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## Absolute pharmacokinetics of heparin in primates

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#### ABSTRACT

Heparin is a commonly used anticoagulant drug, derived from the tissues of animals including pigs, cows, and sheep. Measuring heparin concentration in plasma is challenging due to its complex molecular structure. Existing methods rely on measuring heparin's anticoagulant activity, which provides pharmacodynamic (PD) data but not pharmacokinetic (PK) data, measuring concentration over time. To overcome this limitation, we used liquid chromatography-mass spectrometry (LC-MS) and the multiple reaction monitoring (MRM) method to directly measure heparin's concentration in non-human primates after administering porcine, bovine, and ovine heparin. A protocol was developed to enable an MRM method for application to small plasma volumes without purification. The PK data obtained from LC-MS are then compared with the data obtained using the Heparin Red assay and the PD data determined using biochemical clinical assays. Results showed that LC-MS and Heparin Red assay measurements closely correlated with unfractionated heparin's biological activities, supporting the use of mass spectra and dye-binding assays to determine heparin levels in plasma. This study builds a way for the measurement of heparin concentration in plasma, which could lead to an improved understanding of heparin's metabolism and dosing safety.

## 1. Introduction

Heparin is an animal-derived, heterogeneous, anionic polysaccharide widely used as an anticoagulant in clinical practice. As a member of the glycosaminoglycan family, heparin is composed of repeating disaccharide units made up of hexosamine and uronic acid residues. These disaccharide units undergo modifications with *N*-acetyl or *N*-sulfo and *O*-sulfo groups at various positions, resulting in an incredibly large number of sequence permutations (Esko & Selleck, 2002; Xia et al., 2021). Fig. 1a illustrates a representative chain structure of heparin.

Partially purified heparin became commercially available in the 1920s (Mutt & Blombäck, 2000), predating the establishment of the US FDA in 1937. The regulatory experience with heparin products started with the introduction of low molecular weight heparins (LMWH) in the 1980s (Linhardt, 1991, 2003). Despite the emergence of new oral anticoagulants over the past two decades, heparin remains irreplaceable in certain applications, such as extracorporeal therapy (Onishi et al.,

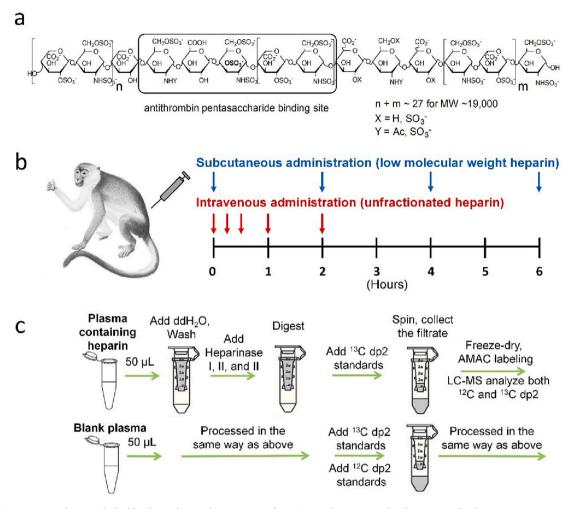
2016).

As heparin is a crucial drug essential to the practice of modern medicine, it is of utmost importance to ensure a stable and secure supply chain. Currently, porcine-derived heparin remains the primary source of heparin but relying on a single animal source poses risks in terms of both quantity and quality of active pharmaceutical ingredients (Guerrini et al., 2008; Kouta, 2021; Liu et al., 2009; Ouyang et al., 2019). Moreover, it raises religious concerns among different populations (Kouta et al., 2019). Thus, alternative sources of heparin, such as bovine and ovine heparins, are being re-evaluated and explored as potential alternatives (Keire et al., 2015). Also, recent advancements in biotechnology have opened new possibilities for producing bioengineered heparins from non-animal sources, an approach that the FDA has encouraged as a long-term strategy to replace animal-sourced heparin (Cress et al., 2019; Jin et al., 2021).

The safe use of different types of heparins requires a thorough understanding of their pharmaceutical kinetics. To achieve this, it is crucial to characterize the relationship between their pharmacokinetics (PK,

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**Fig. 1.** Heparin structure and protocols for blood sampling and preparation of LC-MS samples. **a.** generalized structure of a chain common to porcine, bovine, and ovine unfractionated heparins showing a typical structure for an antithrombin (AT) pentasaccharide binding site; **b.** time points for blood sampling in the PK/PD studies of unfractionated heparins (UFH) and low molecular weight heparin (LMWH). Porcine, bovine, and ovine UFHs were administered *iv* and low LMWHs were administered *sc* to non-human primates, *Macaca mulatta*; **c.** flowchart of the LC-MS sample preparation process. A 50 μL citrated plasma sample was transferred to a 10-kDa spin column, washed, treated with heparinase, and spiked with <sup>13</sup>C disaccharide standards to minimize deviation between samples. The sample was then spun to recover heparin disaccharides, freeze-dried, labeled with AMAC, and analyzed using the LC-MS MRM method for both <sup>12</sup>C and <sup>13</sup>C disaccharides. Additionally, blank plasma samples were spiked with <sup>12</sup>C disaccharide standards to derive calibration curves.

concentration vs. time) and pharmacodynamics (PD, effect vs. time), which is an essential tool in drug discovery and development. However, heparin's chemical complexity resulting from various chain sizes and sequences makes it challenging to analyze and quantify chemically. Therefore, the most common way to assess heparin levels in the body is through bioassays that measure its PD rather than its PK (Mousa et al., 2007; Walenga & Fareed, 1994).

