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Comparative *in vitro* evaluation of a biosimilar enoxaparin candidate and its reference product.

Evaluación comparativa *in vitro* de un candidato biosimilar de enoxaparina y su producto de referencia.

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ABSTRACT

BACKGROUND: Enoxaparin, a low molecular weight heparin, has been widely used to prevent and treat thromboembolic disorders. Following the expiration of the patent of the reference product, biosimilar alternatives were developed, requiring thorough comparability assessments to ensure efficacy and safety. **OBJECTIVE:** To compare the *in vitro* pharmacodynamic and structural properties of Biotexin® and the reference product Clexane®. **METHODS:** The products were evaluated using anti-FXa chromogenic assays, clot-based tests (aPTT, TT), and whole-blood thromboelastometry (ROTEM). Structural analyses included molecular weight profiling and USP potency determination. **RESULTS:** Biotexin® showed anti-FXa activity comparable to that of Clexane®, with overlapping EC_{50} confidence intervals. No statistically significant differences were observed in aPTT or TT assays (P = 0.499 and P = 0.538, respectively). ROTEM analysis confirmed a significant prolongation of clotting time (CT) and clot formation time (CFT) compared to the control (P = 0.0043 and P = 0.0064, respectively), with no differences between the two products. Molecular weight distribution and USP potency were also comparable. **CONCLUSION:** These findings confirm the *in vitro* biosimilarity between Biotexin® and the reference product, supporting their functional equivalence and justifying further pharmacokinetic and clinical studies to establish therapeutic interchangeability.

Keywords: Biosimilar, Enoxaparin, Anticoagulation, Low molecular weight heparin, Thromboelastometry.

RESUMEN

ANTECEDENTES: La enoxaparina, una heparina de bajo peso molecular, ha sido ampliamente utilizada para la prevención y el tratamiento de trastornos tromboembólicos. Tras el vencimiento de la patente del producto de referencia, se desarrollaron alternativas biosimilares que requieren evaluaciones rigurosas de comparabilidad para garantizar su eficacia y seguridad. **OBJETIVO:** Comparar las propiedades farmacodinámicas y estructurales *in vitro* de Biotexin® y el producto de referencia Clexane®. **MÉTODOS:** Se evaluaron ambos productos mediante ensayos cromogénicos anti-FXa, pruebas de coagulación (aPTT, TT) y tromboelastometría en sangre total (ROTEM). Los análisis estructurales incluyeron el perfil de distribución de peso molecular y la determinación de potencia según la Farmacopea de los Estados Unidos (USP). **RESULTADOS**: Biotexin® mostró una actividad anti-FXa comparable a la de Clexane®, con intervalos de confianza de EC_{50} superpuestos. No se observaron diferencias significativas en los ensayos de aPTT y TT (P = 0.499 y P = 0.538, respectivamente). El análisis ROTEM confirmó una prolongación significativa del tiempo de coagulación (CT) y del tiempo de formación del coágulo (CFT) en comparación con el control (P = 0.0043 y P = 0.0064), sin diferencias entre los productos. La distribución de peso molecular y la potencia USP también fueron comparables. **CONCLUSIÓN**: Estos hallazgos confirman la biosimilitud *in vitro* entre Biotexin® y el producto de referencia, respaldando su equivalencia funcional y justificando la realización de estudios farmacocinéticos y clínicos adicionales para establecer su intercambiabilidad terapéutica.

Palabras clave: Biosimilar, Enoxaparina, Anticoagulación, Heparina de bajo peso molecular, Tromboelastometría

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1. INTRODUCTION

Enoxaparin, a low molecular weight heparin (LMWH), is widely used for the prevention and treatment of thromboembolic disorders, including venous thromboembolism and acute coronary syndromes [1]. Derived from unfractionated heparin through controlled depolymerization, enoxaparin offers predictable pharmacokinetics, improved bioavailability, and a lower risk of heparin-induced thrombocytopenia compared to its predecessor [2]. The expiration of patents on the branded formulation, Clexane® (Sanofi, France), has enabled the development of biosimilar enoxaparin products [3–6]several biosimilars of low-molecular-weight heparins (LMWHs, which aim to provide cost-effective alternatives while maintaining therapeutic equivalence [7].

