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ORIGINAL ARTICLE

Structural and activity variability of fractions with different charge density and chain length from pharmaceutical heparins

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Abstract Heparin is a structurally complex polysaccharide used as a clinical anticoagulant. It is comprised of a heterogeneous mixture of polysaccharide chains having a variety of sequences and lengths. The production methods and regulatory controls of pharmaceutical heparins have changed over the years. This study assesses the structural and activity uniformity of the polysaccharide chains comprising two contemporary heparin products. The heparin fractions with different sizes and charges were separated with size exclusion and ion exchange chromatography. The fractions were analyzed for their molecular weight properties, di- and tetrasaccharide compositions, and anti-factor IIa and anti-factor-Xa activities. The distribution of these properties through chains of different lengths and ones with different charge density were compared. The results demonstrate that with the increase in heparin purity, activity and molecular weight required by the current pharmacopeia, the uniformity of pharmaceutical heparin products have increased.

Keywords Heparin · Chromatography · Compositional analysis · Anti-factor IIa activity · Anti-factor-Xa activity

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Introduction

Heparin is a mixture of linear, sulfated polysaccharides comprised of repeating 1-4-linked uronic acid and glucosamine residues [1]. The major structural unit in heparin is the α-L-iduronic acid 2-sulfate (1 → 4) α-D-glucosamine *N*-sulfate, 6-sulfate (IdoA2S (1 → 4) GlcNS6S) disaccharide, corresponding to 60–80% of its structure [1]. Other structural units have reduced levels of *O*-sulfation and the presence of D-glucuronic acid (GlcA) and *N*-acetylation [1]. Rare structures in heparin range from unsubstituted amino groups with no known function [2] to 3-sulfated glucosamine residues, which are critical for heparin binding to antithrombin III (AT) and heparin pharmacological activity [1]. In addition to being heterogeneous, comprised of chains having different saccharide composition and sequence, heparin is also polydisperse having chains of multiple lengths and different weight average and number average molecular weights [1].

Heparin was introduced as a clinical anticoagulant soon after its discovery 100 years ago [3]. As one of the oldest biopolymeric drugs, heparin has undergone many changes over time. Originally prepared from bovine lung tissue, heparin is now primarily obtained from porcine intestinal mucosa [1]. In the 1980's porcine heparin displaced bovine heparin as the major pharmaceutical heparin due to fear of bovine spongiform encephalopathy [4]. In the 1990's the discovery of excessive amounts of chondroitin/dermatan sulfate impurities in heparin lead to changes in manufacturing methods to improve heparin purity [5]. In 2007–8 the heparin adulteration crisis [6] required significant changes in heparin quality control and included the updating of pharmacopoeia monographs [7]. The introduction of bovine intestinal mucosal heparin is currently under active consideration to alleviate potential shortages and to make the supply chain of this essential anticoagulant more secure [4].

Pharmaceutical heparin has changed since its introduction. The anticoagulant activity of heparin, originally measured as sheep blood clotting time but now determined by an amidolytic anti-factor IIa biochemical assay, has increased from 120 to 140 U/mg to 180–200 U/mg [7]. The molecular weight of heparin, originally unspecified, ranged from 9 kDa to 20 kDa, is now specified in the pharmacopoeia at 15 kDa to 19 kDa [7]. These changes, while increasing the uniformity of pharmaceutical heparins, have had a remarkable impact on heparin structure. For example, prior to the most recent changes in the heparin monograph, pharmaceutical heparins were composed 30–40% polysaccharide chains having AT-binding sites that displayed AT-mediated anticoagulant activity (called high affinity chains) and 60–70% polysaccharide chains without AT-binding sites without AT-mediated anticoagulant activity (called low affinity chains) [8]. After the most recent monograph, increasing the minimum activity of heparin to >180 U/mg, the high affinity chains now represent 60–70% of the polysaccharide chains and 30–40% polysaccharide chains having low affinity chains. This study examines the structural features of the polysaccharide chains of two contemporary pharmaceutical heparins as a function of chain size and charge.

