Latin American Journal of Pharmacy
(formerly *Acta Farmacéutica Bonaerense)*
Lat. Am. J. Pharm. **28** (1): 86-90 (2009)

Original Article

Received: September 29, 2008
Accepted: December 8, 2008

Using the *In Vitro* Pyrogen Test in the Validation of Depyrogenation Process by Dry-Heat

Rolando PERDOMO MORALES *[1], Ingo SPREITZER [2], Vivian MONTERO ALEJO [1], Bettina LOESCHNER [2] & Thomas MONTAG-LESSING [2]

[1] *Centro de Investigación y Desarrollo de Medicamentos (CIDEM), Ave 26 N° 1605, Plaza, CP 10600, Ciudad de la Habana, Cuba*
[2] *Paul Ehrlich Institute, Paul-Ehrlich Strasse 51-59, D-63225 Langen, Germany*

SUMMARY. **In the present study, the feasibility to employ the *in vitro* pyrogen test (IPT) in the validation of depyrogenation process is presented. As one of the main advantages of IPT is its ability to determine pyrogen absorbed to the container surface, direct incubation of diluted whole blood with the endotoxin indicator was first attempted. It was not possible to quantify the endotoxin in control indicators due to the high content, which is discussed. However, it was possible to demonstrate that indicators subjected to the depyrogenation process were indeed pyrogen free, a quality that is difficult to assure when the LAL assay is employed in extract of indicators or medical devices. On the other hand, IPT performed as well as LAL when endotoxin was previously extracted from the indicator surface. Finally, some conditions for incubation of whole blood with the test surface and to dilute the supernatant obtained from the incubation are presented.**

INTRODUCTION

Access of endotoxin into the systemic circulation can lead to severe damages to the human health, including fever, shock and death. Endotoxins are exogenous pyrogens located in the outer membrane of Gram negative bacteria. There are other classes of pyrogens such as peptidoglycan, lipoteichoic acid, certain viruses, steroids, and enterotoxins [1].

The production of pharmaceuticals includes depyrogenation procedures in order to assure that parenteral products and medical devices are free of pyrogens. The Limulus Amebocyte Assay (LAL) is widespread employed in the validation of depyrogenation processes and in pyrogen batch release of medical devices. However, the Limulus test has disadvantages such as occurrence of false positives [2], and low and variable recovery when extracts of solid surfaces are tested [3]. The level of endotoxin recovery will depend on endotoxin purity, excipients, the method used to fix the endotoxin to the container surface, and the class of surface (glass or plastic) [3].

Hartung & Wendel [4] described a novel pyrogen test using human whole blood. This test is based on the response of blood cells, mainly monocytes and macrophages, to external pyrogens causing the release of endogenous pyrogens such as IL-1β, TNF-α and IL-6 [5-8]. There are reports suggesting the potential advantages of the novel pyrogen test to assess medical devices [9]. The method has been successfully applied for testing different classes of pyrogens in polymeric surfaces where the use of LAL presents serious disadvantages [10].

The aims of the present study are to evaluate the performance of the new pyrogen test in the validation of depyrogenation processes, and finally to confirm its use as end product pyrogen test for medical devices.

KEY WORDS: Depyrogenation, Endotoxin, *In vitro,* Pyrogen, Validation, Whole blood.

* Author to whom correspondence should be addressed. *E-mail:* rolando.perdomo@infomed.sld.cu

ISSN 0326-2383

MATERIAL AND METHODS

Validation of depyrogenation process

Endotoxin indicators used in this study were produced in the Centro de Investigación y Desarrollo de Medicamentos. Each vial contains E. Coli 055:B5 endotoxin with 2400 EU as determined by LAL assay. In the validation of depyrogenation, the level of endotoxin in control (non-treated), and treated indicators was determined. Treated indicators were those subjected to a depyrogenation cycle by dry heat at 250 °C for 30 min.

In vitro pyrogen test

Blood collection

Five millilitres of blood from human healthy volunteers were drawn using a syringe (Multifly®, Sarstedt) connected to a tube coated with sodium heparin (S-Monovette®, Sarstedt,). This system avoids the contact of the whole blood with the environment and hence reduces the risk of microbial contaminations which can lead to false positives.

