Endotoxin test by recombinant Factor C for 0.9% sodium chloride injection

Teste de endotoxina por Fator C recombinante para solução injetável de cloreto de sódio 0,9%

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ABSTRACT

Endotoxin contamination is a threat to the safety of pharmaceutical products, especially parenteral drugs. Any sterile and/or pyrogen-free pharmaceutical product requires regulatory specifications to ensure safe patient use. This study covers the performance evaluation study of an endotoxin quantitation commercial kit by recombinant Factor C (rFC), Endozyme II® Go, for 0.9% sodium chloride injection. The samples were spiked with endotoxin solutions between 0.0005 and 10 EU/mL and tested by the rFC kit to evaluate precision, accuracy, detection and quantification limits, linearity, and robustness. Each of the six points was assayed at least five times. The relative standard deviation for precision testing ranged from 1.9 to 8.3%. The recovery accuracy values of endotoxin were between 61% and 125% for the range from 0.005 to 10 EU/mL. The results demonstrated that the rFC method allows endotoxin quantification with accuracy, precision, specificity, and linearity for the range of 0.005 and 10 EU/mL for 0.9% sodium chloride injection.

Keywords. Recombinant Factor C, Endotoxins, In Vitro Test, Saline Solution.

RESUMO

A contaminação por endotoxinas é uma ameaça à segurança dos produtos farmacêuticos, especialmente dos medicamentos parenterais. Qualquer produto farmacêutico estéril e/ou livre de pirogênios requer especificações regulatórias para garantir a segurança de uso para o paciente. Este estudo abrange o estudo de avaliação de desempenho empregando o kit comercial Endozyme II® Go para quantificação de endotoxina, por Fator C recombinante (FCr), em amostras de cloreto de sódio 0,9% para uso parenteral. As amostras foram fortificadas com cinco concentrações distintas de soluções de endotoxina na faixa entre 0,0005 e 10 UE/mL. Cada um dos cinco níveis foi testado pelo menos cinco vezes para avaliação dos critérios de precisão, exatidão, limites de detecção e quantificação, linearidade e robustez. O desvio padrão relativo para os testes de precisão variou de 1,9 a 8,3%. Os valores de recuperação de endotoxina para o parâmetro exatidão estiveram compreendidos entre 61% e 125%. Os resultados demonstraram que o método por FCr permite a quantificação de endotoxinas com exatidão, precisão, especificidade e linearidade para a faixa de 0,005 e 10 UE/mL em amostras de cloreto de sódio 0,9% para uso parenteral.

Palavras-chave. Fator C Recombinante, Endotoxinas, Técnicas In Vitro, Solução Salina.

INTRODUCTION

Endotoxin contamination is a threat to the safety of pharmaceutical products, especially parenteral drugs. To ensure the safety of patients, any sterile and/or pyrogen-free pharmaceutical product should be evaluated to verify compliance with regulatory specifications[1]. The presence of substantial amounts of endotoxin in the bloodstream can cause fever, chills, hypotension, breathing difficulties in adults, diffuse intravascular coagulation, and multiple organ failure[2]. In the human bloodstream, the presence of low endotoxin levels, between 0.4 and 0.8 ng/kg, may cause slight depression in the immune system and a mild increase in tumor necrosis factor (TNF) and interleukins (IL-6) levels in the blood. In contrast, a dose range of 2 to 4 ng/kg body weight can cause flu-like symptoms (fever, myalgia, headaches, nausea) and an increase of TNF and IL-6 in the blood, in a similar mechanism observed in sepsis frames[3].

Microorganisms related to water treatment systems are generally Gram-negative bacteria, such as *Pseudomonas* spp, and can grow in nutrient-poor environments, such as water or saline solutions. In these environments, endotoxins can be found at elevated levels, except in highly purified water forms, as the water for injection[4].