The demonstration of biosimilarity follows a stepwise approach, as outlined by regulatory agencies such as the European Medicines Agency (EMA) [8,9]. This process begins with extensive analytical characterization, in which the physicochemical and structural properties of the biosimilar candidate are compared with those of the reference product. Advanced techniques, such as high-performance size-exclusion chromatography (HP-SEC), nuclear magnetic resonance (NMR), and mass spectrometry, are employed to assess critical quality attributes, including molecular weight distribution, sulfation patterns, and structural integrity [7,8]. Following analytical comparability, *in vitro* functional assays are conducted to evaluate the biological activity of the biosimilar, focusing on key anticoagulant properties such as anti-FXa and anti-FIIa activity, clotting assays, and thrombin generation potential [10]. These tests provide essential insights into the biosimilar's mechanism of action and its potential to achieve comparable clinical outcomes [10,11].

Once *in vitro* equivalence is established, the biosimilarity assessment progresses to non-clinical and clinical evaluations [6]. Preclinical studies assess pharmacokinetic and pharmacodynamic properties in suitable animal models, while clinical trials in human subjects are designed to confirm therapeutic equivalence for safety, efficacy, and immunogenicity [5,6,10]but they may not assure similarity in clinical outcomes between biosimilar and branded enoxaparin. This study evaluated the efficacy and safety of biosimilar Cristália versus branded Sanofi enoxaparin in venous thromboembolism (VTE. This comprehensive, stepwise approach ensures that biosimilar enoxaparin meet rigorous standards of comparability before regulatory approval and clinical use.

One notable example of a biosimilar enoxaparin that has completed this process is Inhixa®, manufactured by Techdow Pharmaceutical, which has received regulatory approval in Europe based on comprehensive comparative studies demonstrating its equivalence to Clexane® [6]. This approval highlights Techdow's capability to produce enoxaparin with physicochemical and biological properties that align with stringent regulatory requirements, ensuring its safety and efficacy in clinical practice.

In this study, we aimed to evaluate the *in vitro* comparability of a biosimilar candidate enoxaparin, manufactured by Laboratorios Lasca (Biotexin®, San Lorenzo, Paraguay), with the reference product (Clexane®, Sanofi, France). The raw enoxaparin material used in Biotexin® was supplied by Techdow Pharmaceutical, leveraging their extensive experience in LMWH manufacturing and their established track record in regulatory approvals.

The objective of this study was to assess the biological properties of Biotexin® compared to Clexane®. Specifically, anti-FXa activity, clot-based assays, and thromboelastometry tests were performed to evaluate the biosimilar's structural integrity and anticoagulant activity. This investigation aims to provide critical data supporting the biological similarity of the biosimilar candidate with the reference enoxaparin product.

2. MATERIALS AND METHODS

2.1 Test Products

Two batches of the biosimilar candidate (Biotexin®, Laboratorios Lasca, San Lorenzo, Paraguay), were obtained for analysis: batch 1 (2410311) and batch 2 (2410312). Additionally, two batches of the reference product (Clexane®, Sanofi, France) were analyzed: batch 1 (HS562E) and batch 2 (HS712C). All products were supplied in prefilled syringes containing 60 mg/0.6 mL. The samples were diluted in isotonic saline solution (0.9%) to prepare the working solutions used in the study. The biosimilar and reference test products were used for clot-based assays, the anti-protease assay, and rotational thromboelastometry, whereas the raw material was employed for molecular weight profiling and USP potency.