Materials and methods

Materials

Porcine intestinal heparins were active pharmaceutical ingredients obtained from Celsus Laboratories (Cincinnati, OH, USA) (Heparin 1) and Scientific Protein Laboratories (Waunakee, WI, USA) (Heparin 2) complying with the current United States Pharmacopeia (USP). HiPrep DEAE FF 16/10 column and Superdex 75 increase 10/300 GL were from GE Healthcare Life Sciences (Pittsburgh, PA, USA). Dialysis membranes (500–1000 Da) were from Spectrum Laboratories (Rancho Dominguez, CA, USA). Biophen heparin anti-IIa (2 stages) and Biophen heparin anti-Xa (2 stages) were purchased from Aniara (West Chester, OH, USA). Recombinant *Flavobacterial* heparin lyases I, II and III from *Proteus vulgaris* were expressed in our laboratory using *Escherichia coli* strains provided by Professor Jian Liu (College of Pharmacy, University of North Carolina). Unsaturated disaccharide standards of HS (Di-0S, ΔUA-GlcNAc; Di-NS, ΔUA-GlcNS; Di-6S, ΔUA-GlcNAc6S; Di-UA2S, ΔUA2S-GlcNAc; Di-UA2SNS, ΔUA2S-GlcNS; Di-NS6S, ΔUA-GlcNS6S; Di-UA2S6S, ΔUA2S-GlcNAc6S; Di-triS, ΔUA2S-GlcNS6S) were purchased from Iduron Ltd. (UK). Tetrasaccharides standards (ΔUA-GlcNAc6S-GlcA-GlcNS3S;ΔUA-GlcNAc6S-GlcA-GlcNS3S6S;ΔUA-GlcNS6S-GlcA-GlcNS3S6S; ΔUA2S-GlcNAc6S-GlcA-GlcNS3S6S; ΔUA2S-GlcNS6S-GlcA -GlcNS3S6S) were prepared in our laboratory [9].

Ion exchange chromatography separation of heparin

Heparin (10 mg in 200 μL) was injected to a HiPrep DEAE FF 16/10 column connected to Shimadzu HPLC system (Shimadzu, Kyoto, Japan). Heparin was eluted with a flow rate of 0.5 mL/min by 2.5 mM NaH_2PO_4, pH 3.0 (buffer A) and 2.5 mM NaH_2PO_4 containing 1 M $NaClO_4$, pH 3.0 (buffer B) with 0% B in 20 min and followed by a linear gradient of 35% B in 20 min, kept for 10 min, increased to 40% in 20 min and kept for 10 min, then increased to 45% in 20 min and kept for 10 min, changed to 100% B and kept for 20 min. The eluted heparin was detected at 202 nm. The fraction collector (Model 2110, Bio-Rad) was set to 5 min in conjunction-accumulated separating fractions from HPLC. Buffer salts were removed by 1000 Da dialysis membranes.

Size exclusion chromatography separation of heparin

Heparin (10 mg in 200 μL) was applied to a Superdex 75 increase 10/300 GL column connected to Shimadzu HPLC system with RID-10A refractive index detector. The mobile phase was 0.2 M ammonium carbonate and flow rate was 0.8 mL/min. A sample injection volume was 200 μL (sample concentration was ~50 mg/mL) and fraction collector set to 1 min per tube. Buffer salts were removed by lyophilizing 3-times.

Molecular weight determination using HPLC-GPC

HPLC-GPC was conducted to determine the molecular weight. A guard column TSK SWXL 6 mm × 4 cm, 7 μm diameter was used in series with two analytical columns: TSK G4000 SWXL 7.8 mm × 30 cm, 8 μm in series with TSK G3000 SWXL 7.8 mm × 30 cm, 5 μm (Tosoh Corporation, Tokyo, Japan). The columns were connected to an HPLC system consisting of Shimadzu LC-10Ai pump, a Shimadzu CBM-20A controller and a Shimadzu RID-10A refractive index detector (Shimadzu, Kyoto, Japan). The mobile phase was 0.1 M ammonium acetate with 0.02% (*w*/*v*) sodium azide. Columns and RID were maintained at 30 °C using an Eppendorf column heater (Eppendorf, Hamburg, Germany). A sample injection volume was 20 μL (concentration was ~5 mg/mL) and a flow rate was 0.6 mL/min. For molecular weight determination, USP Heparin Sodium Molecular Weight Calibrant RS (The United States U.S. Pharmacopeial Convention, Inc., MD) was used as a calibrant and USP Heparin Sodium Identification RS (The United States Pharmacopeial Convention, Inc.) was used to confirm system suitability.

Concentration determination of fractions by HPLC

As a standard, a series of dilutions of USP standard heparin of which dried basis amount was known was prepared at 0.625, 1.25, 2.5, 5.0 and 10.0 mg/mL. A testing sample was

dissolved in HPLC-grade water at approximately 5 mg/mL for the analysis. The GPC spectra of the standard and testing sample were obtained by using the same method of the molecular weight measurement. A linear relationship was calculated between heparin concentrations and the peak areas. The acceptance criterion was set as $r^2 > 0.990$.

