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Potency evaluation of unfractionated heparins commercialized in Brazil through anti-factor Xa and anti-factor IIa chromogenic tests and coagulation assay

Avaliação da potência das heparinas não fracionadas comercializadas no Brasil por meio dos ensaios cromogênicos antifator Xa e antifator IIa e teste de coagulação

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ABSTRACT

Introduction: Heparin is a drug that has anticoagulant activity, binding to antithrombin and accelerating the rate of inhibition of several proteases involved in the coagulation process. In the 2000s, the world market faced a troubled period regarding heparins after reports of allergic reactions and deaths caused by its use, requiring more rigorous quality control. **Objective:** The main goal of this work was to perform quality control of unfractionated sodium heparins of porcine origin commercialized in Brazil and heparin raw material on a dry basis, of both porcine and bovine origin, through potency assays. **Methods:** Sixty-four samples of the final product (commercialized) were analyzed: 39 of brand A and 25 of brand B, and six samples of raw materials. Samples were assayed through anti-factor Xa and anti-factor IIa, according to United States Pharmacopeia (USP), and coagulation assay, described in the 5th edition of Brazilian Pharmacopeia (BP). **Results:** In the present study, 40 heparin samples were approved in all potency assays, while 24 samples were non-approved, 23 of brand A and one of brand B. All samples of porcine-origin raw materials were considered approved, while the three of bovine origin showed lower potency. **Conclusions:** Almost all non-approved samples presented potency above 110%, which may represent a bleeding risk for patients. Thus, it is necessary to monitor the quality control of heparins and assess the clinical condition of patients undergoing their use to identify and reduce risks and safeguard public health.

KEYWORDS: Heparin; Anti-factor Xa Assay; Anti-factor IIa Assay; Quality Control; Human Health

RESUMO

Introdução: A heparina é um fármaco que apresenta atividade anticoagulante, ligando-se à antitrombina e acelerando a taxa de inibição de diversas proteases envolvidas no processo de coagulação. Na década de 2000, o mercado mundial enfrentou um período conturbado em relação às heparinas após relatos de reações alérgicas e de mortes causadas pelo seu uso, o que exigiu um controle de qualidade mais rigoroso. **Objetivo:** Realizar o controle de qualidade das heparinas sódicas não fracionadas de origem suína comercializadas no Brasil e da matéria-prima heparina em base seca, tanto de origem suína quanto bovina, por meio de ensaios de potência. **Método:** Foram analisadas 64 amostras do produto final (comercializado), sendo 39 da marca A e 25 da marca B, e seis amostras de matérias-primas. As amostras foram testadas por antifator Xa e antifator Iia, de acordo com a Farmacopeia dos Estados Unidos (USP), e por teste de coagulação, descrito na 5ª edição da Farmacopeia Brasileira (BP). **Resultados:** Quarenta amostras de heparina foram aprovadas em todos os ensaios de potência e 24 amostras não foram aprovadas, sendo 23 da marca

A e uma da marca B. Todas as amostras de matérias-primas de origem suína foram consideradas aprovadas, enquanto as três de origem bovina apresentaram menor potência. **Conclusões:** Quase todas as amostras não aprovadas apresentaram potência acima de 110%, o que pode representar risco de sangramento para os pacientes. Assim, é necessário monitorar o controle de qualidade das heparinas e avaliar a condição clínica dos pacientes em uso para identificar e reduzir os riscos e salvaguardar a saúde pública.

PALAVRAS-CHAVE: Heparina; Ensaio Antifator Xa; Ensaio Antifator Iia; Controle de Qualidade; Saúde Humana

INTRODUCTION

Heparin is the oldest anticoagulant used in medical practice, with great importance in clinics, and is present in the World Health Organization's (WHO) list of essential medicines. Structurally, they are sulfated glycosaminoglycans of variable molecular weight, composed of glucosamine and hexuronic acid units, which alternate through glycosidic bonds. The polymer has large structural heterogeneity due to variable sulfation and acetylation, as well as the distribution of hyaluronic acid units[1,2,3,4].

The main action is the prevention of blood clotting in several risky procedures such as enterotomy and organ transplantation, deep vein thrombosis, or cardiac thrombosis that develops as a result of blood circulatory deterioration[4,5,6]. It is estimated that approximately 20 million patients worldwide use them for venous or arterial thrombosis treatment and prophylaxis. Heparin also has other biological functions, such as anti-inflammatory, antithrombotic, antihyperlipidemic, and anti-arteriosclerotic properties, as well as anticoagulant functions that inhibit thrombin activity[3,4,7].