2.2 Molecular Weight Profiling and USP Potency of Raw Material

The enoxaparin raw material used in the manufacture of biosimilar candidate was supplied by Techdow Pharmaceutical, which performed extensive physicochemical and biological characterization to confirm its physicochemical equivalence to Clexane®. The molecular weight distribution of the raw material was assessed using HP-SEC/TDA (High-Performance Size Exclusion Chromatography with Triple Detector Array), a technique used for characterizing the molecular weight (Mw) and molecular weight distribution (MWD) of polymers, particularly heparins and similar compounds [12]. It combines size exclusion chromatography with a multi-detector system (Refractometer, Right-Angle Laser Light Scattering -RALLS-, and Viscometer) as described elsewhere [12,13]. This method does not require column calibration, making it useful for analyzing molecules where suitable reference standards are unavailable. Briefly, two TSK gel columns in series, G3000 PWXL (7.8 × 300 mm) and G2500 PWXL (7.8 × 300 mm), Tosoh Corp., Tokyo, Japan), were used. Columns, injector, and detectors were maintained at 40 °C. The isocratic mobile phase (0.1 M $NaNO_3$, pre-filtered using a 0.22 µm filter, Merck Millipore, Germany) was used at a flow rate of 0.6 mL/min. Data analysis was performed with OmniSEC software (Viscotek), measuring key parameters such as number-average molecular weight (Mn), weight-average molecular weight (Mw), and the polydispersity index (Mw/Mn). Additionally, the USP potency of the raw material was determined using validated chromogenic assays kits (Aniara Diagnostica, USA) to quantify anti-FXa and anti-FIIa activities. The potency was calculated using the USP standard for enoxaparin. The potency of each product was calculated based on the calibration curve prepared with the USP enoxaparin standard.

2.3 Clot-Based Assays

Serial 1:10 dilutions of each enoxaparin product were prepared in 0.9% sodium chloride solution, achieving a working concentration of 100 µg/mL. For sample preparation, different volumes of the enoxaparin solution were added to citrated plasma obtained from six healthy volunteers with no history of hematological disorders and not undergoing any pharmacological treatment, to obtain final concentrations ranging from 0.0 to 10.0 mg/L in a total volume of 1000 µL.

The anticoagulant activity of the samples was assessed using thrombin time (TT) and activated partial thromboplastin time (aPTT) assays, performed using the coagulometric method on the Sysmex CS-2500® Automated Blood Coagulation Analyzer (Siemens Healthcare Diagnostics, USA) [14,15].

2.4 Anti-Protease Assay

Chromogenic anti-FXa assays were conducted using the Sysmex CS-2500 Automated Blood Coagulation Analyzer (Siemens Healthcare Diagnostics, USA). Citrated plasma, obtained from the same six healthy volunteers who participated in the clot-based assays, was supplemented with enoxaparin working solutions to achieve final concentrations ranging from 0.0 to 10.0 mg/L. The anti-FXa activity was determined using the INNOVANCE® Anti-Xa reagent (Siemens Healthcare Diagnostics, USA), validated for the standardized quantification of both unfractionated heparin (UFH) and LMWH, following WHO standards.

2.5 Rotational Thromboelastometry (ROTEM) Profile in Whole Blood

The viscoelastic properties of clot formation and stability for both enoxaparin products were evaluated using the ROTEM system (Werfen, Germany) [16]. Drug solutions were pre-diluted to achieve a working concentration of 100 IU/mL. Whole blood samples were collected from healthy human volunteers into citrate tubes (4.5 mL) and supplemented with enoxaparin to achieve a final concentration of 1 IU/mL. A 0.9% sodium chloride solution was used as a negative control. The coagulation process was assessed using two ROTEM assays: the intrinsic pathway thromboelastometry (INTEM), which evaluates the activation of the intrinsic coagulation pathway using ellagic acid as an activator, and the heparinase-modified INTEM (HEPTEM), which neutralizes non-fractioned and low molecular weight heparins and serves as a control to differentiate enoxaparin-induced anticoagulation from other abnormalities.

The ROTEM parameters analyzed included clotting time (CT), clot formation time (CFT), maximum clot firmness (MCF), amplitude at 10 minutes (A10), and lysis index at 30 minutes (LI30). Enoxaparin's primary effect was expected to prolong CT and CFT, while MCF was anticipated to remain stable or slightly reduced. A10 and LI30 were not considered primary parameters for biosimilar assessment.