Anticoagulant activities assay

The anti-Xa and anti-IIa activities of fractions were determined using BIOPHEN Heparin Anti-IIa (two stages) and Anti-Xa (two stages) kits. Human AT (R1) and thrombin (R2) for anti-IIa were restored with 1 mL of distilled water and diluted 1:5 in R4 (50 mM Tris, 175 mM NaCl, 7.5 mM EDTA, pH 8.40, containing 0.2% BSA and sodium azide as preservative) buffer to obtain a R1 at 0.25 IU/mL and R2 at 30 μg/mL, respectively. AT (R1) and Factor Xa (R2) for anti-Xa were restored with 1 mL of distilled water and diluted 1:5 in R4 (50 mM Tris, 175 mM NaCl, 7.5 mM EDTA, pH 8.40, containing 0.1% PEG and sodium azide as preservative) buffer to obtain a R1 at 1 IU/mL and R2 at 18 μg/mL. Heparin fractions with different concentrations dissolved in R4 buffer (1, 2.5, 5, 7.5, 10, 12.5 ng in 35 μL) were mixed with 37 °C pre-incubated R1 (35 μL), and incubated at 37 °C for 2 min, then R2 (35 μL) were mixed and incubated for another 2 min. Chromogenic substrate (R3) specific for thrombin [CS-01(38), 1.25 mM, 35 μL] or factor Xa [CS-01(65), 1.2 mM, 35 μL] were added. The reaction mixture was incubated for 1 min for anti-IIa and 2 min for anti-Xa and then stopped with citric acid (20 mg/mL, 70 μL). The absorbance was measured at 405 nm. Anti-IIa and anti-Xa activities were calculated using a standard curve of different concentrations of heparin (0–1 U/mL).

Disaccharides analysis

Heparin fractions (50 μg) were dissolved in 200-μL digestion buffer (50 mM ammonium acetate, 2 mM calcium chloride) and digested by heparin lyases I (5 mU), II (5 mU), III (5 mU) at 37 °C for 2 h. The solutions were boiled for 5 min to stop the reaction and to precipitate proteins. The supernatant was obtained after centrifugation at 15000×*g* for 15 min. Disaccharides analysis was carried out by SAX-HPLC using an analytical Spherisorb S5 column (4 × 250 mm; Waters, Milford, MA, USA) at 232 nm. The sample injection volume was 25 μL. Mobile phase A (MPA): 1.8 mM monobasic sodium phosphate, pH 3.0. Mobile phase B (MPB): 1.8 mM monobasic sodium phosphate, 1 M sodium perchlorate, pH 3.0. MPB was increased from 10% to 100% in 30 min and keep for 5 min, and then changed to 10% and keep for 15 min.

Tetrasaccharide analysis

Heparin fractions (200 μg) were dissolved in 100-μL digestion buffer (50 mM ammonium acetate, 2 mM calcium chloride) and digested by heparin lyase II (20 mU) at 37 °C for 6 h. The solutions were boiled for 5 min to stop the reaction and to precipitate proteins. The supernatant was obtained after centrifugation at 15000×*g* for 15 min. Tetrasaccharide analysis was carried out by SAX-HPLC using an analytical Spherisorb S5 column (4 × 250 mm; Waters, Milford, MA, USA) at 232 nm. Mobile phase A (MPA): 1.8 mM monobasic sodium phosphate, pH 3.0. Mobile phase B (MPB): 1.8 mM monobasic sodium phosphate, 1 M sodium perchlorate, pH 3.0. MPB was increased from 30% to 65% in 35 min, and then increased to 85% in 15 min, changed to 100% and kept for 5 min.

Results

Heparin fractionation by charges and sizes

Heparins 1 and 2 were separated into four fractions (F1-F4) by weak ion exchange chromatography on HiPrep DEAE FF column (Fig. 1). The elution gradient was optimized to get a

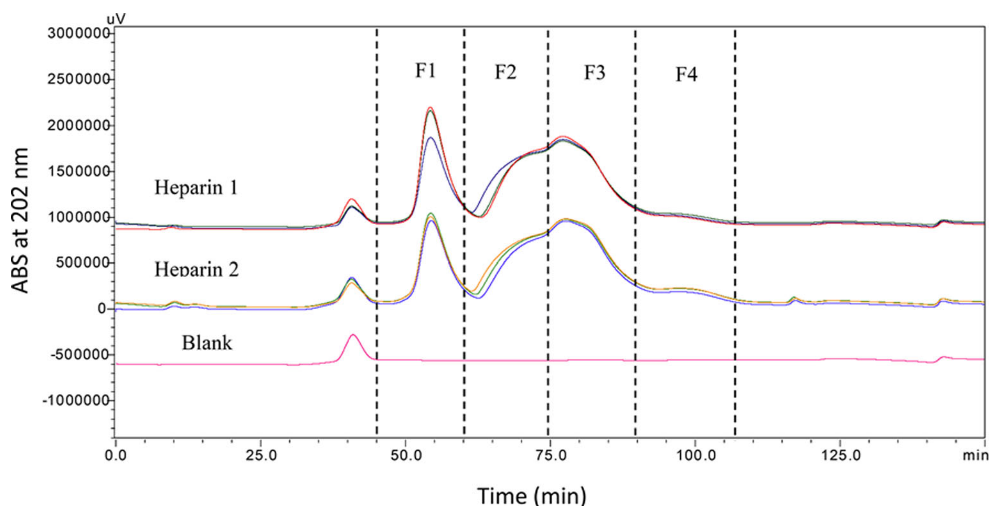


Fig. 1 Ion exchange chromatography of heparins

better separation of different charge state. The size exclusion separation of heparins 1 and 2 are shown in Fig. 2. Each peak was divided into three fractions (f1-f3).