The standard methods for monitoring heparin levels involve tests such as activated thromboplastin time (aPTT), chromogenic anti-factor Xa/IIa assays, or, in cases where higher levels are required, activated clotting time (ACT). Circulating heparin levels determined using these assays have been used to study the PD behavior of heparins in animal models (Brindley et al., 1993; Jeske et al., 2015; Jeske et al., 2018), healthy volunteers (Alban et al., 2002; Mousa et al., 2007), and patient populations (Brancazio et al., 1995; Delavenne et al., 2017). Although these methods have become increasingly sophisticated and sensitive, they only provide information about heparin's biological activity, rather than its chemical presence. Researchers have had to rely on indirect methods that involve measuring the interaction of heparin polyanions with cations to accurately determine heparin PK (Warttinger et al., 2016). However, these methods lack specificity, as they measure the presence of all polyanions, rather than just heparin (Warttinger et al.,

2017). Therefore, a true measure of heparin PK remains an important but unsolved challenge for medical researchers.

Advances in glycoanalysis have led to the use of sensitive and selective LC-MS methods. However, developing an LC-MS method for heparin poses challenges due to its large molecular weight and heterogeneity. Fortunately, modern analytical technology can help address these issues. Heparin lyases are specific enzymes that can convert heparin to smaller disaccharide building blocks. Multiple reaction monitoring (MRM) detection is considered the gold standard for quantitative mass spectrometry (Colgrave et al., 2012), as it enhances sensitivity by removing background signals, particularly when the target molecules are derivatized. MRM methods have been used to quantify glycosaminoglycans from different kinds of biological specimens at the nanogram level (Kaur et al., 2022; Li et al., 2015), but not to determine heparin PK in plasma. The complex plasma matrix affects the MS detection of heparin's disaccharide units. Quantifying glycosaminoglycans in plasma using LC-MS often requires the extraction of glycosaminoglycans (Bratulic et al., 2022), which increases the complexity of the operation and the required amount of plasma sample.

In this study, we developed a protocol for heparin LC-MS (MRM mode) analysis using small volumes of unpurified plasma samples. We investigated the *intravenous* (*iv*) and *subcutaneous* (*sc*) administration, on

both a mass and unit basis, of three different animal-derived heparins in a primate model, representing the gold standard for pharmacological evaluation of heparin *in vivo* (Fareed et al., 1985). We evaluated the PD of the heparins in primates using currently accepted bioassays, and their PK using both LC-MS and Heparin Red, a dye-binding assay. The results of this study provide the first true PK evaluation of *iv*-administered heparin, and allow for a comparison of porcine, bovine, and ovine intestinal mucosal heparins to better understand their *in vivo* efficacy. Furthermore, the compositional changes of heparin revealed by the LC-MS method provide informative insights into the metabolism of heparin *in vivo*.

#### 2. Materials and methods

#### 2.1. Materials

Porcine mucosal unfractionated heparin (Porcine UFH, Lot # PH-13353, 180-195 USP U/mg) was obtained from Medefil Inc., Glendale Heights, IL, USA; bovine mucosal unfractionated heparin (Bovine UFH, Lot # GAG25, 130-140 USP U/mg) was obtained from KinMaster Produtos Ouímicos. Passo Fundo, Brazil: ovine mucosal unfractionated heparin (Ovine UFH, Lot # OHS-160301, 185-200 USP U/mg) was obtained from Ronnsi Pharmaceutical, Suzhou, China; porcine low molecular weight heparin (Porcine LMWH, Lot # Lovenox-7LO10, antifactor Xa and anti-factor IIa potencies in the range of 93-103 U/mg and 30-40 U/mg, respectively) was obtained from Sanofi, Paris, France; bovine low molecular weight heparin (Bovine LMWH, Lot # RX0015-JCJ-003,96-105 U/mg and 30-40 U/mg, respectively), was obtained from Ronnsi Pharmaceutical, Suzhou, China; ovine low molecular weight heparin (Ovine LMWH, Lot # EOS-160403, 94-104 U/mg and 30-40 U/mg, respectively) was obtained from Ronnsi Pharmaceutical, Suzhou, China. The USP reference standard (Heparin Sodium, Lot # FOI187, 9.5 mg - 2144 USP Units) was from the United States Pharmacopeia (USP) Rockville, MD, USA. All heparins were active pharmaceutical ingredients (API). All low molecular weight heparins used in this study were prepared by chemical beta-elimination of the benzyl ester of heparin. The molecular weight profiles of the heparins are provided in Table S1.