Enoxaparin primarily exerts its anticoagulant effect by inhibiting Factor Xa and, to a lesser extent, thrombin (Factor IIa), delaying thrombin generation and impairing fibrin formation. As a result, parameters related to clot initiation and formation were expected to be significantly affected, whereas clot firmness and fibrinolysis remained largely unchanged. CT and CFT were anticipated to be prolonged in INTEM due to the inhibition of Factor Xa, while these effects were expected to be reversed in HEPTEM, confirming that the observed ROTEM alterations were directly attributable to enoxaparin activity.

2.6 Statistical Analysis

To strengthen the statistical evaluation of functional assays, regression analyses of aPTT and TT dose–response curves were performed. In addition to p-values, point estimates for slope and EC_{50} were calculated and presented with their corresponding 95% confidence intervals. For the statistical comparison of regression equations, a cross-fitting analysis (CFA)—a variant of ANOVA adapted to regression models—was employed. CFA allows the simultaneous evaluation of slope and intercept differences between two regression lines while accounting for experimental variability, without assuming a priori homogeneity of regression slopes. This method has been recommended in the literature for the analysis of functional assays in the context of equivalence and similarity testing [17]. Curve fitting was performed using GraphPad Prism 10 (GraphPad Software, USA) to determine whether a single regression model adequately described all datasets, which would indicate statistically indistinguishable in vitro activity.

For the anti-protease assays, inhibitor concentration–response relationships were fitted to an exponential plateau equation:

$$Y = Y_{max} - (Y_{max} - Y_0)e^{kX}$$

where Y_{max} is the maximum percentage of inhibition, Y_0 is the baseline inhibition at the lowest concentration, and k is the rate constant. The EC_{50} value, representing the concentration required to achieve 50% of the maximal inhibitory effect, was obtained through nonlinear regression. Curve-fitting analysis in GraphPad Prism 10 (GraphPad Software, USA), was used to compare primary parameters between products and to assess whether a single curve could describe all datasets.

For the rotational thromboelastometry (ROTEM) analysis in whole blood, comparisons between test and reference products were performed using non-parametric methods, as only a single concentration of two different lots of each product was tested in duplicate. The Kruskal–Wallis test assessed differences in clotting time (CT) and clot formation time (CFT) relative to the control (0.9% sodium chloride). When significant differences were detected ($P < 0.05$), Dunn's post hoc test was applied to identify specific group comparisons.

All tests were performed in duplicate and conducted in independent experiments to ensure reproducibility and minimize variability. Across all assays, statistical significance was defined as $P < 0.05$.

3. RESULTS

3.1 Molecular Weight and Potency Profiling

Analytical results confirmed that the enoxaparin supplied by Techdow exhibited molecular weight and potency profiles comparable to those of the reference product. The number-average molecular weights (Mn) were 3117±103 Daltons and 3172±61 Daltons, the weight-average molecular weights (Mw) were 4526±85 Daltons and 4554±23 Daltons, and the polydispersity indices (Mw/Mn) were 1.45 and 1.44 for the biosimilar candidate and the reference product, respectively. The anti FXa/ anti FIIa was 3.8 for both products.

3.2 Anti-FXa Activity

The inhibition of Factor Xa activity was evaluated using a chromogenic anti-FXa assay (Figure 1). The global fit of the exponential model describing the concentration-response relationship was Y = 87.7 - (87.7 + 65.4) * exp (-0.73 * X), with a P-value of 0.06 in the curve fitting analysis (CFA), indicating no statistically significant differences between the biosimilar candidate and the reference product. The EC_{50} values for the two reference product batches were 1.88 mg/L (95% CI: 1.77–2.01) and 2.12 mg/L (95% CI: 1.98–2.27), while the EC_{50} values for biosimilar candidate batches were 1.91 mg/L (95% CI: 1.79–2.04) and 1.89 mg/L (95% CI: 1.72–2.09). These results demonstrate that the biosimilar exhibits a comparable inhibitory effect on Factor Xa activity to that of the reference product, supporting its functional equivalence *in vitro*.

