and 0.25λ, respectively, in solution B after extraction.

Solution A = solution stemming from endotoxins extraction (from the gel) and being supposed free of detectable endotoxins; Solution B = test for interference; Solution C = control for the labelled lysate sensitivity; Solution D = negative control (water for BET).

The geometric mean end-point concentration of solutions B and C was determined using the expression described for confirmation of the labelled lysate sensitivity. The test was not valid unless all replicates of solutions A and D showed no positive reaction and the results of solution C confirmed the labelled lysate sensitivity. If the sensitivity of the

Table 1: Composition of solutions to be added to LAL reagent at equal volume (100 µl) for the test for interfering factors

Solution	Endotoxins concentration in solution to which endotoxins were added	Dilution factor	Endotoxins concentration	Number of replicates
A	None/Extract			4
B	2λ/Extract	1	2λ	4
		2	1λ	4
		4	0.5λ	4
		8	0.25λ	4
C	2λ/Water for BET	1	2λ	2
		2	1λ	2
		4	0.5λ	2
		8	0.25λ	2
D	None/Water for BET		0	2

Solution A = solution stemming from endotoxins extraction (from the gel) and being supposed free of detectable endotoxins; Solution B = test for interference; Solution C = control for the labelled lysate sensitivity; Solution D = negative control (water for BET).

lysate determined with solution B was not less than 0.5λ and not greater than 2λ, the extract did not contain interfering factors under the experimental conditions and the technique of extraction was validated.

Determination of endotoxin limit concentration in the extract and in the gel

The endotoxin limit concentration (ELC) in the extract was determined according to the procedures of International Pharmacopoeia and European Pharmacopoeia [3,8] using Eq 2.

$$ELC = (K \times C) / M \quad \text{............................} \quad (2)$$

where K = threshold pyrogenic dose of endotoxins per kilogram of body mass in a single hour period; C (concentration of the extract) = sample mass / volume of aqueous phase stemming from extraction; M = maximum recommended dose of product per kilogram of body mass in a single hour period. The endotoxin limit concentration in the gel was calculated as in Eq 3.

$$ELC_{gel} = ELC_{extract} \times V/M \quad \text{..................} \quad (3)$$

where V = volume of aqueous phase stemming from extraction) and M = mass of the gel sample.

Determination of maximum valid dilution

Maximum valid dilution (MVD) is the maximum allowable dilution of the extract at which the endotoxin limit can be determined.

$$MVD = ECL/\lambda \quad \text{...............................} \quad (4)$$

where λ = the labelled lysate sensitivity

Determination of the endotoxins concentration in the gel

Determination of endotoxin concentration in the gels was performed according to European Pharmacopoeia [8], using semi-quantitative test. First, endotoxin concentration of the extract obtained from the gel was determined. Endotoxin concentration in the gel was then computed as in Eq 5.

$$C_{gel} = C_{extract} \times V_{extract} / M_{gel} \quad \text{..............} \quad (5)$$

where C_{gel} = endotoxin concentration of the gel, $C_{extract}$ = endotoxin concentration of the

Table 2: Composition of solutions added to LAL reagent at equal volume (100 µl) for the determination of endotoxins concentration in the extract

Solution	Endotoxin concentration/ solution in which endotoxins were added	Diluents	Dilution factor	Endotoxin concentration	Number of replicates
A_1	None/Extract	Water for BET	1 2 4 8		2 2 2 2
B_1	2λ/Extract	Extract	1	2λ	2
C_1	2λ/Water for BET	Water for BET	1 2 4 8	2λ 1λ 0.5λ 0.25λ	2 2 2 2
D_1	None/Water for BET				2

Solution A_1 = solution of endotoxin extract (from gel) and presumed to be free of detectable endotoxins; Solution B_1 = solution A containing standard endotoxin at a concentration of 2λ (positive product control); Solution C_1 = series of water for BET containing standard endotoxin at concentrations of 2λ, 1λ, 0.5λ and 0.25λ; Solution D_1 = water for BET (negative control)

extract, $V_{extract}$ = volume of the extract, and M_{gel} = weigth of the gel sample.

