Technological research article / https://doi.org/10.15446/rcciquifa.v52n2.106516

Determination of bacterial endotoxins in normal intravenous human immunoglobulin on the replacement of rabbit pyrogen testing in Brazil

Alessandra Vidal Pereira*, Hendro Freitas de Farias, Fernando Faria Fíngula, Sheila Regina Gomes Albertino, Lilia Serodio

Instituto Nacional de Controle de Qualidade em Saúde (INCQS), Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, RJ, Brasil.

*Corresponding author E-mail: lilia.serodio@gmail.com

Received: December 22, 2022
Corrected: March 28, 2023
Accepted: April 3, 2023

Summary

Introduction: The normal intravenous human immunoglobulin is a sterile liquid or lyophilized preparation, with immunoglobulins, especially the immunoglobulin G (IgG). The National Institute for Quality Control in Health (NIQCH) performs analysis of biological products before being distributed to the population. The batches are subjected to physicochemical and biosafety assays. The rabbit pyrogen test (RPT) is a biosafety assay performed to check the contamination by agents that cause fever in the patient and, in cases of high concentration, can lead to death. **Aim:** To compare the results of RPT and bacterial endotoxins assays of ten samples. **Methods:** In RPT, both the product is injected into the rabbit's marginal ear vein and the individual body temperature variations of the animals are evaluated. The bacterial endotoxin assay uses the kinetic chromogenic method, according to the Brazilian Pharmacopoeia and the international compendiums. **Results:** The tests of were plotted in a table for comparison. The tests coincided in 9 samples, except for a sample that presented bacterial endotoxin pyrogenicity. **Conclusion:** Bacterial endotoxin assay must be considered as an alternative method for animal tests.

Keywords: Normal human immunoglobulin, pyrogen, bacterial endotoxin.

Resumen

Determinación de endotoxinas bacterianas en inmunoglobulina humana intravenosa normal en reemplazo de la prueba de pirógenos de conejo en Brasil

Introducción: la inmunoglobulina humana intravenosa normal es una preparación líquida o liofilizada estéril, con inmunoglobulinas, especialmente la inmunoglobulina G (IgG). El Instituto Nacional de Control de Calidad en Salud (INCQS) realiza análisis de productos biológicos antes de ser distribuidos a la población. Los lotes se someten a ensayos fisicoquímicos y de bioseguridad. La prueba de pirógenos en conejo (RPT) es un ensayo de bioseguridad que se realiza para verificar la contaminación por agentes que causan fiebre en el paciente y, en casos de alta concentración, pueden causar la muerte. **Objetivo:** comparar los resultados de los ensayos de RPT y endotoxinas bacterianas de diez muestras. **Métodos:** en RPT, el producto se inyecta en la vena marginal de la oreja del conejo y se evalúan las variaciones de temperatura corporal individual de los animales. El ensayo de endotoxinas bacterianas utiliza el método cromogénico cinético, de acuerdo con la Farmacopea Brasileña y los compendios internacionales. **Resultados:** las pruebas de se trazaron en una tabla para comparación. Las pruebas coincidieron en 9 muestras, excepto en una muestra que presentó pirogenicidad de endotoxina bacteriana. **Conclusión:** el ensayo de endotoxinas bacterianas debe considerarse como un método alternativo para las pruebas en animales.

Palabras clave: Inmunoglobulina humana normal, pirógeno, endotoxina bacteriana.

Resumo

Determinação de endotoxinas bacterianas em imunoglobulina humana intravenosa normal em substituição ao teste de pirogênio em coelhos no Brasil

Introdução: a imunoglobulina humana intravenosa normal é uma preparação líquida estéril ou liofilizada, com imunoglobulinas, principalmente a imunoglobulina G (IgG). O Instituto Nacional de Controle de Qualidade em Saúde (INCQS) realiza análises de produtos biológicos antes de serem distribuídos à população. Os lotes são submetidos a ensaios físico-químicos e de segurança. O teste de pirogênio em coelhos (RPT) é um ensaio de segurança realizado para verificar a contaminação

por agentes que causam febre no paciente e, em casos de alta concentração, podem levar ao óbito. **Objetivo:** comparar os resultados dos ensaios de RPT e endotoxinas bacterianas de onze amostras. **Métodos:** no RPT, o produto é injetado na veia marginal da orelha do coelho, e as variações de temperatura corporal individual dos animais são avaliadas. O ensaio de endotoxina bacteriana utiliza o método cromogênico cinético, de acordo com a Farmacopéia Brasileira e os compêndios internacionais. **Resultados:** os resultados dos ensaios foram plotados em uma tabela para comparação. Os testes coincidiram em 9 amostras, com exceção de 2 amostra que foram discrepantes na comparação entre os dois ensaios. **Conclusão:** o ensaio de endotoxina bacteriana deve ser considerado como um método alternativo para testes em animais.