Mass distribution of pharmaceutical heparin corresponding to fractions collected

The mass distribution of four fractions from the charge separation and three fractions from the size separation were determined by HPLC. The distributions are shown in Table 1. The distribution of Heparin 1 and Heparin 2 were similar in both the charge and the size separations. In ion exchange separation, the F2 chains were the largest and F4 were the smallest. In size separation, the f2 fraction was the major component.

Molecular weight determination

Molecular weight determination is an important identification test for heparin. The Mw of USP heparin should be between 15,000 Da and 19,000 Da, M_{24000} should be no more than 20%, and the ratio of $M_{8000–16000}$ to $M_{16000–24000}$ should be no less than 1.0. The molecular weight of Heparin 1 was 16,400 Heparin 2 was 16,500 (Table 2). The molecular weight increased with charge density as did the M_{24K}.

Anticoagulant activity

USP specification of anti-IIa activity is not less than 180 U/mg with a ratio of anti-Xa/anti-IIa is between 0.9–1.1. The anti-IIa and anti-Xa activities of the fractions prepared from Heparin 1 and 2 are shown in Table 3. The both starting heparins met the USP specifications. In ion exchange fractionation, high charge fractions had the highest anticoagulant activities. In the size exclusion fractionation, the longer chains had higher activities. We also calculated the activities based on molecular weight, the anticoagulant activity based on molecular weight also increased in high-charge fractions. In size separation, fractions the chains in middle fractions (f3) had the highest activities.

Disaccharides analysis

Disaccharides analysis was performed after heparin lyase treatment using SAX-HPLC. The results showed that the TriS was the major component. In the different charge fractions, the TriS increased with high charge status, reaching 80% in F4. The disaccharide components of the three different chain length fractions were nearly the same.

Tetrasaccharides analysis

The content of five major tetrasaccharides ΔUA-GlcNAc6S-GlcA-GlcNS3S (Tetra 1); ΔUA-GlcNAc6S-GlcA-GlcNS3S6S (Tetra 2); ΔUA-GlcNS6S-GlcA-GlcNS3S6S (Tetra 3); ΔUA2S-GlcNAc6S-GlcA-GlcNS3S6S (Tetra 4); ΔUA2S-GlcNS6S-GlcA -GlcNS3S6S (Tetra 5) in each of the fractions were determined by SAX-HPLC. The number of micrograms of each tetrasaccharide in each 25-μg fraction was determined. The total content of 5 tetrasaccharides increased with high charge status or longer chain length (Fig. 3). Tetra 2 and Tetra 5 increased in the charge separations of both Heparin 1 and Heparin 2. In the different size fractions the longer chains had higher tetrasaccharides content.

Discussion

Heparin has changed over the decades of its clinical use. Major changes in this critical drug were made in the past decade with the introduction of new pharmacopeia that regulates, for the first time the level of source and process impurities as well as heparin's molecular weight properties [7].