Solutions A_1, B_1, C_1 and D_1 were prepared as shown in Table 2 and tested according to the procedure for the confirmation of the labelled lysate sensitivity described above. The test was not validated unless the following three conditions were met: both replicates of solution D_1 (negative control) were negative; both replicates of solution B_1 (positive product control) were positive; the geometric mean end-point concentration of solution C_1 was in the range of 0.5λ to 2λ. To determine the endotoxin concentration of solution A_1, the end-point concentration for each replicate series of dilutions was calculated by multiplying each end-point dilution factor by 1λ. The endotoxin concentration in the extract was the geometric mean end-point concentration of the replicates (see Eq 1).

If none of the dilutions of the extract was positive, the endotoxins concentration was less than 1λ. The gel met the requirements of the BET if its endotoxin concentration was less than the endotoxin limit concentration.

Statistical analysis

Statistical analysis was performed using GraphPad PRISM version 2.01 (GraphPad Software Inc., USA). Values of $p < 0.05$ were considered significant. Mann-Whitney test was used to compare the endotoxin concentration of the gel and the theoretical (calculated) endotoxin limit concentration (in the gel).

RESULTS

Confirmation of labelled lysate sensitivity

The results of the gel-clot tests to confirm labelled lysate sensitivity are shown in Table 3. Geometric mean end-point = antilog [(3log 0.015 + log 0.0075) / 4] = 0.0126 IU/ml.

Table 3: Results of the gel-clot tests to confirm the labelled lysate sensitivity (n = 4)

Endotoxin concentration	Tube number			
	1	2	3	4
2λ (= 0,030 IU/ml)	+	+	+	+
1λ (= 0,015 IU/ml)	+	+	+	+
0.5λ (= 0,0075 IU/ml)	-	-	+	-
0.25λ (= 0,0037 IU/ml)	-	-	-	-
0λ (= water for BET)	-	-	-	-

- = absence of formation of viscous gel (negative reaction)
+ = formation of viscous gel (positive reaction)

Table 4: The results of gel-clot tests to verify absence of interfering factors during BET of the gels (n = 4)

Solution	Endotoxin concentration/ solution in which endotoxins were added	Initial endotoxin concentration	Tube number			
			1	2	3	4
A	None/Extract		-	-	-	-
B	2λ/Extract	2λ (= 0,030 IU/ml)	+	+	+	+
		1λ (= 0,015 IU/ml)	+	+	+	+
		0.5λ (= 0,0075 IU/ml)	-	-	-	-
		0.25λ (= 0,0037 IU/ml)	-	-	-	-
C	2λ/Water for BET	2λ	+	+	+	+
		1λ	+	+	+	+
		0.5λ	-	-	-	-
		0.25λ	-	-	-	-
D	None/Water for BET	0	-	-	-	-

- = absence of formation of viscous gel (negative reaction)
+ = formation of viscous gel (positive reaction)

Therefore, the measured sensitivity of the LAL reagent was 0.0126 IU/ml.

Test for interfering factors

Table 4 shows the results of the gel-clot tests verifying absence of interfering factors during BET of the gel. The geometric mean end-point concentration of solutions B and C (i.e., sensitivity of the lysate with solutions B and C) was equal to antilog [(4log 0.015)/4] = 0,015 IU/ml.

Endotoxin limit concentration (ELC)

The maximum recommended dose of the gel per kg body weight (over a 24 h period) was 1.71 g, i.e., 0.0712 g/kg/h. The lowest threshold pyrogenic dose of endotoxins per kg body weight over a single hour period (K) was 0.2 IU. The concentration of the test solution was 100 mg/5 ml, i.e., 20 mg/ml. ELC in the extract = (0.2 IU/kg/h x 20 mg/ml)/ 71.2 mg/kg/h, i.e., 0.056 IU/ml. Therefore, ELC in the gel was (0.056 IU/ml x 5 ml)/ 100 mg, i.e., 0.0028 IU/mg. Thus, the maximum valid dilution (MVD) of the extract was (0.056 IU/ml)/ 0.015 IU/ml, i.e., 3.73.