Palavras-chave: Imunoglobulina humana normal, pirogênio, endotoxina bacteriana.

Introduction

The normal intravenous human immunoglobulin is a sterile liquid or lyophilized, with immunoglobulins, especially immunoglobulin G (IgG) of normal individuals [1, 2].

The production method includes one or several steps that eliminate or inactivate the known infection agents. It should show that the residues in the final product of the substances used in the processes aimed at inactivating the virus do not have any adverse reaction in the patients treated with immunoglobulin [1, 2].

The safety immunoglobulin assays in Brazil are sterility and RPT. However, the European Pharmacopoeia admits bacterial endotoxin assay as an alternative test to the *in vitro* method [2].

Parenteral preparations have to be pyrogen-free because the administration of pyrogens may induce fever, shock, or even death. This safety test is extremely important, because immunoglobulin is used for the treatment of individuals with significant comorbidities such as primary immunodeficiency as well as a number of neurological indications, including chronic inflammatory demyelinating, polyneuropathy, and idiopathic thrombocytopenic purpura, especially in impaired individuals who had received intravenous human immunoglobulin [3, 4]. Endotoxins represent the lipopolysaccharides (LPS) of the cell wall of Gram-negative bacteria (Figure 1).

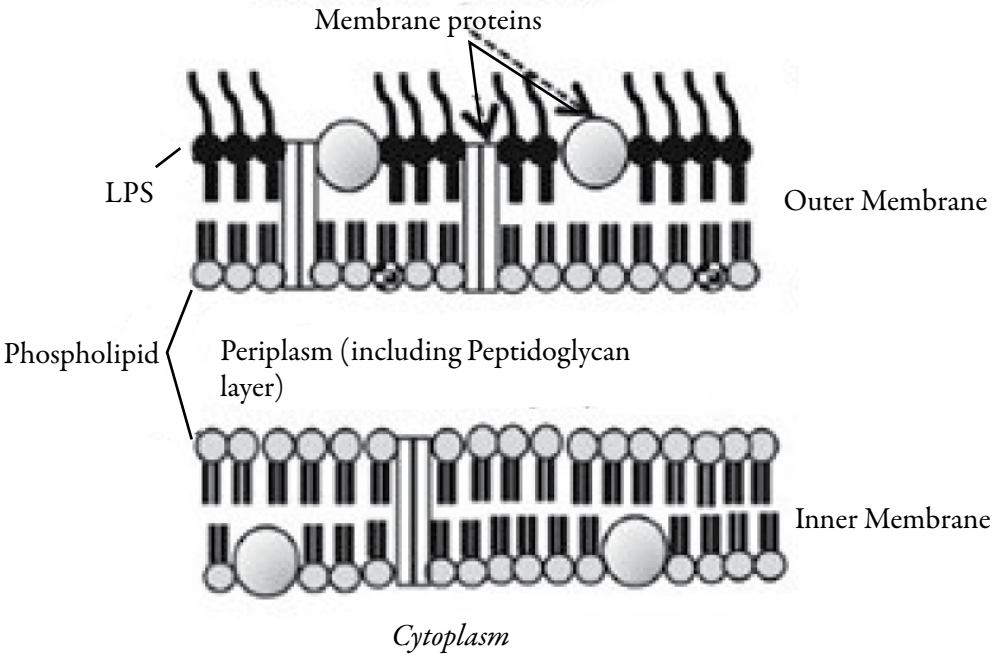

Figure 1. The outer membrane of the Gram-negative cell envelop. Source: McCullough (2019) [4].

Because endotoxins are the most common and potent pyrogens, the LAL testing has successfully replaced the rabbit pyrogen test for many products [5].

Each batch of human immunoglobulin is sent for evaluation at the National Institute for Quality Control in Health (NIQCH), Fiocruz, where biological safety tests are carried out [6].

The *in vivo* pyrogen assay involves raising the temperature of rabbits after intravenous injection of a test solution into the ear vein. In Brazil, the pyrogen test in human immunoglobulin is performed according to the Brazilian Pharmacopeia, and the recommended injection volume is 1 mL/kg of body weight [1].