Therefore, to ensure that all parenteral products for human use are endotoxin-free, quality control testing is mandatory[5]. The pyrogen test in rabbits (Rabbit Pyrogen Test – RPT), developed at the beginning of the 20th century, has proven its utility in establishing drug safety for many years[6].

However, this in vivo test is expensive, ethically questionable, and not suitable for some pharmaceutical products[7]. An alternative test, based on the observation that horseshoe crab hemolymph (*Limulus polyphemus*) clots in the presence of endotoxins, was later developed. The called *Limulus* amebocyte lysate test (LAL) replaced RPT when the potential contaminants are endotoxins. The transition to using the LAL test instead of rabbits represented an incremental step away from laboratory animal-based assays to endotoxin quantification in pharmaceutical products. However, some biologicals, such as plasma fractions or plasma-derived medicinal products, may contain components that interfere with the detection of endotoxins in different LAL testing methods[8-10]. Besides, the LAL test depends on an animal population and can contribute as a significant source of injury and potential mortality for the horseshoe crab population[11], making it difficult to comply with the commitment to replace and reduce animal use when possible[12].

There are alternatives to RPT and LAL tests, considering the importance of reducing animal testing for pharmaceutical products' quality control. A kit for pyrogen testing based on the Monocyte activation test (MAT), which detects IL-6 induced in human blood monocytes stimulated in vitro by pyrogens, was developed[13,14], validated in an international collaborative study[15], and evaluated for the quality control routine of biological products. However, its drawbacks did not make it the ultimate alternative test for pyrogens[16]. Another alternative is the use of the recombinant factor C (rFC). Factor C is a zymogen, part of the horseshoe crab clotting cascade cloned and expressed in cell cultures first in 1997[17]. In the traditional LAL test, the endotoxin is detected by a visible gel clot formation as a result of a cascade of serine protease reactions started by Factor C activation[18]. Some limitations of this method include false-positive results obtained by cross-reaction of LAL with β-glucans interference. In fact, in the presence of endotoxin, the enzyme is activated, and the active enzyme cleaves a synthetic substrate, resulting in a fluorogenic compound.

Although the reagent (rFC) has been commercialized for almost 15 years, its wide use in the pharmaceutical industry is lagging, most likely due to historical concerns related to the few suppliers and the lack of inclusion in most of the global pharmacopeias[19].

According to the results obtained by an industry consortium of biopharmaceutical manufacturers, the ability to rFC detect naturally occurring endotoxins in the presence of the test inhibitors, such as sodium citrate buffers, with a high limit of detection is one of the advantages of using this alternative method[20]. However, limitations to test samples containing proteases that can release fluorophore in the absence of endotoxin were identified by Marius and colleagues[21].

Two suppliers for detection systems based on rFC are available in most global markets, and both reagents rely on the binding of the endotoxins to the rFC and a fluorescent substrate. Pyrogene™ (from Lonza) and Endonext platform (from bioMérieux) detect the endotoxin in the liquid phase by its binding to a fluorescent substrate. For samples with simple matrices, such as WFI or parenteral saline solution, Pyrogene™ or Endozyme II Go® (from the Endonext platform) are the recommended choices.

To evaluate whether the commercial kit Endozyme II Go® can be an option for endotoxin quantitation in 0.9% sodium chloride injection, the study considered all the parameters described by The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH)[22] and by the United States Pharmacopeia[5] for quantitative method validation.

MATERIAL AND METHODS

Kits, reagents, and samples

The 0.9% sodium chloride injection manufactured by Braun (Rio de Janeiro, Brazil – 0.9% Normal saline Ecoflac Plus 1000 mL) was used as a sample to be tested by Endozyme II® Go kit (bioMérieux, Germany). The kit consists of rFC, fluorometric substrate, nonpyrogenic water, substrate buffer, and a pre-coated plate with a standard LPS curve from 0.005 to 50 EU/mL. All the experiments were performed with the same batches of samples and kits. Endotoxin standard 500 EU/mL (bioMérieux, Germany), from *E. coli* O55:B5, was used for spiking the samples. Solutions were prepared in endotoxin-free borosilicate glass tubes (bioMérieux, Germany) with nonpyrogenic water followed by vigorous mixing for at least 3 minutes between each dilution.