Endotoxin concentration in the gel

Table 5 shows the results of gel-clot tests to determine endotoxin concentration in the extract. These results were similar for all the three batches of the gel. The geometric mean end-point concentration of solution C_1 = antilog [(2log 0.015)/2], i.e., 0.015 IU/ml. As neither diluted extract nor initial extract was positive, the endotoxin concentration in the extract was less than λ, i.e., 0.015 IU/ml. Therefore, the endotoxin concentration in the

Table 5: The results of gel-clot tests to determine endotoxin concentration in extracts (n = 4)

Solution	Endotoxin concentration/ solution in which endotoxins were added	Dilution factor	Initial endotoxin concentration	Tube	
A$_1$	None/Extract	1		-	-
		2		-	-
		4		-	-
		8		-	-
B$_1$	2λ/Extract	1	2λ	+	+
C$_1$	2λ/Water for BET	1	2λ	+	+
		2	1λ	+	+
		4	0.5λ	-	-
		8	0.25λ	-	-
D$_1$	None/Water for BET		0	-	-

- = absence of formation of viscous gel (negative reaction)
+ = formation of viscous gel (positive reaction)

gel was less than (0.015 IU/ml x 5ml)/100 mg, i.e., 0.00075 IU/mg.

DISCUSSION

The gel-clot technique for bacterial endotoxins test is based on the gelation of a lysate of amoebocytes (*limulus* amoebocyte lysate) from the horseshoe crab, *Limulus polyphemus* or *Limulus tachypleus*. The addition of a solution containing endotoxins (at least 1λ concentration) to a solution of the lysate produces gelation of the mixture; λ is the labelled lysate sensitivity.

The sensitivity of the lysate (LAL reagent) in the presence or absence of the extract was at least 0.5 times but not more than twice the labelled lysate sensitivity, i.e., between 0.0075 and 0.0300 IU/ml. All negative controls were negative and positive controls were positive for all gel-clot tests (Tables 3 - 5). According to pharmacopoeias [3,7,8], these results confirm the sensitivity of the labelled lysate. The extract neither inhibited nor activated gel formation (see Table 4), suggesting that it was suitable for bacterial endotoxin test. The sensitivity of the lysate remained valid when it was determined with a solution derived from artificially contaminated gel (see results of the test for interfering factors). Therefore, the developed technique

achieved the extraction of the whole endotoxin from the gel.

Due to the physicochemical properties of the gel, we could not extract endotoxin directly with water for BET [10]. It was only feasible after dissolution of the gel in lipidic solvent (isopropyl myristate). The hydrophilic property of the endotoxin enabled them to migrate in the aqueous phase after liquid-liquid phase extraction. Endotoxins, which are lipopolysaccharides, are composed of a hydrophilic polysaccharide moiety, which is covalently linked to a hydrophobic lipid moiety [13].

As monoolein–water liquid crystalline gel of gentamicin is a novel product, their endotoxin limit concentration is not specified in the pharmacopoeias used [3,7,8]. However, the pharmacopoeias state recommendations for determining this parameter. In our study, the endotoxin limit concentration was calculated using parameters that afford the greatest safety to patients. Consequently, the lowest threshold pyrogenic dose of endotoxins per kg body weight over a single hour period (K) suggested by European Pharmacopoeia was used (0.2 IU).

The endotoxin concentration in the gels (< 0.00075 IU/mg) was less than the calculated endotoxin limit concentration (0.0028 IU/mg)

(p < 0.001). The tested gels were apyrogenic and met the requirements of three pharmacopoeias used [3,7,8]. This assertion is justified by the absence of bacterial endotoxin in the product which implies the absence of pyrogenic components [8]. Though pyrogens are a chemically heterogeneous group of fever-inducing compounds, endotoxins from Gram-negative bacteria are of major concern to the pharmaceutical industry due to their ubiquitous sources, stability and highly toxic reactions [6,14].