Although the European Pharmacopoeia already recommends the alternative *in vitro* bacterial endotoxin method, the assay is not yet recommended in the Brazilian Pharmacopoeia as an alternative to the rabbit pyrogen assay in normal human immunoglobulin [1].

In NIQCH, the Kinetic Chromogenic LAL Assay is used, which is a quantitative kinetic assay for the detection of Gram-negative bacterial endotoxin. An endotoxin catalyzes the activation of a proenzyme in the LAL. The initial activation rate depends

on the concentration of the endotoxin. Endotoxin turns a proenzyme into an active enzyme. The enzyme para-nitroanillin (pNA) release produces a yellow color, which is measured photometrically at 405 nm. The intensity of the color corresponds to the activity of endotoxin present in the system [7].

The concentration of unknown samples can then be calculated from a standard curve. It is the chromogenic nature of the Kinetic Chromogenic LAL Assay that makes it the most appropriate choice for testing small volume parenteral solutions, vaccines, antibiotics, and biologicals [8].

This work was aimed at comparing the pyrogenic assays in rabbits and bacterial endotoxins in the immunoglobulins received by the NIQCH.

Methodology

To assess the sensitivity of animals at the threshold dose that causes fever in rabbits and humans, periodic dose-response curves are performed using standard *Escherichia coli* lipopolysaccharide (LPS) solution at non-pyrogenic (0.5 ng/mL), limit (1 ng/mL) and pyrogenic (2 ng/mL) concentrations. In the *in vivo* pyrogen assay, the product is injected into the marginal vein of the ear of three rabbits with a volume of 1 mL/kg, and the individual variations in body temperature of the animals are evaluated. In order for the product to be considered satisfactory, no animal can have a temperature variation equal to or greater than 0.5 °C. If this occurs, a retest is performed using five new rabbits. In this case, out of a total of eight animals, only three can have a temperature variation equal to or greater than 0.5 °C and the sum of the temperatures cannot exceed 3.3 °C. If any of the cases occurs, the product is considered pyrogenic.

The tests on rabbits were authorized by the Ethics Committee for the Use of Animals of the Oswaldo Cruz Foundation, license LW-1/23.

Before LAL assay the Maximum Valid Dilution (MVD) was determined in dilutions 1:50, 1:80 e 1:100 to verify the interference-free dilution, according to the equation [9]:

MVD = Control Standard Endotoxin / the lowest point used in the standard curve the bacterial endotoxin assay; the Lonza Bioscience kit was used. The Kinetic-QCL™ Kinetic Chromogenic LAL Assay is a quantitative kinetic assay used to detect Gram-negative bacterial endotoxin. A sample is mixed with the reconstituted LAL reagent in a 96-well plate and placed in an incubating plate reader that measures absorbance at 405 nm. The reaction is automatically monitored over time for the appearance of a yellow color.

In the presence of endotoxin, the lysate will begin to cleave the chromogenic substrate, causing the solution to become yellow. The concentration of unknown samples can then be calculated from a standard curve and the Kinetic-QCL™ Assay with a sensitivity of 0.005 EU/mL. The assay requires the use of a standard endotoxin termed "positive product control" (PPC), which is a known amount of endotoxin mixed with a test material to confirm the absence of interference, and the endotoxin recovery in positive control (%PPC Recovery) is within the range of 50% to 200%. The maximum specification of bacterial endotoxin considered was inferior to 0.5 EU/mL of immunoglobulin, according to the European Pharmacopoeia [2]. Labeled sensitivity λ was confirmed by using 4 replicates expressed in EU/mL of the lysate reagent before to use in the test. This confirmation was carried out for each lysate batch before being used in the test and this is carried out when the investigators use the batch of lysate for the first time.

Results and discussion

The MDV resulted in a 1:100 dilution, however, we chose to test in lower dilutions, as using the maximum dilution is not routine in our laboratory, where we could run the risk of not detecting endotoxin in the highly diluted sample.

Table 1. Determination of the Maximum Valid Dilution in two batches

Batch	Dilution	Specification (EU/mL)	Result (EU/mL)	%PPC recovery
1	1:100	< 0.5	< 0.500	68%
	1:80		0.283	140%
	1:50		< 0.500	26%
2	1:100	< 0.5	0.375	119%
	1:80		< 0.400	106%
	1:50		< 0.500	30%

The two dilutions 1:80 and 1:100 resulted in a %PPC recovery, showing the absence of interferents. Therefore, we chose to use the 1:80 dilution for all batches. Table 2 shows that, out of a total of 11 samples analyzed, 3 batches were retested in more 5 rabbits and one batch showed fail result, coinciding with the endotoxin assay. However, one sample only failed in the endotoxin assay and the rabbit pyrogen test was negative for the pyrogenic substance. The sample 11 failed in the first pyrogen test on

rabbit and was satisfactory in the retest requested by the distributor. The endotoxin assay was satisfactory from the first test (Table 2).