Unsaturated  $^{12}\text{C}$  disaccharide standards of heparan sulfate (HS, a glycosaminoglycan structurally related to heparin) ( $\Delta\text{UA-GlcNAc}$  (0S);  $\Delta\text{UA-GlcNAc}$  (S);  $\Delta\text{UA-GlcNAc}$  (S);  $\Delta\text{UA-GlcNAc}$  (2S);  $\Delta\text{UA-GlcNAc}$  (2S6S);  $\Delta\text{UA-GlcNAc}$  (2S6S);  $\Delta\text{UA-GlcNAc}$  (2S6S);  $\Delta\text{UA-GlcNAc}$  (2S6S);  $\Delta\text{UA-GlcNAc}$  (2S6S);  $\Delta\text{UA-GlcNAc}$  (2S6S);  $\Delta\text{UA-GlcNAc}$  (1S1S), where  $\Delta\text{UA}$  is 4-deoxy- $\alpha$ -L-threo-hex-4-enopyranosyluronic acid, GlcN is glucosamine, Ac is acetyl and S is sulfo, were purchased from Iduron (UK). The  $^{13}\text{C}$  counterparts of the above unsaturated disaccharide standards were obtained from Jian Liu (College of Pharmacy, University of North Carolina). Recombinant *Flavobacterial* heparin lyases (hepainase) I, II, and III were expressed in our laboratory using *Escherichia coli* strains provided by Jian Liu. 2-Aminoacridone (AMAC), sodium cyanoborohydride were obtained from Sigma-Aldrich (St. Louis, MO, USA). All solvents were HPLC grade. All the centrifugal filters used were from Millipore.

### 2.2. Primate dosing/blood sample collection

Male (n = 6) and female (n = 6) non-human primates (Macaca mulatta) (6.4–10.8 kg) were used in this study. All animal studies were carried out in compliance with the guidelines by the Institutional Animal Care and Use Committee (IACUC), Loyola University Chicago Health Sciences Division and the Guide for the Gare and Gare Ga

Primates were anesthetized by the intramuscular administration of ketamine (10 mg/kg) and xylazine (1–2 mg/kg) based on their most recent charted weight. Following attainment of the appropriate depth of anesthesia (assessed by a lack of response to foot pinch), primates were freshly weighed to accurately determine the dose of the test agent.

Baseline blood samples were collected by venipuncture of the saphenous vein. Bovine, ovine, and porcine UFHs were administered intravenously (*iv*) at an equi-gravimetric dose of 0.5 mg/kg or equi-unit doses of 50 or 100 anti-Xa U/kg through the contralateral saphenous vein. Additional blood samples were collected at 15, 30, 60 and 120 min, post-drug administration (Fig. 1b). Four primates were dosed with each heparin. Bovine, ovine and porcine low molecular weight heparins were administered subcutaneously (*sc*) at an equi-gravimetric dose of 1.0 mg/kg. Additional blood samples were collected at 120, 240 and 360 min, post-drug administration.

All blood samples were collected using a double syringe technique, employing a 21-gauge butterfly needle. After an initial  $\sim\!1$  mL volume (discard blood) was collected, the syringe was changed, and a 2.7 mL sample was drawn and placed into a tube containing 0.3 mL 3.2 % sodium citrate. Citrated blood samples were centrifuged at 1100g for 15 min. The supernatant platelet poor plasma was harvested, and aliquots of plasma were stored frozen at  $-70~^{\circ}\text{C}$ .

To prepare blank monkey plasma, untreated, non-human primates were an esthetized with ketamine (10 mg/kg, intramuscular (im) + xylazine (1–2 mg/kg, im) and blood samples were drawn from the saphenous vein using a 21-gauge im4 inch butterfly needle and a 5 mL syringe. The blood was transferred into a 4.5 mL sodium citrate (3.2 %) BD Vacutainer im6 blood collection tube (Becton Dickinson and Company, Franklin Lakes, NJ) and centrifuged at 4 °C, 2053g for 20 min. Plasma from a minimum of 12 primates was pooled and aliquoted in 10 mL volumes. NMP was frozen in plastic tubes at im80 °C for no im22 months.

#### 2.3. Bioassays: chromogenic anti-Xa and anti-IIa activity measurements

Purified frozen form of human thrombin (Lot # HT 5528PA) reagent and Factor Xa enzyme were obtained from Enzyme Research Laboratories (South Bend, IN) at concentrations of 1000 U/mL. Spectrozyme Xa and Spectrozyme factor IIa substrates were obtained from Sekisui Diagnostics (Stamford, CT). Heparin anti-Xa and anti-IIa USP kits (Biophen Xa and Biophen IIa) were obtained from Hyphen BioMed (Aniara; West Chester, OH). These assays were performed following the instructions included with the USP assay kits.