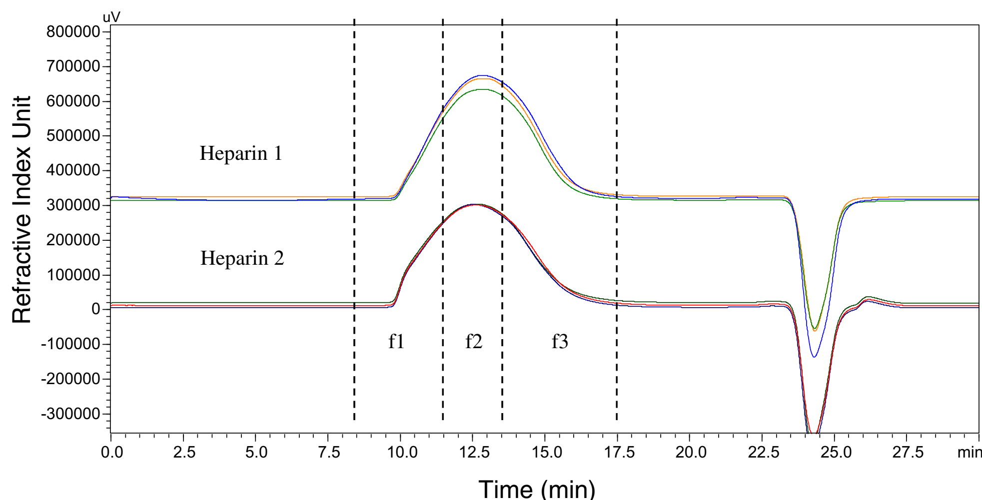
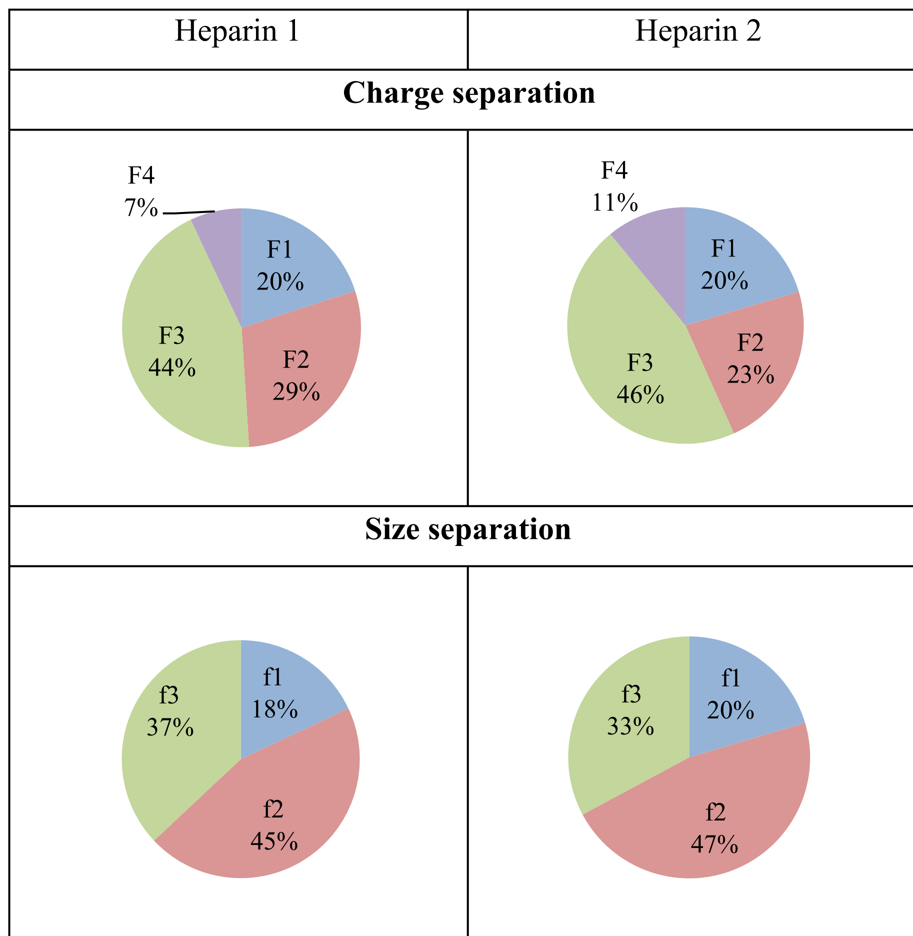


Fig. 2 Size exclusion chromatography of heparins

Table 1 Mass distribution from charge and size separations



Moreover, the minimum specific activity of pharmaceutical heparin has been significantly increased. Despite these changes, detailed studies on the compositional properties of the polysaccharide chains making up this highly heterogeneous and polydisperse product have not been recently reassessed. To our knowledge, the last systematic studies of the composition of chains of various lengths and charged density, prepared from pharmaceutical heparins, was published over 25 years

Table 2 Molecular weight of heparin fractions

Charge separation										
	Heparin 1					Heparin 2				
	Heparin	F1	F2	F3	F4	Heparin	F1	F2	F3	F4
M_W	16,400[a]	11,400	14,000	18,200	24,400	16,500	12,700	15,300	19,800	25,600
M_{24k}	10.0	1.9	2.6	11.6	40.2	13.4	4.1	9.0	22.9	47.9
$M_{8k–16k}/M_{16k–24k}$	1.4	6.8	3.1	0.7	0.1	1.8	3.7	2.3	1.0	0.4
Size separation										
	Heparin 1					Heparin 2				
	Heparin	f1	f2	f3		Heparin	f1	f2	f3	
M_W	16,300	25,800	17,000	11,600		16,900	27,600	17,100	11,000	
M_{24k}	9.3	47.9	5.4	0.2		13.4	55.4	6.5	0.2	
$M_{8k–16k}/M_{16k–24k}$	1.4	0.1	0.9	8.3		1.4	0.1	0.9	10.8	