The pyrogen detection limit does not exceed 50-350 picograms (i.e., 0.5-3.5 EU) of lipopolysaccharide per rabbit kg [10].

The rabbit pyrogen test was studied in the context of the importance of alternatives that could contribute towards its replacement. There are three broad categories of bioassays which are commonly used for biological products: binding assays, cell-based assays, and whole animal assays. These assays can be more variable than binding assays and must be performed carefully to ensure consistent results. Whole animal assays are time consuming and highly variable. These assays are used for pyrogen assays, general safety assays, and potency assays. Binding assays typically have coefficient of variation (CV) in the 5 to 20 % range. Cell and whole animal assays may have variability above 50% [11]. In our tests, a CV of 10% is used.

Table 2. Results from ten human immunoglobulins (HIG) batches evaluated in the rabbit pyrogen and in the endotoxin (LAL) assays.

HIG batch	Rabbit pyrogen test		Endotoxin	
	Total temperature	Number of rabbits	EU/mL	% PPC Recovery
1	Passed (0.65 °C)	3	< 0.400	124%
2	Passed (0.27 °C)	3	0.283	140%
3	Passed (0.45 °C)	3	0.284	175%
4	Passed (0.17 °C)	3	< 0.400	153%
5	Passed (0.19 °C)	3	< 0.400	170%
6	Passed (0.33 °C)	3	< 0.400	117%
7	Passed (0.56 °C)	3	Fail 0.634	136%
8	Passed (0.16 °C)	3	< 0.400	117%
9	Fail (6.37 °C)	8	Fail 39.6	200%
10	Passed (2.09 °C)	8	< 0.400	90%
11	Fail (3,65 °C)	8	< 0.400	106%
11*	Passed (2.72 °C)	8	NR	NR

* Retest requested by the distributor NR – No Retest

The 3Rs – Replacement, reduction and refinement - concept has become a standard in legislation and guidelines concerning animal experimentation in many countries. Replacement involves the replacement of an *in vivo* assay with relevant non-animal-

based methods. The use of biological products must comply with the pyrogen test, according to the regulatory standards of each country. It is a qualitative test in animals, which verifies the absence or presence of contaminants. Among the most relevant factors that can interfere with the final result of *in vivo* tests are the temperature and exhaustion of the test room, sensitivity, infections by microorganisms, and the diet of the animals [12, 13]. The substitution of *in vivo* methods with non-animal assays for the quality control and batch release testing of biological products is already being introduced and recognized by some regulatory authorities [8]. Each monograph needs to be examined individually to verify whether the pyrogen test can be replaced either by the LAL test or by an *in vitro* pyrogen test. The European Pharmacopoeia has set up a Group of Experts to develop *in vitro* alternative tests for pyrogen testing. It is foreseen that when a validated in-vitro pyrogen test is available, it will be included in the European Pharmacopoeia together with appropriate guidelines for validating the *in vitro* for pyrogenic substances assays as a replacement of the pyrogen test [14].

The production process of biological products must be aimed at obtaining products of quality, safety, and efficacy. The processes used must comply with a quality assurance system and Good Manufacturing Practices (GMP) and, therefore, monitoring is necessary during the production stages, including obtaining water, reagents, and containers used for collecting blood, plasma, formulation, and final packaging. Therefore, if all measures to detect the critical steps in obtaining the products are applied, it could significantly reduce the probability of introducing agents that generate pyrogenic contamination [15].

In Brazil, the National Council for the Control of Animal Experimentation (CONCEA) published a Normative Resolution in 2016, recognizing the bacterial endotoxin assay to evaluate the pyrogenic contamination in injectable products [16].

Currently, the NIQCH receives approximately 700 batches of normal human immunoglobulin for tests per year, which requires at least 4,200 rabbits, including manufacturers, to perform the pyrogen test.