Equipments

The equipment Synergy™ HTX Multi-Mode microplate reader for fluorescence (Biotek, United States of America) and MS2 Minishaker 2500 rpm (IKA, Germany) were used.

Study design

This study was performed according to ICH Guidelines for Validation of Analytical Procedures (Q2(R1))[22] and the United States Pharmacopeia[5]. Precision, Accuracy, Detection and Quantification Limits, Linearity, Specificity, and Robustness were evaluated considering the acceptance criteria established in the guidelines[5,22]. For precision and accuracy, the samples were spiked with endotoxin (*E. coli* O55:B5)

solutions at 0.0005 EU/mL, 0.005 EU/mL, 0.05 EU/mL, 0.5 EU/mL, 5 EU/mL and 10 EU/mL. Each point was repeated at least five times, except for 0.005 EU/mL and 0.5 EU/mL points, performed at least ten times by two different analysts on two separate days. For robustness, small changes in the time of enzyme-substrate dispensing were made to check the effect of this change on the results. Each condition (time zero and five minutes) was repeated ten times using the 0.5 EU/mL spiked sample. The test is software auto-validated, i.e., for each dilution, there must be a spiked duplicate with endotoxin at 0.5 EU/mL. The software validates the results according to this spike.

Endotoxin determination

Endotoxin quantification was carried out with the Endozyme II® Go kit according to the supplier's instructions in a BioTek Synergy™ HTX fluorescence microplate reader (380 nm excitation and 445 nm emission). Briefly, samples (spiked or not) were placed on the plate in four replicates. After incubation (5 minutes, 37 °C), the solution containing the rFC and the fluorogenic substrate was added, and the fluorescence was recorded every 5 minutes until 60 minutes of incubation (37 °C). The software (Gen5™ v. 3.05 software for MS Windows™) calculated the curves and estimated the endotoxin concentration based on the standard curve in each pre-filled microplate. The test is valid if the recovery of endotoxin in the positive product control in each test is between 50-200% of the nominal value and if the coefficient of variation between the sample and positive product control replicates is less than 25%[5,22].

Statistical analysis

The statistical analyses were conducted using the Minitab® v.18 software (Minitab Inc., USA) to calculate the RSD for precision and robustness, and the percentage of control endotoxin recovery for accuracy. Whenever needed, the comparisons were made by non-parametric ANOVA considering $p < 0.05$. The linear regression analysis was performed to assess linearity.

RESULTS AND DISCUSSION

With increasingly strict specification limits for bacterial endotoxins in parenteral products, developing and implementing alternative analytical methods that allow the release of reliable results can be a challenge, as before any routine use, whether in quality control or the development of medicines, every assay should be validated. Recombinant Factor C assay is an alternative to BET (Bacterial endotoxin test) enlightened by the 3R's concept[12]. The test requires validation before considering its use for quality control of parenteral products.

As a pioneering approach, in 2021, the European Pharmacopoeia published a specific chapter referring to the endotoxin assay using rFC[23], which guided the inclusion of this methodology as one of the compendial methods listed in the chapter on bacterial endotoxins[24]. With the introduction of this new chapter, the bacterial endotoxin test using rFC can be extended after demonstrating suitability for the intended use, eliminating the need for a full performance assessment. It should be noted, however, that despite the publication of this new chapter, the replacement of an LAL-based method prescribed in the

European monograph with an rFC-based method is considered to be the use of an alternative method. Therefore, until now, only two monographs have been revised and updated to include the rFC-based method as a compendial: water for injections[25] and purified water monographs[26].

Based on this regulatory scenario, the commercial kit Endozyme II® Go was evaluated considering the parameters precision, accuracy, linearity, specificity, and robustness.