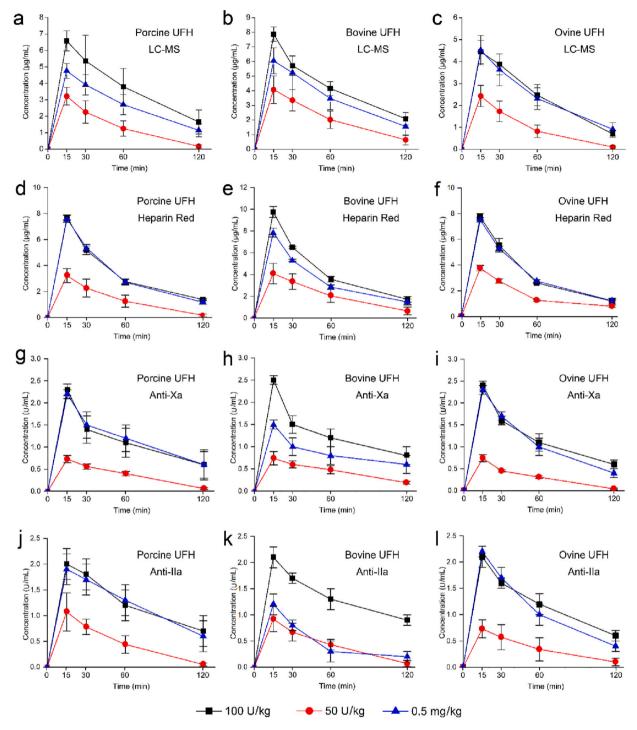
### 2.4. Heparin Red assay

Heparin Red kits (Redprobes UG, Münster, Germany) were used for the quantification of heparin in citrated plasma by a fluorescence assay. This assay measures anionic glycosaminoglycan in mass-based concentration expressed as  $\mu g/mL$ . The Heparin Red assay relies on a cationic molecular probe that changes fluorescence intensity when it binds to anionic polymers like heparin (Warttinger et al., 2016). The assay was carried out following the kit's instruction. Briefly, 20  $\mu$ L of plasma was placed into microplate (black, for fluorescence detection). The Heparin Red and Enhancer solutions were mixed in a 1/111 (V/V) ratio, and 80  $\mu$ L of the mixture was added into the plate well containing plasma. The microplate was immediately introduced in the plate reader, shaken at high speed for 3 min and started fluorescence detection. The calibration curve was derived using the same kind of reference heparin spiked into blank plasma with a concentration range of 1–8  $\mu$ g/mL.

## 2.5. Liquid chromatography-mass spectrometry (LC-MS) assay

#### 2.5.1. Sample preparation

Plasma (50  $\mu L)$  from each specimen was ultrafiltered through a 10-kDa molecular weight cut-off spin tube to remove small molecular compounds. The filter unit was washed twice with 400  $\mu L$  distilled water and then added with 300  $\mu L$  digestion buffer (50 mM ammonium acetate containing 2 mM calcium chloride adjusted to pH 7.0). Recombinant heparin lyase I, II, III (pH optima 7.0–7.5) were added to each filter unit containing sample, mixed well, and incubated at 37 °C for 24 h. Then a mixture of eight  $^{13}\text{C-labeled}$  disaccharide standards (40 ng of each

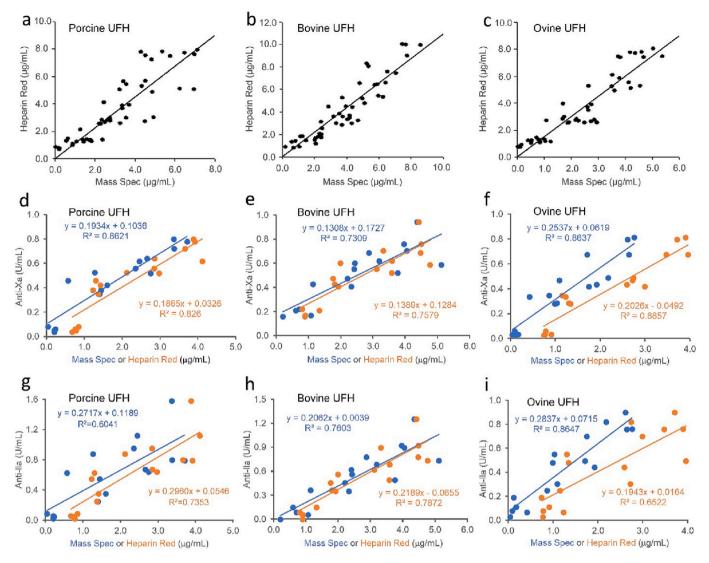


**Fig. 2.** Pharmacokinetic (PK) and pharmacodynamic (PD) time course plots of heparin in plasma after administration of 100 U/kg, 50 U/kg, and 0.5 mg/kg of porcine, bovine, and ovine unfractionated heparins. Plots a—c were determined by LC-MS; plots d—f were determined by Heparin Red assay; plots g-i were determined by anti-Xa assay; and plots j—l were determined by anti-IIa assay. Plots a, d, g, and j correspond to porcine heparin; plots b, e, h, and k correspond to bovine heparin; and plots c, f, i, and l correspond to ovine heparin.

disaccharide) was added into the plasma samples to serve as internal normalization references (Fig. 1c). The enzymatic digestion was terminated by ultrafiltration through the 10 kDa spin tube. The filtrate was collected, and the filter unit was washed twice with 200  $\mu L$  distilled water. All the filtrates containing the disaccharide products were combined and freeze dried.

Besides the above samples, calibration references were prepared using the blank plasma and normal  $^{12}$ C disaccharide standards. One set of blank plasma (50  $\mu$ L) was supplemented with only  $^{13}$ C-labeled

standards as described above. Another two sets blank plasma (50  $\mu L$  each) were supplemented with premixed  $^{12}C$  disaccharide standards (7.5  $\mu M$  for each disaccharide) at a lower amount (5 or 10  $\mu L$ ) and a higher amount (30 or 50  $\mu L$ ), respectively. This step was carried out after the digestion was completed and the  $^{13}C$ -labeled disaccharide standards were added, and then all the reference samples were subjected to the same collection procedure as mentioned above. The protocol for sample preparation is exhibited in Fig. S1.



**Fig. 3.** Correlations of plasma heparin quantification using different methods. Plots a–c, the correlation between LC-MS analysis and the Heparin Red assay; Plots d–f, the correlation between the anti-IIa assay and the LC-MS/Heparin Red assays; Plots g–i, the correlation between the anti-IIa assay and the LC-MS/Heparin Red assays. The data for plots a, d, and g correspond to porcine heparin, plots b, e, and h correspond to bovine heparin, and plots c, f, and i correspond to ovine heparin. For plots a–c. the correlations include all time points for primates treated with any of the *iv* doses (0.5 mg/kg, 50 U/kg, 100 U/kg). For plots d–i, the correlations include all time points for primates treated with the *iv* dose of 50 U/kg.