This substance can be found in various animal tissues such as the lung, liver, blood, and intestinal tissues of higher animals, especially in mast cells. To be used as a drug, heparin is extracted from the porcine intestinal mucosa or the bovine lung as by-products of the meat production process and transformed into calcium or sodium salt through enzymatic and chemical treatment[3,4,5]. Brazilian Pharmacopeia (BP) considers heparins sourced from porcine or bovine mucosa as distinct active pharmaceutical ingredients (APIs)[5,6,8,9].

During the isolation and extraction process, partial degradation of its glycosaminoglycan chains occurs, producing a formulation formed by fragments of heterogeneous molecular weights, ranging from 3,000 to 30,0000 Da, known as unfractionated heparin, conventional heparin, or simply heparin[4,5]. Low molecular weight heparins are fractions of unfractionated heparin produced by controlled depolymerization of their polysaccharide chains, either chemically or by an enzymatic reaction. In this process, the final product's molecular weight is reduced to an average of 4,000 to 6,000 Da[4].

The therapeutic responses to heparin are very variable among individuals. Thus, it is essential to monitor its effectiveness and safety for the patient through laboratory assays, such as the Partial Activated Thromboplastin Time (aPTT), used to evaluate all coagulation factors, except platelets, by determining the time required to form a fibrin thrombus in a plasma sample[10].

Due to its risk of causing significant harm to patients due to failure in its usage process, Niccolai et al.[11] drew attention to the association of unfractionated heparin (UFH) with a high rate of problems related to its inherent pharmacological properties or frequently caused by medication errors. They also warned that the Institute for Safe Medication Practices classified UFH as a high-inerting drug. In 2008, the Institute for Healthcare Improvement (IHI) ranked UFH as one of the ten high-risk or potentially dangerous drugs categories. The IHI also advised that these drugs were related to most adverse events in a hospital setting[12].

Bezerra and Silva[13] also evidenced this fact in 2008 after analyzing 100 reports of adverse events in a sentinel hospital in the Midwest region of Brazil. The authors observed that sodium heparin and other anticoagulants appear as one of the drugs most frequently involved in adverse events, ranging from 3.0 to 5.7% of all drug-related adverse events. Junqueira et al.[14] observed in a study conducted in 2011 that 5.0 to 10.0% of patients using UFH have some bleeding, such as hematuria, upper gastrointestinal bleeding, hemoptysis, epistaxis, hematomas, and melenas. The main concern is the bleeding events caused using heparin that will vary in degree of risk according to the site and volume of blood involved leading to hemodynamic instability, ventilation, hospitalization, and mortality[10].

Coronavirus Disease 2019 (COVID-19), caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), has spread rapidly worldwide at a high transmission rate. Severe COVID-19 is marked by thrombotic complications associated with multiple organ failures and increased mortality. Applying unfractionated and low molecular weight heparins as anticoagulant drugs have significantly reduced disease severity and mortality induced by COVID-19, as heparin is a multifunctional agent[15]. This drug is recommended by expert consensus for patients with severe COVID-19. They further point out that, although heparin may be beneficial in treating coagulopathy in COVID-19 (i.e. direct antiviral and anti-inflammatory effects), the balance between its benefits and risks should be considered. Quality control of the potency of this drug is of great importance for successful treatment and preservation of a patient's life[16,17,18].

Another source of concern regarding the quality of commercial heparins is the possibility of fraudulent adulteration of their composition with substances such as supersulfated chondroitin sulphate. In 2008 the world market faced a troubled period regarding the reliability the quality of heparin[19]. Episodes reported in the United States of America (USA) and Europe identify batches

of non-fractionated heparin contaminated with supersulfated chondroitin sulphate, resulting in the death of hundreds of patients[20,21]. To monitor the quality of heparins commercialised in the USA, the United States Pharmacopeia (USP) published in 2019 the analytical methodology for quality control of potency, identification, and purity of this medication on 42 - NF 37 The USP and National Formulary[22].

A striking episode occurred in Brazil after the interruption of the commercialisation of specific heparin widely used in cardiovascular surgery services for intravenous use from Roche Laboratory (Liquemine®). The other brands available in the market caused complications and increased rates of reoperation for bleeding after cardiac surgeries recorded by the Brazilian Society of Cardiovascular Surgery (BSCVS)[3,14].

After 2008, international pharmacopoeias started a review of heparin monographs, and analytical methods for the detection of contaminants have been introduced to the list of mandatory quality control assays. Methodologies such as capillary electrophoresis and nuclear magnetic resonance were developed to detect impurities and contaminating species and were published in those documents. Impurity assays had their limits reduced to control the polydispersity of heparin through molecular weight analysis[21,23].