Incubation conditions

First, an experiment was designed to evaluate the level of IL-1β obtained from the whole blood incubation with the indicators with or without shaking at 37 °C. In addition, as IL-1β is not a quite stable protein, it was decided to evaluate diluents such as physiological saline and human plasma to produce the different dilutions of the protein. Hundred microlliliters of whole blood and 1.1 ml of saline were added into four indicator vials and were incubated 24 h at 37 °C. Three vials were incubated with shaking while the other three were resting. Twenty-four hours later, the content of each vial was homogenized, and centrifuged at 12000g 3 min. The supernatant was diluted either with physiological saline solution or human plasma to 1/5, 1/10 and 1/50 to determinate IL-1β by ELISA. Result were analyzed using one way ANOVA to compare the optical density of samples incubated with or without shaking, and diluted with saline or plasma.

Determination of IL-1β level obtained by direct incubation of whole blood with the indicator surface

Hundred microlliter of whole blood and 1.1 ml of saline were added to each endotoxin indicator. Each vial was incubated at 37 °C for 24 h. The supernatant was obtained as described above and diluted in two fold dilution step with saline for IL-1β determination.

Determination of IL-1β response in indicators extracts.

The extracts were obtained using the same methodology used to test in Limulus assay [11]. Briefly, one milliliter of pyrogen free water was added to each indicator (treated and control) and vortex for one minute each ten minute during an hour. In control indicators, the extract was diluted 1/1000 with pyrogen free water, and thereafter two fold dilutions were prepared. Treated indicator extracts were directly diluted in a two fold serial dilution. Hundred microlliters of each extract were incubated with 100 µl whole blood and 1 ml saline for 24 h at 37 °C. Whole blood incubation was carried out as described above. Supernatants were directly employed in IL-1β determination.

ELISA determination of IL-1 β

Determination of IL-1β level was conducted using ELISA Endosafe-IPT (InVitro Pyrogen Test) according to the manufacturer instruction. Endotoxin quantification was conducted using the supernatant of a 1 log. endotoxin standard curve incubated with the whole blood. Valid quantitative assays required a regression coefficient of the plot between OD and endotoxin concentration higher than 0.98. Ranges studied were 0.25-2.5 EU/ml and 0.5-5 EU/ml.

RESULTS

The results of the study encompassing the level of IL-1β produced vs. the incubation mode with whole blood are displayed in Table 1. The IL-1β level produced, as assessed by the OD values, were higher when the vial is incubated without shaking. Significant differences ($p < 0.05$) between the IL-1β levels produced between the shaking modes were found. However, there was no significant difference ($p < 0.05$) in the IL-1β level (see OD values in Table 1) when the supernatant is diluted with saline or plasma. Therefore, in the assays where the IL-1β level is determined by direct incubation of diluted blood with the container surface conditions without shaking were selected. In addition, saline was used to dilute the IL-1β in the supernatant if necessary.

The regression coefficient obtained for 0.25-2.5 EU/mL endotoxin curve was 0.82. However, that for 0.5-5 EU/mL was 0.983. Therefore, for the quantitative approach this last curve was employed.

Table 2 describes the endotoxin concentration obtained when indicators were incubated directly with diluted whole blood. It was not

Dilutions	Supernatant diluted with saline		Supernatant diluted with plasma	
	Incubation with shaking (OD)	Incubation without shaking (OD)	Incubation with shaking (OD)	Incubation without shaking (OD)
Undiluted	2.291 ± 0.0085	3.225 ± 0.198	2.181 ± 0.019	2.872 ± 0.284
1/5	0.497 ± 0.010	0.755 ± 0.030	0.492 ± 0.006	0.659 ± 0.08
1/10	0.276 ± 0.014	0.408 ± 0.024	0.269 ± 0.006	0.352 ± 0.045
1/50	0.091 ± 0.0079	0.117 ± 0.010	0.092 ± 0.003	0.106 ± 0.005

Table 1. Evaluation of the incubation mode of whole blood with the container surface on the IL-1β level. OD: Optical density. Result are the average value of three vials ± SD.

Dilutions	Control EU/ml	Depyrogenated EU/ml
Undiluted	-	≤ 0.5
1/2	5.75	≤ 0.5
1/4	1.48	≤ 0.5
1/8	≤ 0.5	≤ 0.5
1/16	≤ 0.5	≤ 0.5
1/32	≤ 0.5	≤ 0.5
1/64	≤ 0.5	≤ 0.5
1/128	≤ 0.5	≤ 0.5

Tabla 2. Endotoxin concentration in supernatants obtained by direct incubation of diluted whole blood with the indicator surface. EU: Endotoxin Units. One Endotoxin Unit (EU) represent the activity in the LAL assay of 0.1 ng of endotoxin from *E. coli* 0113:h10:k, which is the endotoxin primary standard from FDA, USP, WHO and EP.

possible to quantify the endotoxin on undiluted sample due to its high content that was above the higher standard concentration (5 EU/ml). Endotoxin concentration fails inside the standard curve for 1/2 and 1/4 dilutions. After correct for the dilution factor, average concentration in control vials was 8.7 EU/ml or, considering the volume of incubation mixture (1.2 ml; see material and methods), 10.4 EU/vial. The endotoxin indicator used through the present study contains 2400 EU/vial as determined by LAL assay. Therefore, using the direct incubation of endotoxin indicator with diluted whole blood, only around 0.4% endotoxin content seems to be recovered compared to that obtained when using extracts evaluated by LAL assay.