#### 2.5.2. AMAC labeling

The dried samples were AMAC-labeled by adding 10  $\mu L$  of 0.1 M AMAC in dimethyl sulfoxide/acetic acid (17/3, V/V) incubating at room temperature for 10 min, followed by adding 10  $\mu L$  of 1 M aqueous sodium cyanoborohydride and incubating for 1 h at 45 °C. After the AMAC-labeling reaction, the samples were centrifuged, and each supernatant was recovered and diluted with the same volume of dilution solvent (water/dimethyl sulfoxide/acetic acid = 20/17/3, V/V) for MS analysis.

### 2.5.3. LC-MS operation

LC was performed on an Agilent 1200 LC system at 45 °C using an Agilent Poroshell 120 ECC18 (2.7 mm,  $3.0\times50$  mm) column. Mobile phase A (MPA) was 50 mM ammonium acetate aqueous solution, and the mobile phase B (MPB) was methanol. The mobile phase passed through the column at a flow rate of 300  $\mu L/min$ . The gradient was 0–10 min, 5–45 % B; 10–10.2 min, 45–100 % B; 10.2–14 min, 100 % B; 14–22 min, 100–5 % B. Injection volume is 5  $\mu L$ . A triple quadrupole mass spectrometry system equipped with an ESI source (Thermo Fisher Scientific, San Jose, CA) was used a detector. The online MS analysis

utilized the Multiple Reaction Monitoring (MRM) mode. MS parameters: negative ionization mode with a spray voltage of 3000 V, a vaporizer temperature of 300  $^{\circ}$ C, and a capillary temperature of 270  $^{\circ}$ C.

## 2.6. Calculation method for LC-MS assay

Based on our previous studies, we have found that the detection of MS can be significantly affected by the plasma matrix. We spiked normal  $^{12}\mathrm{C}$  disaccharide standards into blank plasma to construct a calibration curve for the quantification of plasma samples. By using the  $^{12}\mathrm{C}$  disaccharide standards at the same level as the disaccharides digested from heparin, the calibration curve can be adapted for quantification of plasma samples.

 $^{13}\mathrm{C}\text{-labeled}$  disaccharide standards were spiked in, as the plasma matrix of each sample may differ from one another, to minimize deviation in each individual sample. The MRM method was used to record the peak area of all  $^{12}\mathrm{C}\text{-}$  and  $^{13}\mathrm{C}\text{-labeled}$  disaccharides. For each sample, the peak areas of the  $^{12}\mathrm{C}$  disaccharides were normalized. The normalization process utilized the read peak area of each  $^{12}\mathrm{C}\text{-}$  and its  $^{13}\mathrm{C}\text{-labeled}$  counterpart, as well as the average peak area of  $^{13}\mathrm{C}\text{-labeled}$  disaccharide

 $^{12}$ C Peak Area<sub>Normalized</sub>= $^{12}$ C Peak Area<sub>Read</sub> ×  $\left(^{13}$ C-labeled Peak Area<sub>Average</sub>/ $^{13}$ C-labeled Peak Area<sub>Read</sub> $\right)$ 

The background (endogenous) HS was then removed by subtracting the  $^{12}$ C peak area of the blank plasma from the normalized data of the non-blank samples. Using the calibration curve, each heparin disaccharide was calculated, and the amount of heparin was determined by summing up all eight disaccharide building blocks. The four primates dosed with the same heparin were used as duplicates for the quantification.

# 2.7. Calculation method for Heparin Red assay, bioassays, and pharmacokinetic parameters

The calibration curves for Heparin Red assay and bioassays were created by supplementing porcine, bovine, and ovine heparins into the blank plasma. The graphing software, SigmaPlot for Windows version 12.3 (Systat Software, San Jose, CA) was employed to generate the best-fit curves, plotting the concentrations of different heparins against optical densities or fluorescence intensity. The area under the curve (AUC) and half-life were calculated using the PKSolver® add-in software for Microsoft Excel (Zhang et al., 2010). Results are presented as mean  $\pm$  SD.

#### 3. Results

# 3.1. Pharmacokinetics and pharmacodynamics of intravenously administered unfractionated heparin

Fig. 2a–c and Table S2 present the pharmacokinetics (PK) of unfractionated heparin (UFH) as determined by LC-MS. All three types of UFH showed similar concentration curves for doses of 100 U/kg and 0.5 mg/kg, with a curve of half the height for a 50 U/kg dose. Following administration of a 50 U/kg dose, each type of UFH displayed an elimination half-life ranging from 20 to 40 min, whereas doses of 100 U/kg and 0.5 mg/kg exhibited extended half-lives (Table S3).

Plasma heparin levels were also determined using Heparin Red assay, which is based on cationic dye binding to ionic heparin and results in fluorescence quenching. The time course of heparin levels assessed using the Heparin Red assay is shown in Fig. 2 d–f. The curves for UFH at doses of 100 U/kg and 0.5 mg/kg were similar, with peak heparin levels of approximately 8 to 10  $\mu g/mL$  and similar areas under the curve (AUC) (Table S4). When each heparin was administered at a dose of 50 U/kg, the AUC was proportionately reduced. The mean half-life of the various heparins did not depend on the dose administered when determined using the Heparin Red assay.