The RSD from precision results between 0.0005 and 10 EU/mL were lower than 30%, in compliance with the guidelines[5,22] (**Table 1**). As expected, the highest value of RSD was obtained for the lowest level of the endotoxin sample.

Table 1. Precision and intermediate precision results

Spike (EU/mL)	Replicate number (n)	Spiked sample			Positive Product Control (0.5 EU/mL)		
		Calculated mean (EU/mL)	Standard deviation (EU/mL)	RSD (%)	Calculated mean (EU/mL)	Standard Deviation (EU/mL)	RSD (%)
0.0005	5	0.009	0.002	18.6	0.406	0.086	21.2
0.005	10	0.006	0.001	8.3	0.363	0.023	6.3
0.05	5	0.042	0.001	3.3	0.424	0.027	6.3
0.5	10	0.381	0.007	1.9	0.739	0.024	3.2
5	5	3.505	0.226	6.4	3.940	0.189	4.8
10	5	6.146	0.242	3.9	6.611	0.382	5.8
Spike (EU/mL)	Replicate number (n)	Spiked sample			Positive Product Control (0.5 EU/mL)		
		Calculated mean (EU/mL)	Standard deviation (EU/mL)	RSD (%)	Calculated mean (EU/mL)	Standard Deviation (EU/mL)	RSD (%)
0.005	20	0.006	0.001	13.3	0.517	0.214	21.3
0.5	20	0.388	0.018	4.7	0.7251	0.038	5.3

As the analyst differences may explain the variability of measurements, the repeatability and reproducibility (R&R) study was carried out. The detected fluorescence values obtained by analysts A and B for samples spiked with 0.005 EU/mL and 0.5 EU/mL were used for conducting the statistical analysis (**Table 1**). The ANOVA test results were more significant than 0.05 for the p-values related to each endotoxin concentration evaluated ($p_{0.005 \text{ EU/mL}} = 0.75$; $p_{0.5 \text{ EU/mL}} = 0.07$), indicating that the interaction between day and analyst was not significant at the level of significance of 5%. The RSD from intermediate precision results were 13.3% (n = 20) and 4.7% (n = 20) for 0.005 EU/mL and 0.5 EU/mL, respectively, in compliance with the acceptance criteria[5,22].