On the other hand, the OD values obtained in treated or depyrogenated vials were beneath the lower endotoxin standard (0.5 EU/ml), and at the same level of the blank (data not shown), producing negatives values of concentration with the standard curve used. Therefore, it was possible to demonstrate with this methodology

Dilution	Control EU/ml	Depyrogenated EU/ml
Undil	1.05	≤ 0.5
1/2	0.48	≤ 0.5
1/4	≤ 0.5	≤ 0.5

Tabla 3. Determination of endotoxin concentration in indicator extracts. Extracts obtained from control indicators were firstly diluted 1/1000, while treated indicators were two fold diluted directly from the extract.

that there was no endotoxin in treated vials when the indicator is incubated directly with diluted whole blood. These cases will be referred as lower or equal than 0.5 EU/ml in the rest of the study, as it showed in the tables.

Endotoxin concentrations in extracts of endotoxin indicators in treated and control vials using the *in vitro* pyrogen test are displayed in Table 3. It was possible to calculate the endotoxin concentration at undiluted and 1/2 dilutions. After correct for the dilution factor (x 1000), control vials contained approximately 1050 EU/ml, while treated vials contain less than 0.5 EU/ml. Therefore, it is possible to determine the logarithmic reduction of the depyrogenation cycle (log. Endotoxin concentration in controls - log. endotoxin concentration in treated vials). The value obtained was 3.6 log., higher than the lower limit (3 log) defined by regulator entities such as the United State Pharmacopoeia.

DISCUSSION

For safety reasons, quality control of medical devices designed to be in contact with the systemic circulation, such as catheters, needles and implants, claim for end product pyrogen testing. Currently, the Limulus test is widely employed for such purpose. United State pharmacopoeia established that each device should contain 20 EU/unit or less than 0.5 EU/ml in water extracts

[12]. Using of LAL test is also described by FDA [13] and by ISO 10993-11. However, special cases require the use of the rabbit pyrogen test, which is regulated in ISO 10993-11, the European guideline for medical device (Guideline 93/42/EEC) and the European Standard normative (EN).

On the other hand, good manufacturing practice of parenteral products includes the validation of depyrogenation cycles. The use of endotoxin indicator is described in the United State pharmacopoeia. Validation is successful if a 1000 fold reduction of endotoxin content in treated endotoxin indicators is achieved compared to non treated indicators [14]. Validation of depyrogenation cycles and pyrogen test on medical devices, share that for both it is necessary to elute or to extract the endotoxin from the container surface. A homogenous phase is required, i.e. these assays are limited to the soluble endotoxin. Therefore, pyrogenic contaminants have to be extracted from the original material. It is well know that endotoxin tends to adhere tightly to container surfaces. Endotoxin recovery ranged between 12-89 % in polystyrene while less than 1 % has been recovered from polypropylene. In glass surfaces, like soda lime and borosilicate, recoveries ranged from 8-63% and 3-60%, respectively, being borosilicate the most variable [3].

Among the properties of the novel *in vitro* pyrogen test, the direct contact between the relevant cells with the surface of a material (and/or probably bound Endotoxin) is the initial event which might lead to the production of endogenous mediators. In fact, the basic physical-chemical principle of the assay is a heterogeneous phase. Studies using *in vitro* pyrogen test have shown an increased detection limit for endotoxin in polystyrene and polypropylene surface compared to LAL 10. This result suggests the usefulness of the novel method to determine pyrogens on plastic surfaces, where LAL assay use is troublesome.

Taking together the stated above, the performance of the IPT in validation, control and monitoring of depyrogenation process was evaluated. In the depyrogenation validation study, where the diluted human whole blood was incubated directly with the indicator surface, no suitable results were obtained. Production of IL-1β by endotoxin activated monocyte/macrophage present a dose response range between 10-100 pg/ml of endotoxin. Further increase of endotoxin in several orders above this range will only slightly increase the IL-1β production [6]. Endotoxin indicators are designed to be used in conjunction with the LAL assay, and should contain at least 1000 UE/vial (0.1 µg endotoxin per vial), which is a definitively high concentration, out of the range where a dose-response in IL-1β production vs. endotoxin content may occur. Hence, it is not possible to employ the *in vitro* pyrogen test by using the supernatant obtained from the incubation of the whole blood with the container surface of the endotoxin indicator. However, it is possible to employ extracts of indicators to evaluate the IL-1β production after its incubation with whole blood. Therefore, this approach is as suitable as LAL assay to evaluate endotoxin content during a validation of depyrogenation process as is currently designed.