In Brazil, the National Health Surveillance Agency (Anvisa) conducted a project to review the monographs of both calcium and sodium heparin of the BF. Under these new guidelines, the laboratories that produced injectable heparin sodium in Brazil compulsorily analysed their products, and no contamination by supersulfated chondroitin sulphate was detected. However, dermatan sulphate was also observed, a compound with probably no toxic effect but which shows poor quality control. Samples also were chemically degraded and with a significant change in molecular weight. A difference in potency values between UFH of bovine origin and those of porcine origin was also calculated and showed a significantly reduced anticoagulant activity. The authors warned that this decreased anticoagulant activity may be responsible for consumption coagulopathy during cardiopulmonary bypass (CPB) and may be mistakenly translated by a clinical picture of blood dyscrasia[24].

In the 5th edition of the BF, those assays to detect impurities and contaminating species, using the nuclear magnetic resonance spectroscopy technique, capable of identifying contaminants such as dermatan sulphate, were published on the monographs of calcium and sodium heparin raw materials. In addition, anti-factor IIa (anti-FIIa) activity assaying and anticoagulant activity assays for potency determination were also published for quality control of raw materials[25]. In 2016, the First Supplement of Brazilian Pharmacopoeia 5th edition was finally approved. Currently, the methodologies for quality control analyses of heparin sodium and injectable calcium solutions are the anti-FIIa activity assay for heparin sodium and the anti-factor Xa (anti-FXa) activity and anti-FIIa activity assays for low molecular weight heparins. Both have specific values for the different types of low molecular weight heparin: enoxaparin, tinzaparin, dalteparin, and nadroparin[26,27].

The current study had the main objective of analyzing, for two years from February 2014 to March 2016, unfractionated porcine-origin sodium heparins potency of different brands commercialized in Brazil and raw material on a dry basis of porcine and bovine origin using chromogenic assays described in USP and BP. The heparin samples were collected as part of a Anvisa study for quality control of those drugs[25,26]. The results were compared to the coagulation assay described in BP, using the WHO 6th International Heparin Standard as a reference.

METHOD

Sampling

During a period of two years between February 2014 and March 2016, the Laboratory of Physiology of the Department of Pharmacology and Toxicology of the National Institute for Quality Control in Health (INCQS) of Oswaldo Cruz Foundation (Fiocruz) received 70 heparins samples: 64 samples of the pharmaceutical form of injection solution from unfractionated sodium heparins of porcine origin with 5,000 IU/mL vial or 5,000 IU/0.25 mL ampoule of two national brands. Thirty-nine belonged to brand A, and 25 samples belonged to brand B. Other six samples of raw heparin material on a dry basis of porcine and bovine origin material.

Anti-FXa and anti-FIIa activity chromogenic assays

Sample preparation

Finished products samples were diluted with tris(hydroxymethyl) aminomethane (TRIS) buffer solution (0.050 M Tris, 0.0075 M ethylenediamine tetraacetic acid (EDTA), 0.175 M NaCl, 1% polyethene glycol 6000 [PEG 6000]) at pH 8.4. A concentration of 20 IU/mL was obtained. Subsequently diluted to a concentration of 2 IU/mL and then diluted for the assay concentrations using a digital precision scale (Edutec, EEQ9003F-B, Brazil). One mg of raw material was weighed and then reconstituted with 1 mL deionized water estimating a 180 IU/mg concentration, which is the minimum potency established at BP per mg on a dry basis. After that, they were diluted to 20 IU/mL, following the same procedure described for finished products.

Standard preparation

WHO 6th International Standard for Unfractionated Heparin from National Institute for Biological Standards and Control (NIBSC), code: 07/328 (here in this article called just as “standard”) was used. This preparation had a certificated concentration of 2.145 IU/ampoule. For the standard preparation, one ampoule was reconstituted in 100 mL of deionized water and aliquoted in tubes with 1 mL at a final concentration of 21.45 IU/mL. For the assay, the aliquots were diluted in Tris buffer solution pH 8.4 (0.050 M Tris, 0.0075 M EDTA, 0.175 M NaCl, 1% PEG 6000) to obtain a concentration of 2 UI/mL and subsequently obtain concentrations used in the assays.

Anti-FXa activity assay

For the anti-FXa activity assay, a 96-well microplate was used, pre-heated to 37ºC for 15 minutes in a microplate incubator

(Thermo Shaker, Agimaxx) with 30 µL of the standard solution in four concentrations (0.02, 0.04, 0.07, and 0.10 IU/mL, selected based on the linear region), and 30 µL of samples in duplicate to establish a dose-response curve. Thirty µL of antithrombin 1 IU/mL (Anti-thrombin III 1.5 mg, HYPHEN BioMed) was added and gently shaken for 2 minutes at 37ºC so that heparin and antithrombin could bind. After the incubation period, 60 µL of FXa 10 nKat (Bovine Xa factor, HYPHEN BioMed) was added, and once again, it was gently shaken at 37ºC for 2 minutes. Finally, 60 µL of 1.30 mM specific FXa chromogenic substrate (S-2222 25 mg, Chromogenix) was added, incubated at 37ºC, and gently shaken for another 5 minutes. The reaction was interrupted with 30 µL of 20% acetic acid solution. The absorbance was obtained in a microplate reader (BioTek, Epoch) at an endpoint of 405 nm.

