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Isolation and charaterization of hexasaccharides derived from heparin. Analysis by HPLC and elucidation of structure by ^{1}H NMR ☆

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Abstract

Four hexasaccharides representing major structural sequences of heparin were isolated and characterized after degradation of heparin by heparinase. The structures were determined from two-dimensional ^{1}H NMR spectroscopy including TOCSY (total correlated spectroscopy), COSY (correlated spectroscopy), and ROESY (rotating frame nuclear Overhauser enhancement spectroscopy) methods, providing new data on hexasaccharides. One of the hexasaccharides, the last eluting component from anion exchange chromatography, was derived from the tri-sulfated repeating disaccharide, α-L-idopyranosyluronic acid 2-sulfate-(1 → 4)-2-amino-2-deoxy-D-glucopyranose 6,N-disulfate, and having the structure ΔUA*p*2S-(1 → 4)-α-D-GlcN*p*2S6S-(1 → 4)-α-L-IdoA*p*2S-(1 → 4)-α-D-GlcN*p*2S6S-(1 → 4)-α-L-IdoA*p*2S-(1 → 4)-α-D-GlcN*p*2S6S. The second hexasaccharide contained a nonsulfated D-glucuronic acid unit instead of the L-iduronic acid adjacent to the reducing end, and having the structure ΔUA*p*2S-(1 → 4)-α-D-GlcN*p*2S6S-(1 → 4)-α-L-IdoA*p*2S-(1 → 4)-α-D-GlcN*p*2S6S-(1 → 4)-β-D-GlcA*p*-(1 → 4)-α-D-GlcN*p*2S6S. The last two hexasaccharides were obtained in lower yield and they have not been isolated and characterized before. The structure of the third saccharide corresponded to a trimer of the repeating disaccharide except for the lack of a 6-*O*-sulfate group at the reducing end glucosamine residue; ΔUA*p*2S-(1 → 4)-α-D-GlcN*p*2S6S-(1 → 4)-α-L-IdoA*p*2S-(1 → 4)-α-D-GlcN*p*2S6S-(1

☆ Abbreviations used. IdoA*p*, idopyranosyluronic acid; GlcA*p*, glucopyranosyluronic acid; ΔUA*p*, 4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid; GlcN*p*, 2-deoxy-2-aminoglucopyranose; S, sulfate; Ac, acetate; TOCSY, total correlated spectroscopy; COSY, correlated spectroscopy; ROESY, rotating frame nuclear Overhauser enhancement spectroscopy; ROE, rotating frame nuclear Overhauser enhancement; NOE, nuclear Overhause enhancement; AT III, antithrombin III; HPLC, high performance liquid chromatography; GPC, gel permeation chromatography; SAX, strong anion exchange.

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→ 4)-α-L-IdoA*p*2S-(1 → 4)-α-D-GlcN*p*2S. The fourth and last hexasaccharide was less sulfated and the following structure was established ΔUA*p*2S-(1 → 4)-α-D-GlcN*p*2S6S-(1 → 4)-α-L-IdoA*p*2S-(1 → 4)-α-D-GlcN*p*2S6S-(1 → 4)-α-L-IdoA*p*-(1 → 4)-α-D-GlcN*p*Ac6S. Analysis of the ROESY spectra revealed conformational difference of the glucosidic linkage α-L-IdoA*p*-(1 → 4)-α-D-GlcN*p* between the hexasaccharides and longer heparin chains.

Keywords: Heparin; ^{1}H NMR; Heparinase of *Flavobacterium heparinum*; Porcine intestinal heparin

1. Introduction

Heparin is known to modulate various biological systems as it binds to many different proteins. Best known is its clinical use as an anticoagulant agent exerting its effect by binding to and thus increasing the activity of serine protease inhibitors of the blood coagulation cascade [1,2], the most important being the inhibitor antithrombin III (AT III) [3]. A specific pentasaccharide sequence has been identified as a structural requirement for the interaction of heparin with AT III [4–8]. Besides heparin is known to release lipoprotein lipase and hepatic lipase (reviewed by Olivercrona and Bengtsson-Olivercrona [9], to inhibit complement activation [10,11], to bind several growth factors [12,13] and to participate in the regulation of cell proliferation [14,15], to inhibit angiogenesis and tumour growth [16,17], and to have antiviral activity [18,19]. Due to the many biological systems that heparin interacts with, detailed information about the entire structure of the polysaccharide is necessary to extend the study of structure–activity relationship and the understanding of the biological effects of heparin.