[a] The molecular weight data was the mean of two injections

Table 3 Anticoagulant activity of heparin fractions

	Heparin 1 Original	F1	F2	F3	F4	Heparin 2 Original	F1	F2	F3	F4
Charge separation										
Anti-IIa (U/mg)	196 ± 1	77 ± 2	193 ± 1	293 ± 2	395 ± 3	198 ± 1	72 ± 2	145 ± 1	313 ± 3	410 ± 2
Anti-IIa (mU/M_w)	11.9 ± 0.1	6.7 ± 0.2	13.8 ± 0.1	16.1 ± 0.2	16.2 ± 0.2	12.0 ± 0.1	5.7 ± 0.2	9.5 ± 0.1	15.8 ± 0.3	16.0 ± 0.2
Anti-Xa (U/mg)	202 ± 2	71 ± 3	196 ± 2	310 ± 4	428 ± 2	223 ± 1	77 ± 2	166 ± 3	342 ± 4	457 ± 2
Anti-Xa (mU/M_w)	12.3 ± 0.2	6.2 ± 0.3	14.0 ± 0.1	17.0 ± 0.3	17.5 ± 0.2	13.5 ± 0.1	6.1 ± 0.2	10.8 ± 0.3	17.3 ± 0.4	17.9 ± 0.2
Anti-Xa/ Anti-IIa	1.0	0.9	1.0	1.1	1.1	1.1	1.1	1.1	1.1	1.1
Size separation										
	Heparin 1 Original	f1	f2	f3		Heparin 2 Original	f1	f2	f3	
Anti-IIa (U/mg)	200 ± 1	250 ± 2	224 ± 2	184 ± 3		205 ± 1	297 ± 2	256 ± 3	196 ± 1	
Anti-IIa (mU/M_w)	12.3 ± 0.1	9.7 ± 0.2	13.2 ± 0.2	15.9 ± 0.3		12.1 ± 0.1	10.8 ± 0.2	15.0 ± 0.3	17.8 ± 0.1	
Anti-Xa (U/mg)	189 ± 1	258 ± 3	224 ± 2	138 ± 1		198 ± 1	297 ± 3	226 ± 3	139 ± 2	
Anti-Xa (mU/M_w)	11.6 ± 0.1	10.0 ± 0 .3	13.2 ± 0.2	11.9 ± 0.1		11.7 ± 0.1	10.8 ± 0.3	13.2 ± 0.3	12.6 ± 0.2	
Anti-Xa/ Anti-IIa	0.9	1.0	1.0	0.8		1.0	1.0	0.9	0.7	