Anti-FIIa activity assay

A 96-well microplate was used, pre-heated to 37ºC for 15 minutes in a microplate incubator (Thermo Shaker, Agimaxx). Fifty µL of four standard concentrations (0.003, 0.005, 0.010, and 0.020 IU/mL selected based on the linear region) and 50 µL of the sample in the same concentrations were duplicated to establish a dose-response curve. The microplate was then incubated at 37ºC and gently shaken for 2 minutes with 100 µL of antithrombin 0.125 IU/mL (Anti-thrombin III 1.5 mg, HYPHEN BioMed) so that heparin and antithrombin could bound. Afterwards, 25 µL of thrombin 5 IU/mL (Thrombin, Sigma Aldrich) was added, incubated at 37ºC, and gently shaken for two minutes. Finally, 50 µL of chromogenic specific substrate for FIIa 1.25 mM (S-2238 25 mg, Chromogenix) was added, incubated at 37ºC, and gently shaken for 5 minutes. The reaction was interrupted with 50 µL of 20% acetic acid solution. The absorbance was obtained in a microplate reader (BioTek, Epoch) at 405 nm.

Coagulation assay (Partially Activated Thromboplastin Time - aPTT)

Sample preparation

The finished product samples were diluted with saline solution (0.9% NaCl) to obtain a 20 IU/mL concentration that was subsequently diluted in 0.8, 0.92, and 1.05 IU/mL concentrations, selected based on the linear region. For the raw material, 1 mg was weighed on a precision digital scale (Edutec, EEQ9003F-B, Brazil) and then reconstituted with 1 mL deionized water, with an estimated concentration of 180 IU/mg. After that, they were diluted following the same procedure described for the finished products.

Standard preparation

The same standard previously described was used. To perform the coagulation assay, aliquots with 21.45 IU/mL concentration were diluted with saline solution (0.9% NaCl) to 0.8, 0.92, and 1.05 IU/mL concentrations.

Assay procedure

For the coagulation assay, a coagulometer was used (Stago, STart®) for incubation at 37ºC and time counting. Three independent assays were performed, each in duplicate. For each assay, 50 µL of sheep plasma (obtained from the Federal University of Santa Maria, Rio Grande do Sul, Brazil) and 50 µL of heparin (standard or sample) were gently shaken and incubated at 37ºC for 800 seconds. After that, 50 µL of cephalin (Stago, CK PREST®) was added and incubated at 37ºC for another 2 minutes. Fifty µL of 0.025 M CaCl2 solution (Stago) was added, and the time required for coagulation was monitored. The control recalcification time was measured at the beginning and the end of the procedure, replacing the heparin dilutions with a saline solution (0.9% NaCl).

Statistical analysis

Chromogenic assays were analyzed using parallel lines. For each series, the absorbance regression or absorbance change per minute was calculated against the concentrations in the logarithm of the Sample and Standard solutions and the potency of the sample was calculated using statistical methods for parallel line assays. The potency of low molecular mass heparin was expressed in IU/mg. A validated statistical package, Combistats™ (EDQM reference: 0000061710, 2017), was used to perform the calculations. Linearity, parallelism and regression were analyzed.

To calculate the results, the finished product's estimated potency value in each assay should correspond from 90% to 110% of the declared potency value. Raw material potency must not be less than 180 IU/mg in each assay. Estimated potency confidence limits must not be less than 80% and not more than 125% of the declared value (P = 0.95). A sample acceptance criterion is a ratio of anti-factor Xa activity to anti-factor IIa activity between 0.9 and 1.1. Assays were considered valid when linearity and parallelism presented $p > 0.05$, and regression $p < 0.05$, respectively.

The three coagulation assays performed for each sample were calculated separately using the Parallel Lines statistical model and combined using the CombistatsTM statistical package (reference EDQM: 0000061710, 2017). Linearity and regression were also analyzed. The assays were valid when linearity and parallelism presented $p > 0.05$ and regression $p < 0.05$.