Considering the lowest endotoxin concentration evaluated (0.0005 EU/mL), the average recovery value for accuracy was higher than the established acceptance criteria[5,22], namely between 50 and 200% (**Figure 1**). This endotoxin concentration is one order of magnitude (1/10) lower than the detection limit test reported by the kit manufacturer. Therefore, the results obtained were above the detection limit.
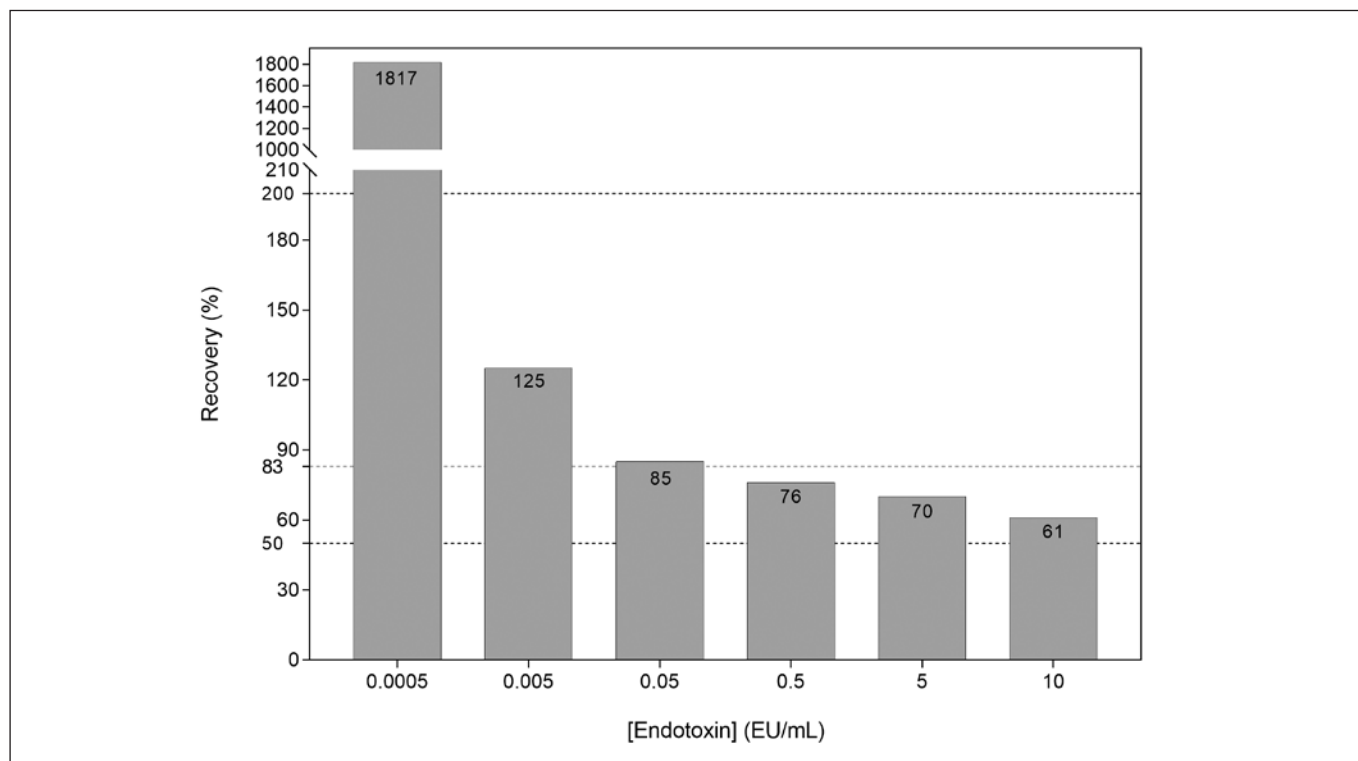
Figure 1. Average endotoxin recovery from spiked samples. Numbers on the column indicate the recovery for each endotoxin spike. The dotted line at 83% represents the overall average for endotoxin recovery (disregarding 0.0005 EU/mL). Limits between 50% and 200% are considered valid

For the concentration levels between 0.005 and 10 EU/mL, the recovery values of endotoxin were greater than 61% and lower than 125% (**Figure 1**). The average was 83% of endotoxin recovery (0.0005 EU/mL was not considered).

The concentration of 0.0005 EU/mL was used to show that concentrations below the minimum of the standard curve tend to give incorrect results, as demonstrated by the high recovery value obtained (**Figure 1**), i.e. below 0.0005 EU/mL the response is not linear, and the results may not be considered. The assay includes quadruplicate negative controls and calculations are only performed if the controls fluoresce below the most dilute point on the standard curve, as per the manufacturer's recommendations.

Based on the standard deviation of the blank, the detection and quantification limits of the method were calculated (**Table 2**). Although the concentration level of 0.0005 EU/mL presented positive detection in all replicates, the lowest endotoxin concentration assessed that met the precision, accuracy, and linearity criteria was 0.005 EU/mL. For all these reasons, the detection and quantification limits were established at 0.005 EU/mL.