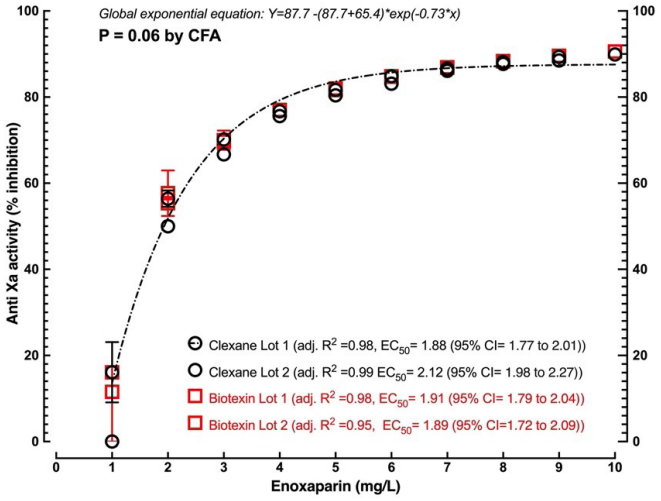

Figure 1. Inhibition of factor Xa activity by two enoxaparin products. The test product, Biotexin (Laboratorios Lasca), is depicted by red lines, while the reference product, Clexane (Sanofi), is depicted by black lines.

3.3 Clot-Based Assays (aPTT and TT)

The anticoagulant effect of the biosimilar candidate and the reference product was evaluated using activated partial thromboplastin time (aPTT) and thrombin time (TT) assays. Regression analyses of the dose–response curves showed highly comparable profiles between both products.

For aPTT (Figure 2), both regression models displayed excellent linearity (adjusted R^2 = 0.99). The slopes and EC_{50} values were closely aligned, with overlapping 95% confidence intervals (slope: 2.47 [2.40–2.54] vs. 2.37 [2.31–2.43]; EC_{50}: 5.73 [5.45–6.02] vs. 5.63 [5.38–5.88]).

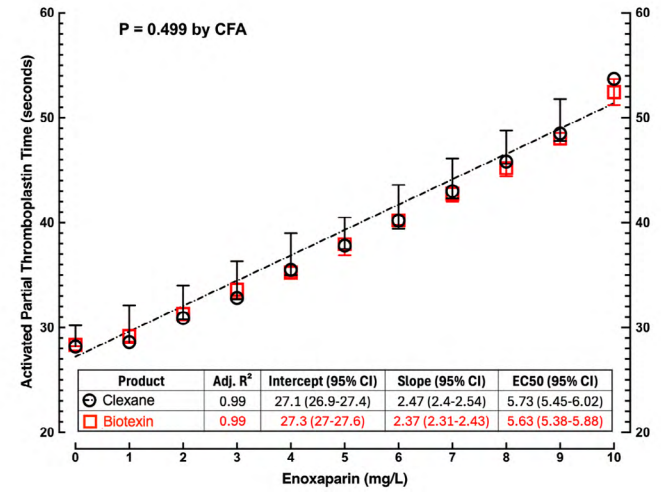

Figure 2. Activated partial thromboplastin time (aPTT) of two enoxaparin products. The test product, Biotexin (Laboratorios Lasca), is depicted by red lines, while the reference product, Clexane (Sanofi), is depicted by black lines.

For TT (Figure 3), linear regression models yielded adjusted R^2 values of 0.97 for the reference product and 0.94 for the biosimilar candidate. The slope and EC_{50} estimates, with 95% confidence intervals, also showed close agreement (slope: 1.86 [1.65–2.08] vs. 1.79 [1.46–2.13]; EC_{50}: 5.86 [5.48–6.24] vs. 6.33 [5.68–6.97]). Cross-fitting analysis (CFA) detected no statistically significant differences in slopes or intercepts for either aPTT (p = 0.499) or TT (p = 0.538).