RESULTS

Finished products potency determination

The mean, range, and standard deviation values of the coagulation assay (aPTT), chromogenic anti-FIIa and anti-FXa activity assays, and the anti-FXa/anti-FIIa activity ratio for porcine origin finished products potency determination are described in Table 1. Regarding chromogenic anti-FXa and anti-FIIa activity assays, 48 samples were approved, which means that they were approved individually in both assays, with a potency limit between 90 and 110%, and presented an anti-FXa/anti-FII activity ratio between 0.9 and 1.1. A total of 16 samples were non-approved because they failed at least one of the assays. The mean potency value for non-approved samples in the anti-FXa activity assay and anti-FIIa assays were 117.92% and 127.34%, respectively. In comparison, the mean potency value obtained

Table 1. Values of mean, range, and standard deviation of chromogenic anti-FXa and anti-FIIa activity assays, anti-FXa/anti-FIIa activity ratio, and coagulation assay for potency* determination of commercial heparin collected from 2014 to 2016 in Brazil (IU/mL).

Assay	Sample		Year of samples collection		
			2014	2015	2016
Anti-FIIa (IU/mL)	Brand A	Mean	5,364.904	5,786.39	5,912.67
		Range (± SD)	4605.28 - 6882.04 (567.48)	3938.96 - 6888.10 (884.01)	4961.18 - 8165.92 (1515.09)
		n	14	21	4
	Brand B	Mean	5,314.21	4990.72	4975.46
		Range (± SD)	4,909.28 - 5689.97 (321.29)	4,679.76 - 5,443.31 (297.10)	4,604.77 - 5,220.22 (231.47)
		n	4	16	5
Anti-FXa (IU/mL)	Brand A	Mean	5,271.097	5,385.84	5,365.09
		Range (± SD)	4,839.66 - 6,556.61 (419.27)	4,769.34 - 6,042.15 (379.82)	5,126.38 - 5,699.65 (252.69)
		N	14	21	4
	Brand B	Mean	5,169.61	5,076.64	4,930.88
		Range (± SD)	5,001.99 - 5,429.78 (183.35)	4,652.73 - 5,461.13 (230.37)	4,959.71 - 5,378.27 (267.54)
		n	4	16	5
Anti-FXa/Anti-FIIa activity ratio	Brand A	Mean	1	1	1.02
		Range (± SD)	0.912 - 1.064 (0.041)	0.886 - 1.100 (0.06)	0.944 - 1.063 (0.07)
		n	14	21	4
	Brand B	Mean	1.01	1.02	0.99
		Range (± SD)	0.970 - 1.050 (0.04)	0.945 - 1.100 (0.06)	0.950 - 1.030 (0.04)
		n	4	16	5
Coagulation (aPTT) (IU/mL)	Brand A	Mean	5,461.75	5,700.31	5,540.96
		Range (± SD)	4,968.06 - 5,973.09 (336.72)	4,921.67 - 6704.6 (382.66)	5,246.95 - 5855.07 (258.47)
		n	14	21	4
	Brand B	Mean	5,480.58	5,152.91	5,096.09
		Range (± SD)	5,426.15 - 5,508.82 (38.13)	4,887.56 - 5,479.83 (177.06)	4,933.12 - 5,209.43 (111.44)
		n	4	16	5

Source: Elaborate by the authors, 2021.
*Heparin potency value was calculated from the anti-factor IIa and anti-factor Xa activity assay values, coagulation assay (aPTT) value, and the ratio between anti-factor Xa and anti-factor IIa assay values.
USP reference values: Heparin potency = 5,000 IU/mL, anti-FXa/FIIa activity ratio = between 0.9 and 1.1.
n: analyzed sample number; SD: standard deviation; aPTT: Partial Activated Thromboplastin Time.

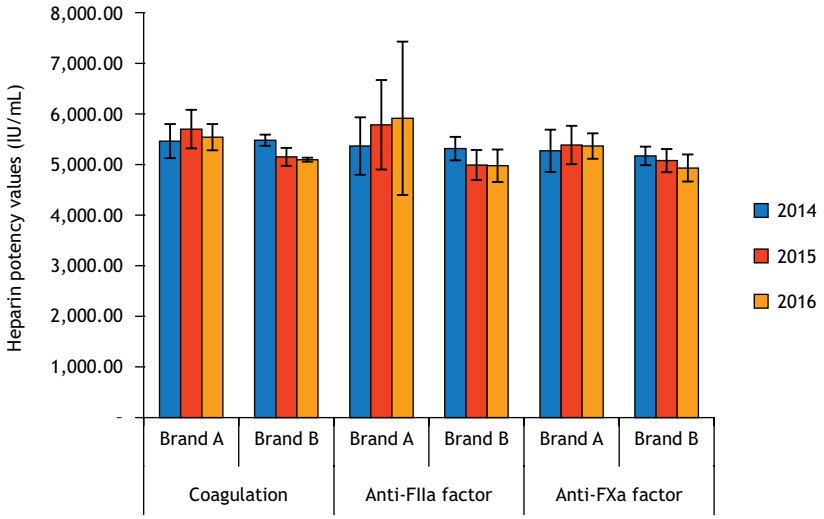
for samples approved in the anti-FXa activity assay and anti-FIIa activity assay were 102.48% and 101.94%, respectively.