Table 2. Detection and quantification limit results

	Replicate number (n)	**dRFU Mean**	**SD**
Blank	4	2.2	5.3
0.0005 EU/mL	5	5.9	1.7
0.005 EU/mL	10	23.1	4.4

Notes: dRFU = relative fluorescence unit (RFU) difference between first and last assay read. SD = standard deviation. Limit of detection = dRFU Mean(0.005 EU/mL) – dRFU Mean(Blank) ≥ 3.3 x SD (Blank). Limit of quantification = dRFU Mean(0.005 EU/mL) – dRFU Mean(Blank) ≥ 1.6 x (SD(Blank) + SD(0.005 EU/mL))

The regression analysis has shown a linear correlation ($R^2 = 1.00$) between the detected fluorescence values and the endotoxin concentration for the range from 0.005 to 10 EU/mL, as shown in **Figure 2**.
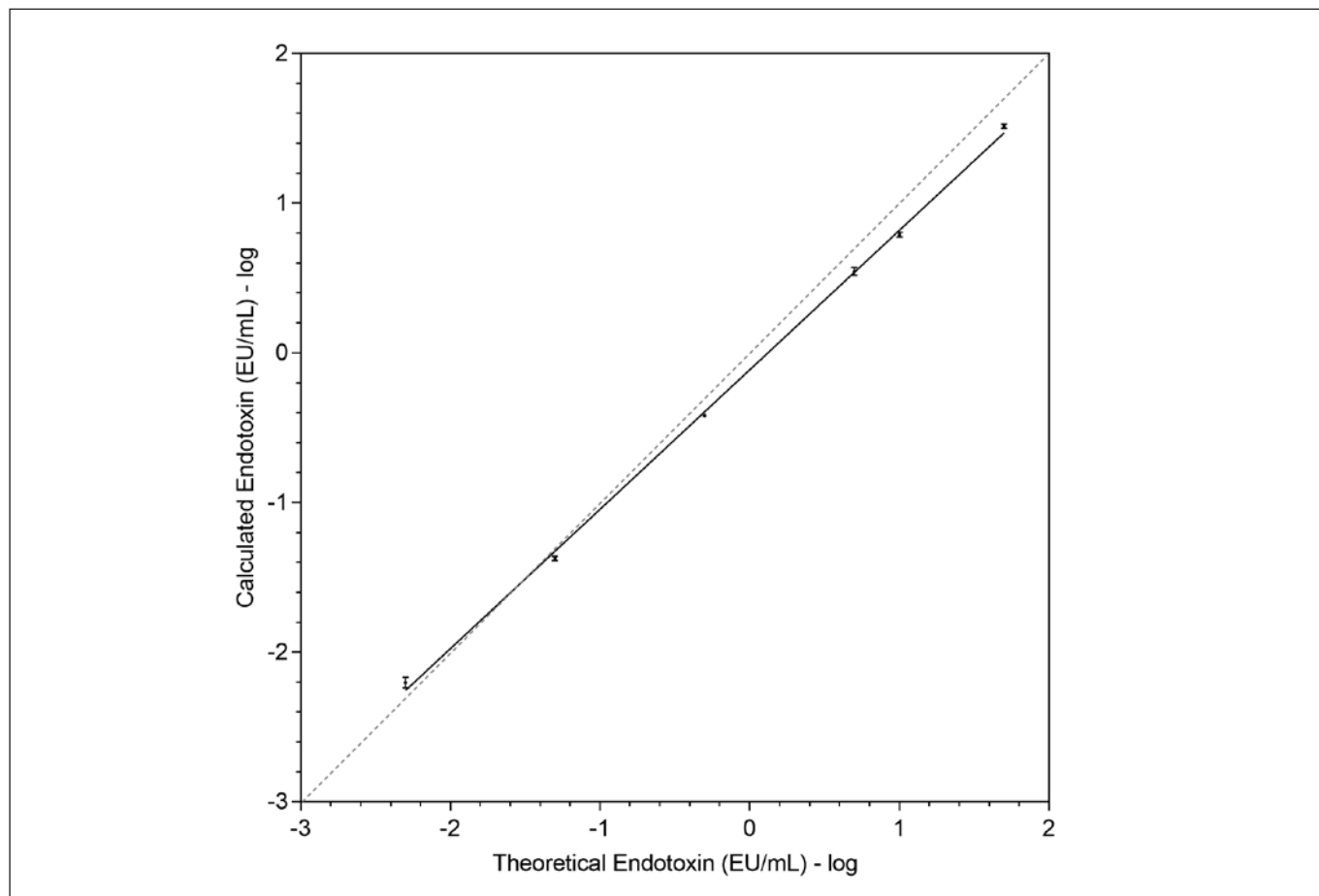
Figure 2. Linear regression analysis from 0.005 to 10 EU/mL: measured vs. nominal endotoxin concentration. Y = 0.9316x - 0.1134. Where Y = Calculated Endotoxin (EU/mL) log and x = Theoretical Endotoxin (EU/mL) log. $R^2 = 0.9991$