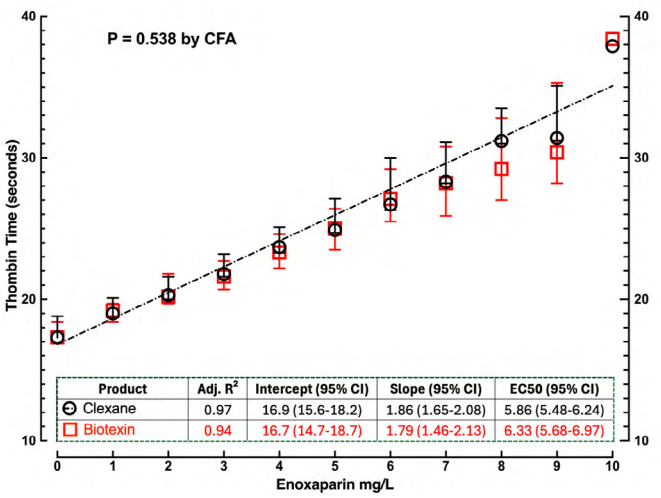

Figure 3. Clotting profile: thrombin time (TT) of two enoxaparin products. The test product, Biotexin (Laboratorios Lasca), is depicted by red lines, while the reference product, Clexane (Sanofi), is depicted by black lines.

3.4 Rotational Thromboelastometry (ROTEM) Analysis

The ROTEM analysis provided additional insights into the anticoagulant effect of Biotexin® and Clexane®, particularly through the intrinsic pathway thromboelastometry (INTEM) and heparinase-modified INTEM (HEPTEM) assays.

In the INTEM assay, clotting time (CT) and clot formation time (CFT) were significantly prolonged in both biosimilar and reference product groups compared to the control. The Kruskal-Wallis test revealed statistically significant differences in CT (P = 0.0043) and CFT (P = 0.0064) between the enoxaparin-treated groups and the 0.9% sodium chloride solution control, confirming the expected anticoagulant effect. However, no significant differences were observed between the test and reference product, supporting their comparability (Table 1).

The median CT values for the biosimilar candidate were 402 s (Lot 1) and 439.5 s (Lot 2), while for the

reference product, they were 458 s (Lot 1) and 430 s (Lot 2). Similarly, the median CFT values were 141.5 s (Lot 1) and 159 s (Lot 2) for test, and 155.0 s (Lot 1) and 175.5s (Lot 2) for reference. The observed prolongation of CT and CFT aligns with enoxaparin's Factor Xa inhibition, further supporting the functional comparability between the biosimilar and the reference product.

The maximum clot firmness (MCF) remained largely unchanged across products, with values within the expected range (55–59 mm) for all samples. Amplitude at 10 minutes (A10) and Lysis Index at 30 minutes (LI30) also remained stable, reinforcing that enoxaparin primarily affects clot initiation rather than clot stability.

In the HEPTEM assay, which includes heparinase to neutralize enoxaparin's effect that depends on its molecular integrity, both the CT and CFT values were substantially reduced, returning to levels similar to those of the control group. This confirms that the ROTEM alterations observed in INTEM were specifically due to enoxaparin activity and no other coagulation abnormalities.

Table 1. Comparative thrombelastograph (ROTEM) assessment of biosimilar candidate and reference product anticoagulant activity.