The mean and standard deviation distribution for the values obtained during the evaluation were presented in Figure 1 and 2 comparing brands A and B. Sixty-six point sixty-seven percent and 58.34% of the samples showed values of more than 100% for calculated potency in the anti-FXa and anti-FIIa activity assay, respectively when compared to the standard.

Considering non-approved samples, nine failed in both assays, with potency higher than 110%; six samples obtained values higher than 110% only in the anti-FIIa activity assay, and only one sample obtained a value lower than 90% in the anti-FIIa activity assay.

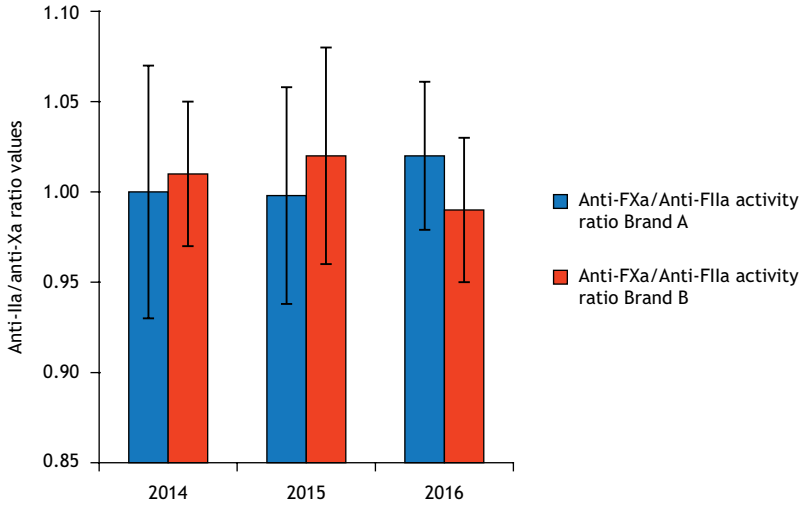
Comparing samples by brand (brand A and brand B), it was possible to observe quality superiority from B to A. In a total of 64 samples analyzed, 39 belonged to brand A, with 24 approved (62.00%) and 15 non-approved (38.00%), while 25 samples belonged to brand B, with 24 approved (96.00%) and one non-approved (4.00%), as shown in Figure 3.

Potency determination established with a coagulation assay (aPTT) demonstrated that, among the 64 samples previously



Source: Elaborate by the authors, 2021.
Reference values: Heparin potency = 5,000 IU/mL. For brand A, n = 14, 21 and 4 in 2014, 2015 and 2016, respectively. For brand B, n = 4, 16 and 5 in 2014, 2015, and 2016, respectively.

Figure 1. Distribution of means and standard deviation values obtained from chromogenic anti-FXa, anti-FIIa activity assays, and coagulation assays for commercial heparin collected from 2014 to 2016 in Brazil.



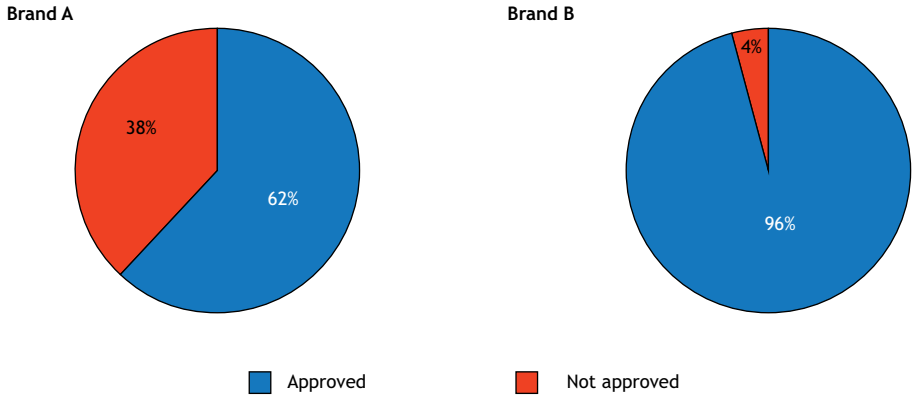
Source: Elaborate by the authors, 2021.
Reference values: anti-FXa/anti-FIIa activity ratio = between 0.9 and 1.1. For brand A, n = 14, 21, and 4 in 2014, 2015, and 2016, respectively. For brand B, n = 4, 16, and 5 in 2014, 2015 and 2016, respectively.