Heparin levels were also assessed using anti-Xa and anti-IIa assays (Fig. 2 g-i and j-l), which represent the pharmacodynamics (PD) of UFH. At the 100 U/kg dose, each heparin displayed peak heparin levels of approximately 2.5 U/mL as measured by the anti-Xa assay. The curves for the 0.5 mg/kg dose were similar to those for the 100 U/kg dose, except for bovine UFH. Similar curves were observed when heparin levels were determined based on anti-factor Xa and anti-factor IIa activities. Consistent with previous reports, the half-life of heparin was longer following the administration of higher doses (Table S3), likely contributing to the disproportionate increase in AUC observed with increasing dose from 50 to 100 U/kg (Table S4).

LC-MS and Heparin Red assays determine absolute heparin levels in different ways. Nonetheless, the correlation between heparin levels determined using each assay was good, with correlation coefficients >0.9 for each heparin ( $r^2=0.929$  for porcine;  $r^2=0.960$  for bovine;  $r^2=0.959$  for ovine) (Fig. 3a–c). The regression lines for each heparin exhibited slopes slightly over 1, indicating that the Heparin Red assay tended to report higher heparin levels compared to LC-MS. This effect was most apparent for ovine heparin (slope ovine = 1.497; slope porcine = 1.123; slope bovine = 1.096).

The LC-MS and Heparin Red assays also correlated to the anti-Xa and anti-IIa bioassays (Fig. 3 d–i). The correlations between the chemical and bioassays were lower (ranging from 0.6041 to 0.8857) than those between the two chemical assays. Interestingly, the correlations between the chemical and bioassays were lowest in the case of bovine heparin, which had the lowest specific activity.

#### 3.3. Variation of disaccharide proportion of UFH in plasma

The disaccharide composition of heparin from different animal sources has been investigated by Wang et al. (2022) and Zhang et al. (2023). In the current study, plasma from treated primates was used to observe the disaccharide composition at an early time point ( $T_{max}$  of 15 min), which was found to be similar to previous reports. Our findings are in agreement with Zhang's report (Zhang et al., 2023) and largely consistent with those presented by Wang et al. (2022), with the exception of slightly reduced TriS ( $\Delta$ UA2S-GlcNS6S) and increased NS2S ( $\Delta$ UA2S-GlcNS) percentages. Among the different heparins, ovine heparin has the highest proportion of TriS disaccharides (Fig. 4), while bovine heparin is less sulfated than porcine and ovine heparins and has a higher percentage of NS2S. The weight percentages of each disaccharide building block for the different heparins are provided in Table S5.

As time passed after administration of an iv dose of UFHs, all groups showed a decrease in TriS proportion and an increase in NS2S and 0S proportions (Fig. 5), while NS6S percentages remained stable across all groups. The low-dose (50 U/kg) porcine and ovine groups exhibited a higher rate of decrease in TriS proportion than the high-dose groups

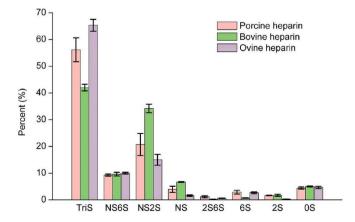


Fig. 4. The disaccharide composition of the unfractionated heparins (100 U/kg) in plasma at 15 min ( $T_{max}$ ). The bar graph shows the weight percentage of the disaccharide building blocks for each heparin, with the total disaccharide content of each heparin adding up to 100 %. Error bars represent the standard deviation for  $n \geq 3$ .

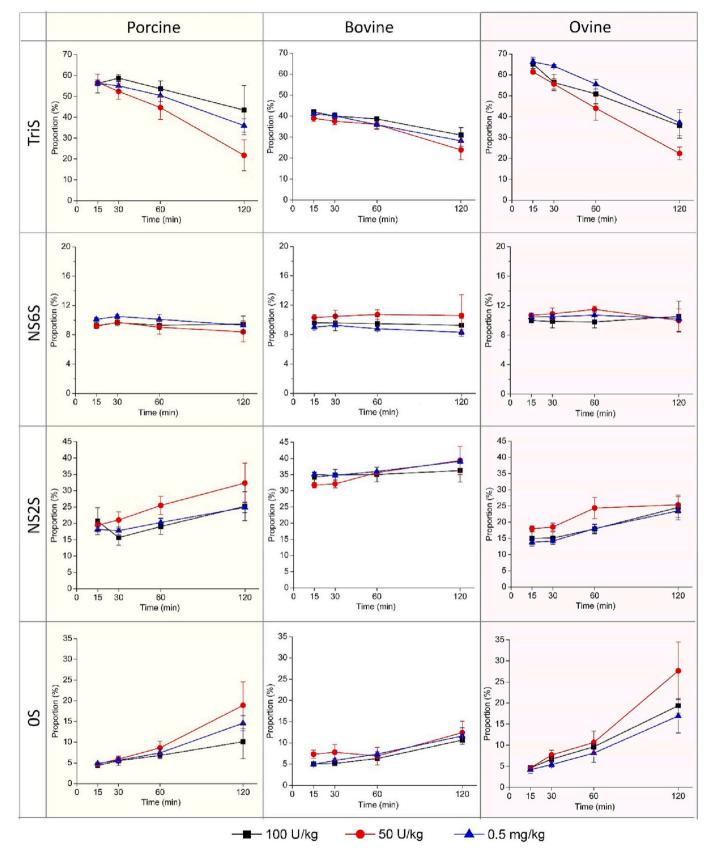


Fig. 5. Variation of the proportion of major disaccharides in primate plasma following administration of porcine, bovine, and ovine unfractionated heparin. The types of the disaccharide (TriS, NS6S, NS2S, OS) and the animal origin of heparin (porcine, bovine, and ovine) are labeled for each row and column.