			Biotexin®						Clexane®					
ROTEM parameter*	Reference Values	Control	Lot 1 (2410311)			Lot 2 (2410312)			Lot 1 (HS562E)			Lot 2 (HS712C)		
			Sample 1	Sample 2	Median	Sample 1	Sample 2	Median	Sample 1	Sample 2	Median	Sample 1	Sample 2	Median
INTEM **														
CT	100-240	218	423	381	402	438	441	439,5	467	448	458	505	355	430
CFT	30-110	98	149	134	141,5	161	157	159	171	139	155	199	152	175,5
MCF	50-72	56	57	58	57,5	56	56	56	55	55	55	53	57	55
A10	44-66	51	47	48	47,5	45	45	45	45	48	46,5	42	46	44
LI30	94-100	98	100	100	100	100	100	100	100	98	99	100	100	100
HEPTEM ***														
CT	100-240	219	236	224	230	238	224	231	236	234	235	254	236	245
CFT	30-110	93	112	108	110	110	108	109	110	100	105	107	101	104
MCF	50-72	57	57	58	57,5	58	57	57,5	58	57	57,5	59	59	59
A10	44-66	52	49	51	50	50	49	49,5	51	51	51	53	52	52,5
LI30	94-100	98	100	100	100	100	100	100	100	100	100	100	100	100

*The CT and CFT are in seconds, MCF and A10 are in mm, and LI30 are a percentage of MCF. **INTEM (intrinsic pathway activation). *** HEPTEM (intrinsic activation with inhibition of heparin)

DISCUSSION

Enoxaparin, a low molecular weight heparin, is widely used for its anticoagulant properties. It is produced through the depolymerization of heparin and has been the subject of various studies comparing its efficacy and safety to biosimilar versions [10]. The present study evaluated the *in vitro* comparability of this potential biosimilar enoxaparin, with the reference product through a comprehensive stepwise assessment of anticoagulant activity. The results demonstrated a high degree of similarity between both products across multiple analytical and functional assays, including chromogenic anti-FXa activity, clot-based assays (aPTT and TT), and whole-blood thromboelastometry (ROTEM®). These findings provide robust evidence supporting the functional equivalence of Biotexin® to Clexane®, reinforcing its potential as a biosimilar enoxaparin.

The anticoagulant activity of enoxaparin is primarily driven by its ability to enhance the action of antithrombin III, thereby inhibiting thrombin formation. Its efficacy is closely associated with the preferential inhibition of factor Xa over factor IIa, with factor Xa inhibition being the main contributor

to its anticoagulant effect [3]several biosimilars of low-molecular-weight heparins (LMWHs. In this study, factor Xa inhibition was determined using a chromogenic anti-FXa assay. The EC_{50} values obtained for biosimilar and reference product were within overlapping confidence intervals, with no statistically significant differences in curve fitting analysis (CFA, P = 0.06). These results indicate that the potential biosimilar enoxaparin exhibits a comparable inhibitory potency against Factor Xa, a key determinant of enoxaparin's anticoagulant effect. The preservation of Factor Xa inhibition is critical for biosimilarity, as even minor deviations could result in altered pharmacodynamic responses and potential differences in clinical efficacy [18,19].

In vitro studies comparing anticoagulant activity underscore the importance of statistically evaluating clotting time (CT), thrombin time (TT), and activated partial thromboplastin time (aPTT) in bioequivalence assessments. Consistent with previous *in vitro* findings that demonstrated similar TT and aPTT profiles—showing indistinguishable prolongation between test and reference products [10,20,21]—our study revealed closely aligned mean TT and aPTT curves for both products (Figures 2 and 3). Linear regression analysis of enoxaparin concentration versus clotting time yielded nearly identical slopes and intercepts, further supporting the similarity between the formulations. The comparative evaluation of aPTT and TT assays demonstrated that the biosimilar candidate and the reference product exhibit equivalent anticoagulant activity. The consistency of regression parameters, including slopes and EC_{50} values with overlapping 95% confidence intervals, together with the absence of statistically significant differences by CFA (p = 0.499 for aPTT; p = 0.538 for TT), confirm that both products induce comparable prolongation of clotting times. Collectively, these findings demonstrate comparable effects on thrombin generation and fibrin clot formation, reinforcing the functional biosimilarity of the biosimilar candidate to the reference enoxaparin, in line with the requirements for demonstrating pharmacodynamic equivalence.