Figure 2. Distribution of anti-FXa/anti-FIIa activity ratio means and standard deviation values obtained from commercial heparin collected from 2014 to 2016 in Brazil.

analyzed by chromogenic assays, 44 were approved, with potency values between 90 and 110%. The other 20 samples were considered non-approved. Ninety point nine% of the approved samples had a potency value greater than 100%, and all the non-approved samples had a potency value greater than 110%. The average potency values of approved and non-approved samples were 104.80% and 114.90%, respectively.

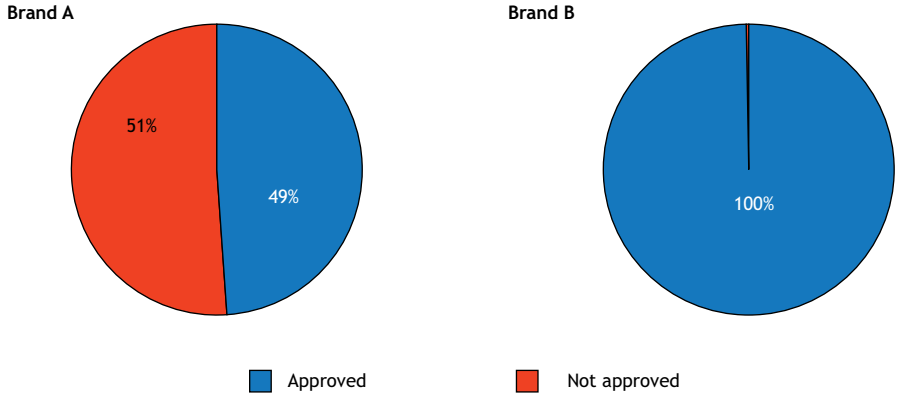
Comparing the results in this assay of brands A and B, it was possible to observe that 20 samples of brand A were non-approved, and 39 samples approved (51.00%), while all 25 samples of brand B were approved (Figure 4).

Regarding the 20 samples considered non-approved in the coagulation assay (aPTT), eight were also non-approved in the two chromogenic assays, four were non-approved only in the anti-FIIa activity, and eight were non-approved only in the coagulation assay. Considering brands, A and B samples together, 24 between 64 samples analyzed had at least one negative result in one of the assays performed (anti-FXa activity, anti-FIIa activity, or coagulation assay [aPTT]).



Source: Elaborate by the authors, 2021.

Figure 3. Percentage of approved and non-approved in anti-FXa and anti-FIIa chromogenic assays of commercial heparin collected from 2014 to 2016 in Brazil, separated by brands.



Source: Elaborate by the authors, 2021.

Figure 4. Percentage of approved and non-approved coagulation assay (aPTT) of commercial heparin collected from 2014 to 2016 in Brazil, separated by brands.

Table 2. Values of chromogenic anti-FXa and anti-FIIa activity assays and coagulation potency for heparin raw materials on a dry basis of porcine and bovine origin collected from 2014 to 2016 in Brazil.

Origin		Anti-FXa activity (IU/mg)	Anti-FIIa activity (IU/mg)	Anti-FXa/Anti-FIIa activity ratio	Coagulation potency (IU/mg)
Porcine	1	193.157	193.651	0.997	197.538
	2	181.886	182.325	0.998	186.908
	3	184.043	191.201	0.963	204.701
Bovine	1	107.795	169.427	0.636	169.660
	2	85.320	81.088	1.052	119.755
	3	140.488	137.928	1.018	165.186

Source: Elaborate by the authors, 2021.

Potency determination of heparin raw material

Six samples of heparin raw material were analyzed, three of porcine origin and three of bovine origin. All three porcine origin samples showed at least 180 IU/mg in one chromogenic assay, and the anti-FXa/anti-FIIa activity ratio value was between 0.9 and 1.1; therefore, they were approved. The three samples of bovine origin presented a potency value below 180 IU/mg in both chromogenic assays. The three porcine-origin samples also showed a potency value between 186 and 204 IU/mg in the coagulation assays (aPTT). In contrast, bovine-origin samples presented a potency value between 119 and 169 IU/mg (Table 2). Despite the anti-FXa/anti-FIIa

activity ratio of two bovine samples being between 0.9 and 1.1, this single criterion is insufficient to consider these samples approved.

DISCUSSION

This research demonstrated that 24 of the 64 samples of finished products analyzed were non-approved, at least in one of the assays described, totaling 37.5% of non-approved samples. This means that the assayed heparin samples do not meet the values described in the monographs of UFH sodium present in the USP (USP 42 - the NF 37) and the 5th edition of the BP for chromogenic assays, besides the coagulation assay (aPTT.) This last one, described in this research, was performed only for comparative purposes since, moments before the completion of the experiments; it was removed from both pharmacopoeias due to the lack of correlation between activated clotting time and plasma heparin[28,29].