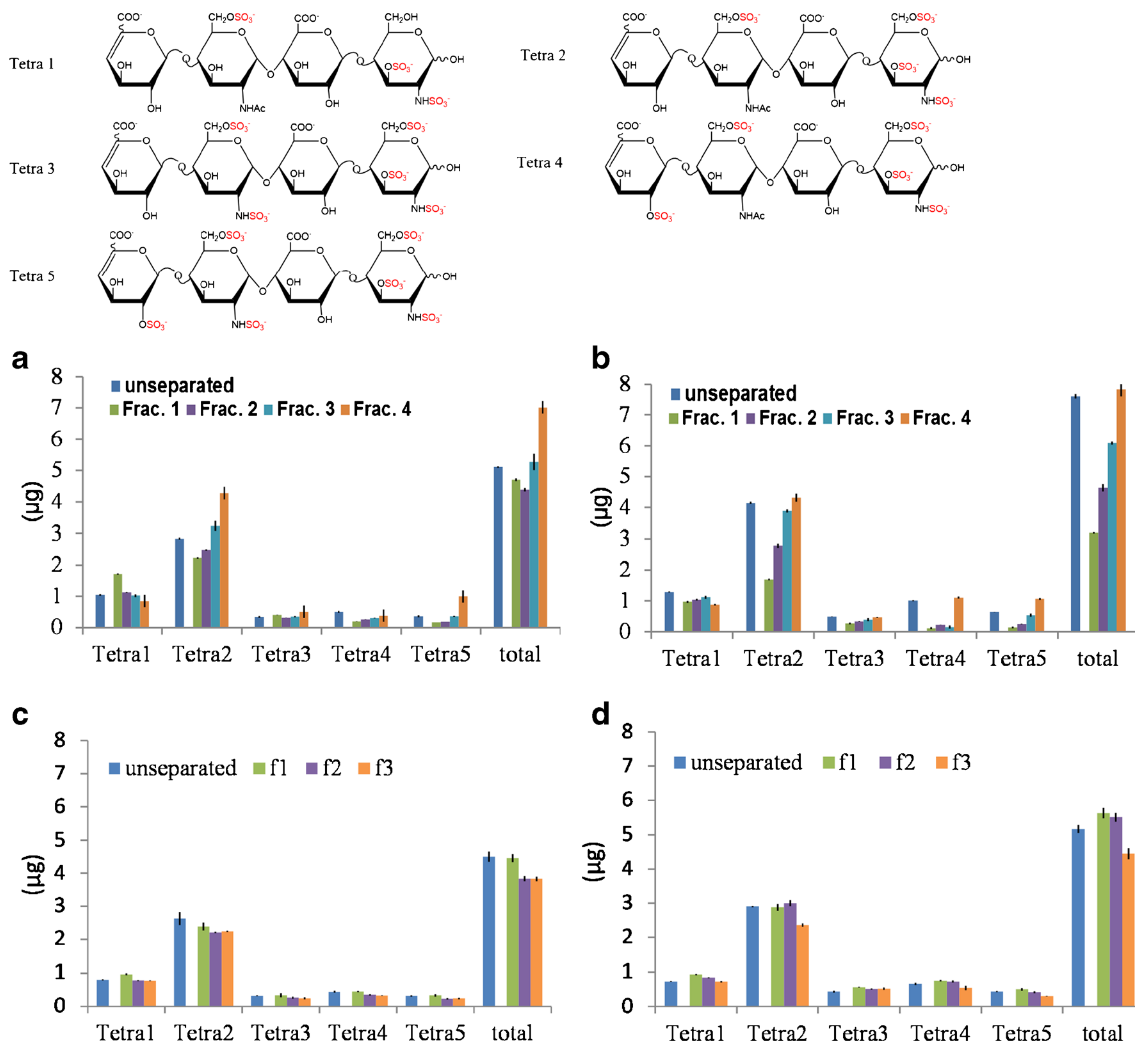
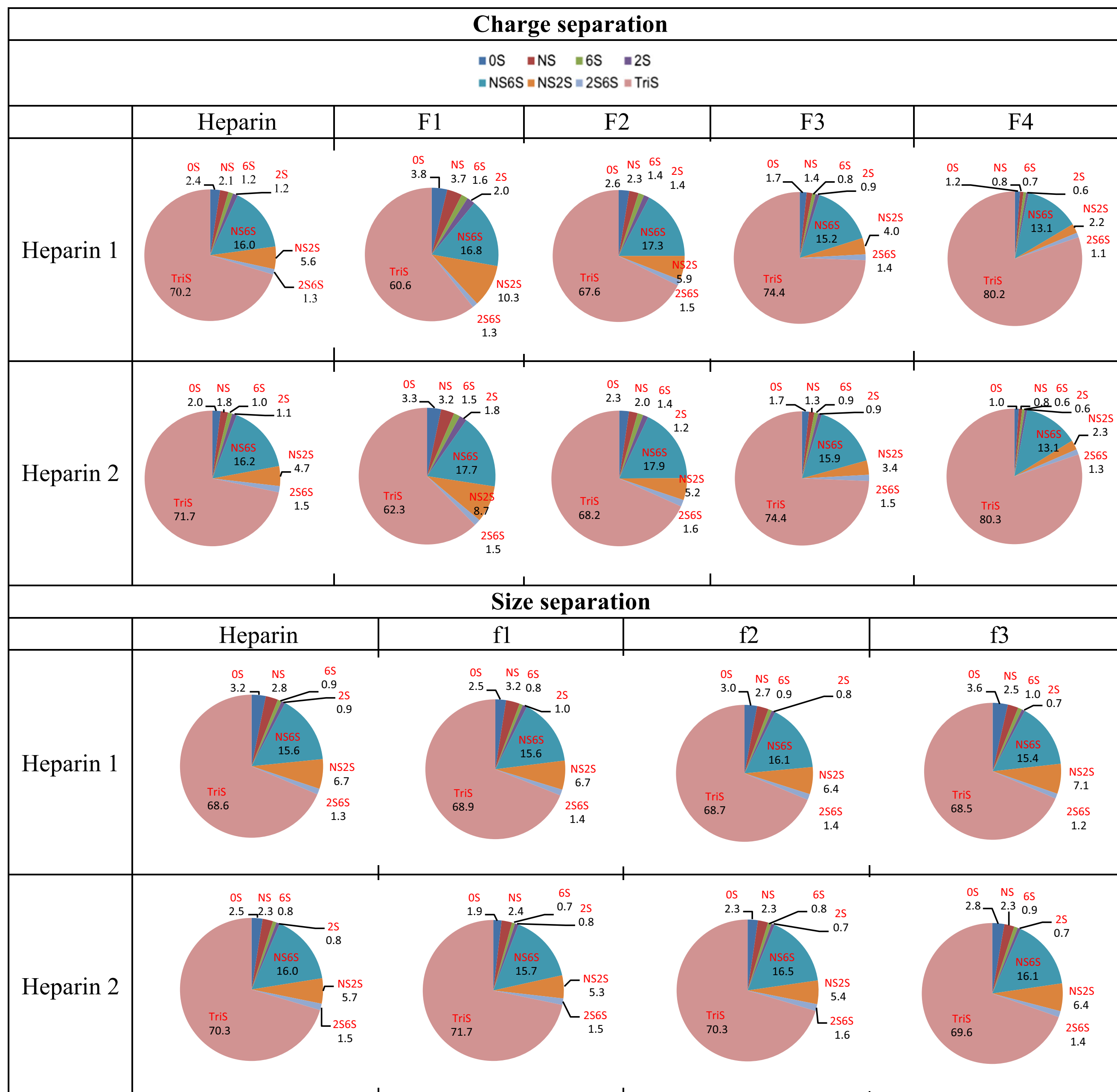