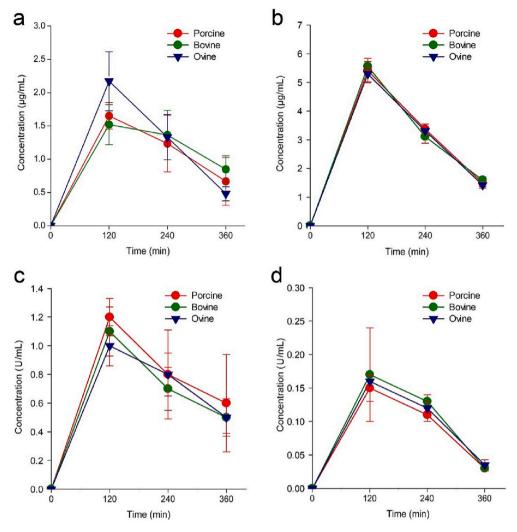


Fig. 6. Pharmacokinetic (PK) and pharmacodynamic (PD) time course plots for low molecular weight heparin (LMWH) in plasma after subcutaneous administration of 1 mg/kg of porcine, bovine, and ovine LMWHs. LMWH levels were determined using four different methods: a, LC-MS analysis; b, Heparin Red assay; c, anti-Xa assay; and d, anti-IIa assay.

(100 U/kg and 0.5 mg/kg).

# 3.4. Pharmacokinetics and pharmacodynamics of subcutaneously administered LMWHs

LMWHs produced from different species of heparin were administered to primates at a dose of 1 mg/kg subcutaneously. Each LMWH exhibited comparable time course trends regardless of which assay was used to determine heparin level (Fig. 6). Unlike the unfractionated heparins, the concentrations measured by the Heparin Red and LC-MS assays differed substantially, with the Heparin Red assay consistently measured higher levels (Fig. 7).

### 4. Discussion

Heparin is currently the most complex and heterogeneous drug in use, which presents significant challenges in analyzing it in biological fluids like plasma. Throughout the hundred years of heparin's medical use, it has been monitored solely using bioassays. In the current study, we present a protocol for the direct analysis of plasma heparin using the MRM method with LC-MS, and compare the results with data derived from three less direct quantification methods: a chemical dye-binding method using Heparin Red, and two anticoagulant-bioactivity-based methods.

The PD and PK curves obtained for UFH administered iv to primates using different assays were qualitatively similar, but the concentrations determined by each assay differed (Fig. 2). We applied four methods to quantify UFH, anti-Xa, anti-IIa, Heparin Red, and MRM analysis. Each assay works on different principles, and each has certain limitations. The UFH studied have different molecular weight properties, different levels of sulfation, different amounts of antithrombin (AT) binding sites (affording different amounts of resistant tetrasaccharides not measured by MRM) and different disaccharide compositions. The anti-Xa and anti-IIa assays, used for PD determination, measure the bioactivities of select components within UFH. The anti-Xa assay is sensitive for chains of all sizes (greater than a pentasaccharide) containing AT binding sites, whereas the anti-IIa assay is most sensitive to larger chains with AT binding sites. The Heparin Red assay, used for PK determination, is more responsive to highly sulfated chains. These three assays rely on external standard curves and provide information on the relative amount of heparin present. In contrast, the MRM assay, while more intricate to perform, directly measured the mass of UFH present in a biological sample, providing the best absolute quantification.

The PK curves for the mass-based, iv dose (0.5 mg/kg) of porcine, bovine, and ovine UFHs measured by LC-MS and Heparin Red assays were very similar (blue curves Fig. 2a–f) with peak plasma concentrations of 4.5 to 8  $\mu$ g/mL. The conversion of units to milligrams relied on the manufacturer's activities: porcine UFH, 180–195 USP U/mg; bovine

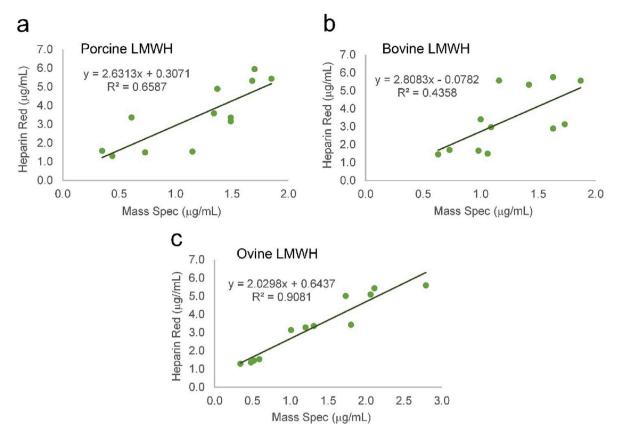


Fig. 7. The correlations of the quantification of porcine (a), bovine (b), and ovine (c) low molecular weight heparins measured by LC-MS and Heparin Red assays.