Conclusion

A wide range of animals is used for the manufacture or quality control of biological products. The National Institute of Quality Control in Health presented criteria to use bacterial endotoxin assay as an alternative method for the use of the rabbit pyrogen test in determining the pyrogen substances in order to comply with the CONCEA in the substitution of animals according to the 3 Rs. The Brazilian Pharmacopoeia should be updated, as the use of rabbits for pyrogen substance identification will be prohibited in

the end of 2024. Both methods (LAL test and RPT) used for endotoxin detection can be applied for various biological products including therapeutic proteins, vaccines, and other biological products. However, the LAL test is more accurate, fast, economical, and sensitive than RPT. Thus, the use of the bacterial endotoxin assay would be a feasible alternative for animal test to detect pyrogenic substances in normal intravenous human immunoglobulin on the replacement of rabbit pyrogen testing.

Conflict of interest

All authors report that they do not have any conflicts of interest.

References

1. ANVISA, Farmacopéia Brasileira, 6ª edição, HD018-00, Resolução de Diretoria Colegiada RDC nº 298, 2019.

2. European Pharmacopoeia, Human normal immunoglobulin for intravenous administration, 2022, Vol. 10.8, p. 6929-6930.

3. C. Zervos, T.P. Zimmerman, T. Willis, G. Flexman, J. Srivastava, R. Silverstein, M. Williams, P. Vandeberg, J.L. Culp, D. Burns, V. Barham, A. Durham, D.A. Malinzak, Immunoglobulin G from single plasma donor in immune globulin intravenous causes false positive pyrogen test, *Biologicals*, **59**, 12-19 (2019). Doi: https://doi.org/10.1016/j.biologicals.2019.04.001

4. K.Z. McCullough, "Fit for purpose" endotoxin analytes, *American Pharmaceutical Review*, 2019. URL: https://www.americanpharmaceuticalreview.com/Featured-Articles/557587-Fit-For-Purpose-Endotoxin-Analytes/

5. European Medicines Agency, *Guideline on the replacement of rabbit pyrogen testing by an alternative test for plasma derived medicinal products*, 2009. p. 1-6.

6. ANVISA, Resolução RDC nº 73 de 21/10/2008.

7. S. Iwanaga, Biochemical principle of Limulus test for detecting bacterial endotoxins, *Proc. Jpn. Acad. Ser. B*, *Phys. Biol. Sci.*, **83**(4), 110-119 (2007). Doi: https://doi.org/10.2183/pjab.83.110

8. M. Halder, Three Rs potential in the development and quality control of immunobiologicals, *ALTEX*, **18**(Suppl 1), 13-47 (2001).

9. Food and Drug Administration – FDA, *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*, 1987

10. M.A. Elkhateeb, W. Elkhatib, M. Aboulwafa, An expert review on current approaches for endotoxin detection in various biological products, *Archives of Pharmaceutical Sciences Ain Shams University*, **3**(2), 142-153 (2019). Doi: https://doi.org/10.21608/APS.2019.13058.1000

11. World Health Organization, A WHO guide to good manufacturing practice (GMP) requirements. Part 2: Validation, 1997.

12. P. Castle, Alternatives to animal testing: Achievements and recent developments in the European Pharmacopoeia, *Developments in Biological Standardization*, **86**, 21-29 (1996).

13. T. Hartung, I. Aaberge, S. Berthold, G. Carlin, E. Charton, S. Coecke, S. Fennrich, M. Fischer, M. Gommer, M. Halder, K. Haslov, M. Jahnke, T. Montag-Lessing, S. Poole, L. Schechtman, A. Wendel, G. Werner-Felmayer, Novel pyrogen tests based on the human fever reaction: The Report and Recommendations of ECVAM Workshop 43. European Centre for the Validation of Alternative Methods. European Centre for the Validation of Alternative Methods, *Alternatives to Laboratory Animals*, **29**(2), 99-123 (2001). Doi. https://doi.org/10.1177/0261192901029002

14. E. Charton, P. Castle, Reduction, replacement and refinement of animal tests in the European Pharmacopoeia: Recent developments for monographs on biological substances and preparations, *PHARMEUROPA*, **13**(1), 94-96 (2001).

15. ANVISA, Resolução RDC nº 658, 30 de março de 2022.

16. CONCEA, Resolução Normativa no 31, 18 de agosto de 2016.

How to cite this article

A. Vidal-Pereira, H. Freitas de Farias, F. Faria-Fíngula, S.R. Gomes-Albertino, L. Serodio, Determination of bacterial endotoxins in normal intravenous human immunoglobulin on the replacement of rabbit pyrogen testing in Brazil, *Rev. Colomb. Cienc. Quim. Farm.*, **52**(2), 816-825 (2023). https://doi.org/10.15446/rcciquifa.v52n2.106516