As a highly complex heterogeneous product, the pharmacokinetic behaviour of heparin has been studied as an alternative to its pharmacodynamic properties. Anti-FXa and anti-FIIa levels in plasma, which express potency in terms of antithrombin activities, can quantify the pharmacokinetic behaviour[30,33]. The results described represent a potential risk for patients submitted to the use of this drug.

An essential data obtained from this study was that almost all unapproved samples obtained more than 110% potency in at least one of the trials. It has been estimated that 5 to 10% of patients undergoing treatment with UFH have some type of bleeding, such as haematuria, upper gastrointestinal bleeding, haemoptysis, epistaxis, ecchymosis, and hematomas. Haemorrhagic events, such as complications from the use of continuous infusion of sodium heparin, are worrisome because, depending on the site or organ affected and the volume of blood involved, it may increase hemodynamic instability, and ventilation, increase mortality, length of hospital stay, besides requiring intervention measures[33]. Thus, heparins that have a higher potency than stated may potentiate these effects, especially when dealing with risk groups, including the elderly and patients with hypertension or renal failure[31,32].

Research developed by Walenga et al.[30] analysed enoxaparin, a complex, biologically derived low-molecular-weight heparin, branded (Lovenox; Sanofi, US), compared to generic enoxaparin. The potency of the products determined in biochemically defined systems was similar in the anti-FXa assay (IC50 value: 0.61 + 0.08 vs 0.67 + 0.11, for branded and generic products, respectively) and the anti-FIIa assay (IC50 value 0.53 + 0.06 for the branded product and 0.62 + 0.10 for the generic product). A significant difference in fibrinokinetics was observed throughout the clot formation period in the effect of branded enoxaparin compared with generic enoxaparin. This difference was stable in all drug concentrations and tested product batches. Branded enoxaparin consistently showed a more potent anticoagulant effect demonstrated by slower clot formation with a weaker final clot structure.

Tan and Cui[33] studied varying Lovenox or generic Enoxaparin concentrations in the coagulation test (aPTT). The aPTT results indicated that the clotting times for both Enoxaparins were concentration dependent; the higher the concentration, the longer the clotting time, and indicated that there were no statistically significant differences.

In another study conducted in 2020, anti-factor Xa and anti-factor IIa assays showed similar inhibitory responses with derived low-molecular-weight heparin. All agents produced a concentration-dependent inhibition of factor Xa and factor IIa. The IC50 of all drugs in anti-Xa was 2.5 mg/mL. The IC50 was much higher in anti-IIa assays, in which Heparinox batches showed a value of 9.4 mg/mL and 9.6 mg/mL, while Lovenox showed a value of 7.6 mg/mL. The anti-Xa and anti-IIa ratios of the biosimilar and branded low-molecular-weight heparins (LMWH) were comparable and in the range of 3.1-3.4. The aPTT results of the two brands analysed were similar and concentration-dependent. The aPTT response was in the range of 75-85 seconds at 10 mg/mL concentration[34].

The raw materials analysis showed that all samples of UFH of porcine origin passed, according to the USP and BP limits, i.e. they obtained at least 180 IU/mg measured on a dry basis. Moreover, the results obtained in the three assays were consistent in at least two samples. Regarding those of bovine origin, we can observe that they have less potency than porcine ones, as shown in the literature[14,20]. However, the assay was performed using standard porcine heparin of non-bovine origin. Therefore, we cannot categorically classify them as approved or not approved.

CONCLUSIONS

In this work, we obtained a potency values overview of the unfractionated sodium heparins commercialized in Brazil using potency assays. In general, most of the samples were approved by the Pharmacopoeias requirements. However, non-approved samples that presented potency greater than 110% may represent a risk of bleeding for patients, in addition to potentiating other risks, such as heparin-induced thrombocytopenia. Thus, it is necessary to monitor heparins continuously to reduce the risks inherent in their use and to safeguard public health.

Monitoring the potency of commercial heparins is of great importance for health surveillance because, in addition to verifying their compliance with current regulations, they can also guide Anvisa's and other regulatory agencies' strategic actions regarding the safe use of this product.

Nevertheless, laboratory monitoring of patients through the coagulation assay (aPTT) is essential to assess heparin therapeutic response. It can help to detect changes caused by eventual quality failures, ensuring its effectiveness and safety.

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Authors' Contribution

Martins JF, Medeiros RJ, Machado TSC – Conception, planning (study design), acquisition, analysis, interpretation of results and writing of the work. Gonçalves NP, Magalhães MM – Acquisition, analysis, interpretation of results and writing of the work. All authors approved the final version of the work.

Conflict of Interest

Authors have no potential conflict of interest to declare, related to this study's political or financial peers and institutions.