CONCLUSION

We have demonstrated that it is possible to perform *in vitro* endotoxin test (BET) on liquid crystalline gels. Application of this developed technique enabled us to perform *in vitro* endotoxins test of a monoolein–water liquid crystalline gel intended for use as an implant. The tested batches of gel were apyrogenic under the conditions of the study. The *in vitro* endotoxin test that we developed may be used to assess the safety of a wide range of insoluble parenteral drugs.

ACKNOWLEDGMENT

REFERENCES

1   Avis EK, Lieberman AH, Lachman L. *Pharmaceutical dosage forms: parenteral medication. Volume 2.* 2nd ed., Marcel Dekker, Inc, New York, 1993

2   Poole S, Mistry Y, Ball C, Rose E, Gaines Das REG, Opie LP, Tucker G, Patel M. A rapid 'one-plate' in vitro test for pyrogens. J Immunol Methods, 2003; 274: 209– 220.

3   The International Pharmacopoeia, World Health Organization (WHO), Geneva, 4th ed, 2006; 30-32.

4   Zijlstra S, Gerken P, Rechin C, Wortmann R, Notohamiprodjo G. Validation of the limulus amebocyte(LAL) test for routine PET Radiopharmaceuticals Lysate. Appl. Radiat Isot, 1997; 48(1): 51-54.

5   Eperon S, De Groote D, Werner-Felmayer G, Jungi TW. Human monocytoid cell lines as indicators of endotoxin: comparison with rabbit pyrogen and Limulus amoebocyte lysate assay. J Immunol Methods, 1997; 207: 135-145.

6   Hoffmann S, Peterbauere A, Schindler S, Fennrich S, Poole S, Mistry Y, Montag-Lessing T, Spreitzer I, Lfschner B, Van Aalderen M et al. International validation of novel pyrogen tests based on human monocytoid cells. J Immunol Methods, 2005 ; 298 : 161– 173.

7   United States Pharmacopeia, 25th ed. Rockville, USA: United States Pharmacopeial Convention, Inc, 2002; 1829-1830.

8   European Pharmacopoeia, 5th ed, Paris: Jouve, 2005; 89-96. .

9   Rowe RC, Sheskey PJ, Weller PJ. Handbook of pharmaceutical excipients. 4th ed., The pharmaceuticals society of Great Britain and the American pharmaceutical association, Washington, London, 2003.

10 Ouédraogo M, Semdé R, Somé TI, Traoré-Ouédraogo R, Guissou IP, Henschel V, Dubois J, Amighi K, Evrard B. Monoolein–Water Liquid Crystalline Gels of Gentamicin as Bioresorbable Implants for the Local Treatment of Chronic Osteomyelitis: In Vitro Characterization. Drug Dev Ind Pharm, 2008; 34: 753–760.

11 Ouédraogo M, Sanou M, Ramdé N, Goumbri O, Somé TI, Semdé R, Ouédraogo R, Guissou IP, Henschel V, Evrard B, Amighi K, Dubois J. In vivo Biocompatibility and Toxicity Assessment of a Gentamicin-Loaded Monoolein Gel Intended to Treat Chronic Osteomyelitis. J. Pharmacol. Toxicol., 2008; 3(5): 386-393.

12 Ouédraogo M, Nacoulma WC, Semdé R, Somé TI, Guissou IP, Henschel V, Evrard B, Amighi K, Dubois J. In Vitro Biocompatibility and Genotoxicity Assessment of a Gentamicin-Loaded Monoolein Gel Intended to Treat Chronic Osteomyelitis. J. Pharmacol. Toxicol., 2008; 3(5): 386-393.

13 Magalahaes PO, Lopes AM, Mazolla PG, Rangel-Yagui C, Penna TCV, Pessoa Jr A. Methods of Endotoxins Removal from Biological Preparations: a Review. J Pharm Pharmaceut Sci. 2007; 10(3): 388-404.

14 ennrich S, Fischer M, Hartung T, Lexa P, Montag-Lessing T, Sontag HG, Weigandt M, Wendel A. Detection of endotoxins and other pyrogens using