Fig. 3 Tetrasaccharides analysis results on heparin fractions. **a** ion exchange fractions of Heparin 1; **b** ion exchange fractions of Heparin 2; **c** size exclusion fractions of Heparin 1; **d** size exclusion fractions of Heparin 2

Table 4 Disaccharides analysis results



ago [10]. This study examined polysaccharide chains prepared from porcine pharmaceutical heparin (avg. MW 14,000, 171 U/mg) that had been fractionated based on their charge density and molecular size. The charge-fractionated porcine intestinal mucosal heparin chains (lower to higher charge density) showed increasing levels of trisulfated disaccharide repeating unit, increasing AT-mediated anti-factor IIa activity, ranging from 17 U/mg to 540 U/mg, and increasing polysaccharide molecular weight, ranging from 7.6 kDa to 20.5 kDa. The size-fractionated porcine intestinal heparin chains (longer to shorter chain length) showed decreasing molecular-weight, ranging from 25.3 kDa to 4.0 kDa, AT-mediated anti-factor IIa activity, which peaked in the intermediate sized chains at 130 and 196 U/mg with low activity in the longest and shortest chains, and the trisulfated disaccharide repeating unit also peaked in the intermediate sized chains.

The current study utilized a similar approach to characterize two contemporary pharmaceutical heparins, both prepared by US manufactures from porcine intestinal mucosal tissues. Fractionation, relying on weak anion exchange chromatography, afforded four fractions F1-F4 (Fig. 1) and fractionation, relying on a size exclusion chromatographic separation

afforded three fractions (f1-f3) (Fig. 2). The distribution of pharmaceutical heparin within the fractions collected was variable with the highest charge density and longest chain length fractions representing the smallest component of both pharmaceutical heparins (Table 1). The molecular weight of the fractions increased with charge density, ranging from 11.4 kDa to 24.4 kDa and decreased in size fractionation, ranging from 16.3 kDa to 11.6 kDa. Thus, these modern heparin polysaccharide chains were larger than those of earlier pharmaceutical heparin and showed lower molecular weight variability. The anticoagulant activities, both anti-IIa and anti-Xa, increased with chain charge density from ~70 U/mg to ~400 U/mg, again showing enhanced activity and a narrower range than earlier pharmaceutical heparin (Table 3). Little activity variability was detected in the size fractions but again the highest activity was observed in chains of intermediate size (Table 1). As expected disaccharide compositional analysis of the charge fractions showed enhanced trisulfated disaccharide composition, ranging from 60 to 80% (Table 4). In contrast, few if any differences were detected in the disaccharide composition of the size fractions (Table 4).

Recent advances in analytical technologies now allow for the assessment of the structural variability of AT-binding sites through the analysis of lyase-resistant tetrasaccharides [9]. The charge fractions increased in the composition of the total of all five AT-binding site structural variants (Fig. 3) as expected from their observed activity increase (Table 3). Interestingly, tetra-1, corresponding to the lowest sulfated AT-binding site, was present at the highest concentration in the fraction having the lowest charge density (Fig. 3). In contrast, all the other tetrasaccharides, tetra-2,-3,-4, and −5, all increased as the polysaccharide charge density increased. This suggests that most of the AT-binding sites are found in increasing amounts as a function of polysaccharide charge density. The size fractionated chains showed little or no variability in their content or distribution of AT-binding site structural variants.

In conclusion, contemporary pharmaceutical heparins show enhanced activity corresponding to the presence of AT-binding sites in most of their polysaccharide chains. Moreover, the structure and activity variability of the polysaccharide chains in contemporary pharmaceutical heparins are lower than those observed 25 years ago. This suggests that as a result of greater care in heparin manufacturing processes and increased regulatory control, pharmaceutical heparin has become a more uniform product.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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