Further evaluation of the performance of IPT on control of pyrogens in medical devices was conducted using the treated indicators which were apparently depyrogenated. Optical density obtained in such samples was at the same level as blank, therefore no IL-1β was produced and it is possible to define the product as pyrogen free. In quality control for pyrogens in medical devices, where high levels of pyrogens (like those found in the artificially spiked endotoxin indicators) are not expectable, the use of the *in vitro* pyrogen test approach seems to be advantageous. This benefit is based on the ability of the method to detect pyrogens adhered to the surface, avoiding the extraction step and its known restrictions. The best way to carry out this is incubating the device with diluted whole blood without shaking at 37 °C for 24 h. In addition it is possible to dilute the supernatant with physiological saline before the IL-1β determination by ELISA (if required).

CONCLUSION

Because of the characteristic of the *in vitro* pyrogen test, incubation between the device and blood partly mimics the physiological conditions in which the device will be employed. Hence, the pyrogen free condition could be guaranteed, no matter what class of pyrogen could be involved. On the other hand, because it is possible to quantify more than 1000 fold reduction in endotoxin content, it is possible to employ IPT for validation of depyrogenation process when extract of indicator are used. However, with this approach the main advantages to IPT are missed. This issue would require further studies, probably focusing in the new design of endotoxin indicators.

REFERENCES

1. Perdomo Morales, R. (2004) *Rev. Cubana. Farm.* **38**: 33-8.
2. Rolansky, P.F., M.E. Dawson & T.J. Novitsky (1991) *J. Parent. Sci. and Technol.* **45**: 83-7.
3. Novitsky, T.J., J. Schmidt-Gengenbach & J.F. Remillard (1986) *J. Parent. Sci. Technol.* **40**: 284-6.
4. Hartung, T. & A. Wendel (1996) *In Vitro Toxicol.* **9**: 353-9.
5. Hartung, T., S. Fennrich, M. Fischer, T. Montag-Lessing & A. Wendel (2000) *"Prevalidation of an alternative to the rabbit pyrogen test based on human whole blood",* in "Progress in the reduction refinement and replacement of animal experimentation", (M. Balls, A.M. van Zeller & M.E. Halder, eds.) Elsevier Science, pp. 991-9.
6. Fennrich, S., M. Fischer, T. Hartung, P. Lexa, T. Montag-Lessing, H.G. Sonntag, M. Weigandt & A. Wendel (1999) *"Detection of endotoxins and others pyrogen using human whole blood",* in *"Alternatives to animals in the development and control of biological products for human and veterinary use",* (F. Brown, C. Hendriksen & D. Sesardic, eds.) Karger, Basel, pp. 131-9.
7. Jahnke, M., M. Weigand & H.G. Sonntag (2000) *Eur. J. Parent. Sci.* **5**: 39-44.
8. Poole, S. & R.E. Gaines (2001) *Eur. J. Parent. Sci.* **6**: 63-4.
9. Fennrich, S., A. Wendel & T. Hartung (1999) *ALTEX.* **16**: 146-9.
10. Petri, E., A. van de Ploeg, B. Habermaier & S. Fennrich (2000) *"Improved detection of pyrogenic substances on polymer surfaces with an ex vivo human whole-blood assay in comparison to the Limulus amoebocyte lysate test",* in "Progress in the reduction refinement and replacement of animal experimentation", (M. Balls, A.M. van Zeller & M.E. Halder, eds.) Elsevier Science, pp. 339-45.
11. Dawson, M.E. (1983) *LAL Update* **11**: 1-4.
12. The United States Pharmacopeia 27 (2003) *"Transfusion and Infusion assemblies and similar medical devices"* <161>. Rockville: The United States Pharmacopeial Convention, pp. 2195-6.
13. Food and Drugs Administration (1987) *"Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products and Medical Devices".* U.S. Department of Health and Humans Services, Public Health Service, December, 1987.
14. The United States Pharmacopeia 27 (2003) *"Sterilization and sterility assurance of compendial articles"* <1211>. Rockville: The United States Pharmacopeial Convention, pp. 2616-21.