Whole-blood ROTEM analysis provided additional insights into the anticoagulant effect of biosimilar candidate and reference product under physiological conditions, particularly through the intrinsic pathway (INTEM) and heparinase-modified INTEM (HEPTEM) assays. The observed prolongation of clotting time (CT) and clot formation time (CFT) in INTEM aligns with the expected anticoagulant effect of enoxaparin, confirming its mechanism of action through Factor Xa inhibition. The Kruskal-Wallis test demonstrated that CT and CFT prolongation were significantly different compared to the control (P = 0.0043 for CT and P = 0.0064 for CFT) but not between both products, further reinforcing their comparability.

Notably, the maximum clot firmness (MCF) remained stable across both enoxaparin products, suggesting that their anticoagulant action is mainly exerted on the early phase of coagulation by delaying clot initiation, rather than affecting the mechanical stability of the formed clot. This observation is relevant from a clinical perspective, as it implies that enoxaparin preserves the final clot strength while modulating thrombin generation and fibrin polymerization kinetics. These findings are in agreement with previous studies that have identified ROTEM as a sensitive and reliable tool to detect the anticoagulant effects of heparins, including subtle differences in clotting time and initiation parameters without substantial changes in clot firmness [22]. Furthermore, the reversal of CT and CFT prolongation in HEPTEM, where heparinase neutralizes enoxaparin activity, confirms that the observed ROTEM alterations were specifically mediated by enoxaparin rather than other coagulation abnormalities [23].

Structural comparability is a critical prerequisite for establishing biosimilarity, since enoxaparin is a heterogeneous mixture of sulfated oligosaccharides with distinct molecular weight distributions that influence its pharmacokinetics and pharmacodynamics. In this study, we used two products with comparable molecular weight distributions and similar anti-Xa/anti-IIa activity ratios, which correlate with similar biological activity in *in vitro* tests.

Regulatory agencies, including the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA), require a rigorous stepwise approach for the approval of biosimilar enoxaparin products, involving detailed analytical, *in vitro*, and clinical studies [24]. The findings of the present study strongly support the *in vitro* similarity of Biotexin® to Clexane®, fulfilling part of the biosimilarity assessment. While these *in vitro* results provide substantial evidence of equivalence, additional pharmacokinetic/pharmacodynamic (PK/PD) studies and clinical trials could be necessary to confirm therapeutic interchangeability [25].

The results of this study are consistent with previous investigations of enoxaparin biosimilars that have successfully demonstrated functional equivalence *in vitro*, leading to regulatory approval and clinical adoption. Notably, Inhixa, an enoxaparin biosimilar also manufactured by Techdow Pharmaceutical, was approved in Europe based on a similar stepwise demonstration of biosimilarity, highlighting the feasibility of this approach [6].

It is important to acknowledge certain limitations of this study. The comparability assessment was performed using only two production batches, which constrains the ability to evaluate inter-batch variability and reduces the statistical robustness of the analysis. Including additional batches would provide a more comprehensive assessment of potential manufacturing variability, thereby enhancing the analytical robustness and increasing the credibility of the conclusions regarding biosimilarity.

CONCLUSION

The results of this study provide strong evidence supporting the *in vitro* biosimilarity of Biotexin® to Clexane®, based on comparable Factor Xa inhibition, clot-based assay responses, and ROTEM anticoagulation profiles. These findings suggest that the biosimilar candidate is functionally equivalent to the reference product, fulfilling the essential criteria for biosimilar development. Future PK/PD and clinical studies will be necessary to confirm its therapeutic interchangeability in a clinical setting.

DECLARATIONS

Ethics approval and consent to participate.

Informed consent was obtained from all individual participants to obtain the blood samples used for *in vitro* analysis.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

AFZ has received honoraria for advisory boards and lectures not related to the content of this paper from Abbvie, Amgen, Bayer, Janssen, Lilly, Merck, Novartis, Novo Nordisk, Pfizer, Roche, and Sanofi. IJT and AH declare no conflicts of interest.

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Authors' contributions

AFZ and IJT assisted in conceptualization, project drafting, searching, and manuscript writing. AH assisted in experimental procedures. All authors review and approve the final manuscript.

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