To understand the limits of the operating parameters of the method, the time for the addition of the enzyme-substrate was chosen to be evaluated as a robustness parameter because it is a critical point mentioned in the Endozyme II® Go manufacturer's instructions for use. The ANOVA test, used to assess the statistical differences (p-value ≥ 0.05) between the results obtained in two different conditions on enzymatic reagent dispensing (**Table 3**), showed that the differences observed between conditions were not significant ($p = 0.61$).

Table 3. Robustness results: changes in time of enzyme-substrate dispensing

Condition	0.5 EU/mL Spiked sample			Positive Product Control (0.5 EU/mL)		
	Endotoxin (EU/mL)	Standard deviation (EU/mL)	RSD (%)	Endotoxin (EU/mL)	Standard deviation (EU/mL)	RSD (%)
Time zero	0.377	0.037	9.7	0.744	0.070	9.4
After five minutes	0.385	0.023	6.1	0.750	0.036	4.9

According to the kit's validity criteria, a critical issue considering endotoxin assays is the rate of invalid results. Results were considered invalid when Positive Product Control (PPC) recoveries were less than 50% or more than 200%, or if the RSD for sample/PPC replicates were more than 25%[27]. There was 8.0% (n = 7) of invalid data in this validation study: 4.6% (n = 4) related to sample/PPC recovery and 3.4% (n = 3) related to sample/PPC RSD, a higher value than that observed by Marius et al[28].

Large-volume parenteral solutions do not have a specific dosage, as they are diluents or irrigation solutions. The administration of large volumes of this category of products combined with the levels of endotoxins in medications administered together can expose patients to a high risk of poisoning. Therefore, implementing alternative methods that allow low-level quantification of bacterial endotoxins, such as rFC methods, has great relevance in ensuring the safety and quality of large-volume parenteral pharmaceutical products.

According to recent European Pharmacopoeia publications[23-26] establishing endotoxin testing employing rFC as a compendial method and including this methodology in updated monographs, along with moves by the American Pharmacopoeia[29] in the same direction, the use of recombinant reagents for endotoxin detection offers an opportunity to the pharmaceutical area to modernize procedures and significantly contribute to the conservation of horseshoe crabs and reducing the use of rabbits in testing.

CONCLUSION

The commercial rFC alternative method, Endozyme II® Go, met the accuracy, precision, and linearity criteria from 0.005 to 10 EU/mL for 0.9% sodium chloride injection samples. Limits of detection and quantification were set at the lowest concentration (0.005 EU/mL). Robustness tests showed that deviations in enzyme-substrate addition times and the binding times did not affect the results.

Despite its advantages, such as improved sensitivity, reduced occurrence of false-positive results for glucans, the benefits linked to the conservation of horseshoe crabs, and adherence to the 3Rs, additional studies need to be developed to assist the sector in the face of reliability and risk assessment, to ensure patient safety.

PRESENTATION NOTE

The results of this article are part of Ellen Gameiro Hilinski's doctoral thesis, entitled "Alternative methods for evaluating endotoxins: challenges and international trends for patient safety", which will be defended in 2024 by the Postgraduate Program in Pharmaceuticals and Medicines from the Faculty of Pharmaceutical Sciences of the University of São Paulo.

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