UFH, 130–140 USP U/mg and ovine UFH, 185–200 USP U/mg. As expected, the 100 U/kg and 0.5 mg/kg curves for these UFHs are similar (black and blue curves Fig. 2a–f) with the 50 U/mg curve (red curves Fig. 2a–f) only half as high with peak plasma concentrations of 2.4 to 4  $\mu$ g/mL. Thus, both LC-MS and Heparin Red assays proved to be reliable measures of PK. The PD curves (Fig. 2g–l) basically paralleled the PK curves. The 0.5 mg/kg curves were lower than the 100 U/kg curves for bovine UFHs (blue and black curves in Fig. 2b, e, h, and k) due to its lower U/mg specific activity.

The correlations of plasma concentrations of UFHs, measured by the four different assays, were next evaluated. The best correlations were for the LC-MS and Heparin Red assays of the three UFHs, although the LC-MS afforded slightly lower concentrations than Heparin Red assays (Fig. 3a, b and c). As expected, the correlations between the activity and mass-based assays were weaker.

MRM method relies on the disaccharide detection of the heparin being analyzed. The disaccharide composition of each UFH measured at the earliest time point (15 min) (Fig. 4) is comparable to previously reported compositions of each drug (Wang et al., 2022; Zhang et al., 2023). Surprisingly, we observed changes in the amounts of certain disaccharides over time (Fig. 5). Changes, particularly in TriS and 0S disaccharides in porcine and ovine heparin over time, suggest some selective uptake or metabolism of certain chains might take place but this requires further studies to confirm.

The PK and PD curves obtained using each assay for porcine, bovine and ovine LMWHs were similar (Fig. 6). As expected, the height of the PD curves, as measured by anti-Xa and anti-IIa assays, differed by 5- to 8-fold due to the low anti-IIa activity associated with these LMWHs. Plasma concentrations of LMWHs, as measured by the mass-based assays, showed that LC-MS again measured less LMWH in each sample. Heparin Red assay detected over twice the mass of LMWH than the LC-MS assay. Moreover, variable and sometimes poor correlations were observed between LC-MS and Heparin Red assays (Fig. 7). Heparin

contains resistant structures for digestion as well as some reducing/non-reducing end structures that are not detected in MRM mode (Zhang et al., 2023). In the LMWHs, the proportion of the reducing/non-reducing end structure, some of which are modified sugars as artifacts of depolymerization, would increase due to the reduced chain length. The inability to detect these structures in MRM assay might explain the difference between MRM and Heparin Red assay.

This study introduces the use of LC-MS MRM for quantifying plasma heparin in PK studies. This method has not been previously used for direct quantification of plasma heparin without first recovering and purifying the heparin. Thus, we explored the factors that might compromise the direct quantification of plasma heparin using MRM. We found that disaccharide detection of plasma heparin showed a 70 % improvement by recovering disaccharides from the plasma using a centrifugal filter and that a 10kD pore size was optimal (Figs. S2 and S3). While plasma proteins can either bind to heparin and impede the heparinase digestion or impact MS detection by influencing the ionization of the derivatized saccharides, we found that pretreatment with a nonspecific protease did not improve heparinase digestion or heparin quantification. Heparinase digestion treatment directly in the centrifugal filter without transferring the sample to a second tube afforded good disaccharide recovery (Fig. S2) Furthermore, careful AMAC derivatization improved the recovery of different disaccharides. The doping of disaccharide standards into the blank plasma was used to build a calibration curve and internal  $^{13}\mathrm{C}$  disaccharide standards controlled for differences in ionization efficiencies (Fig. S4). We added various amounts of standard disaccharides to determine the concentration range of disaccharides in plasma suitable for our MRM method. The resulting concentration-peak area curve exhibited a good linear relationship, ranging from 0.055 µg/mL to 39.9 µg/mL for each disaccharide in

Different quantification methods we used in this research have their own advantages and limitations. Bioassays directly reflect the heparin's

pharmaceutical activity *in vivo*. The Heparin Red method is a fast, convenient, and sensitive chemical method for mass-based heparin quantification in plasma. However, Heparin Red assay lacks specificity towards heparin as the presence of the other polyanions in the biologic fluid may also interact with the cation reagent and it varies in response to heparin chains of different sulfation degree and molecular mass. The slopes of Heparin Red/LC-MS correlation for ovine, porcine, and bovine UFH are 1.497, 1.123, and 1.096, respectively, consistent with their sulfation levels. Although a calibration curve can be constructed using the administered heparin, selective clearance, or endogenous mammalian enzymes, such as heparanase (Mao et al., 2014; Rabelink et al., 2017) or sulfatases (Uchimura et al., 2010), can alter the molecule's weight or sulfation levels, thereby introducing bias into quantitative results.

We summarize the merits and limitations of the four analytical methods used in this study in Table S6. The results of the current study show that LC-MS and the Heparin Red method gave similar heparin levels for unfractionated heparin but differed somewhat in the detection of low molecular weight heparin. Both the chemical methods measured heparin levels correlated to the unfractionated heparin's biological activities. Thus, the results presented support the use of chemical methods for the determination of unfractionated heparin levels in plasma.

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#### CRediT authorship contribution statement

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 $\label{thm:conceptualization} \mbox{ Jawed Fareed: Conceptualization, Supervision, Reviewing, Funding acquisition.}$ 

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#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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