The overall structure of heparin is now well established. It is a polydisperse, highly sulfated, linear polymer of alternating (1 → 4)-linked glucosamine and uronic acid residues. The major repeating unit is a trisulfated disaccharide; → 4)-α-L-idopyranosyluronic acid 2-sulfate-(1 → 4)-2-amino-2-deoxy-α-D-glucopyranose 6*O*,2*N*-disulfate, but many structural variants in terms of uronic acid composition, *N*- and *O*-sulfation and *N*-acetylation are observed [20,21]. To elucidate the detailed sequences of heparin, the structures of heparin-derived hexasaccharides have been studied. Previously, the components of the tetrasaccharide mixtures have been investigated in detail and structures of several tetrasaccharides have been elucidated [22–29]. However, the mixture of the hexasaccharides is more complex and so far studied to a much lesser extent. Relatively few hexasaccharides have been isolated and characterized [27,28,30–33]. We report herein the isolation and characterization of four hexasaccharides of which two are characterized for the first time. In addition, analyses of the ROESY spectra provided new information about the conformation of the glycosidic linkages in hexasaccharides.

2. Materials and methods

Preparation of heparin samples.—The starting material was a side fraction from the manufacture of low molecular weight heparin (Tinzaparin sodium lot. No. 894701), which was produced by Novo Nordisk A/S, Denmark, by enzymatic depolymerization

of porcine intestinal mucosal heparin using heparinase from *Flavobacterium heparinum*. The side fraction obtained from ethanol precipitation had a peak maximum M of 1900, an anti-factor Xa activity of 33.6 units/mg and an anti-factor IIa activity of 2 units/mg. The starting material was first fractionated by ethanol precipitation. Ethanol was added in portions to a heparin solution (200 mg/mL) in 2% sodium chloride, pH 7.0, and the mixture was allowed to stand at 4°C while precipitating. The precipitate at each ethanol concentration was isolated and analyzed by GPC–HPLC for molecular mass distribution. The fraction precipitating at ethanol concentrations between 70 and 80% (v/v) consisted mainly of hexasaccharides.

Preparative GPC of heparin-derived oligosaccharides.—A sample of heparin (1.5 g) was reconstituted in 5 mL 0.1 M sodium acetate, pH 7.0, and then applied on two columns (2.5 × 100 cm) connected in series, packed with Sephadex G-50 (Pharmacia, Sweden) and eluted at 0.3 mL/min with 0.1 M sodium acetate. Elution was monitored by recording the absorbance at 254 nm. Fractions homogeneous by size were pooled and recovered from ethanol precipitation after an adequate number of runs were performed.

Analytical GPC of heparin-derived oligosaccharides.—The oligosaccharides obtained from fractional precipitation and preparative gel filtration were applied (0.5 mg in 50 μL) onto Protein-Pak 60 and Protein-Pak 125 columns, [0.78 × 30 cm columns in series with a 0.4 × 2 cm guard column, Waters/Millipore (USA)] and eluted with 0.5 M sodium sulfate at a flow rate of 0.5 mL/min. The elution was monitored by refractive index detection (Knauer, Germany). GPC was performed on a HPLC system consisting of a Hitachi L-6200 Intelligent Pump (Japan) and a 710 B Wisp injector from Waters/Millipore (USA). Data were collected and analyzed by GPC/SEC-software from Polymer Laboratories (UK).

Preparative SAX HPLC of hexasaccharides.—Strong anion exchange (SAX) chromatography was done on an HPLC system including a Hitachi L-6200 Intelligent Pump (Japan), a 712 Wisp injector from Waters/Millipore (USA), and a Tunable Absorbance detector 486 from Waters/Millipore (USA). Millinnium software also from Waters/Millipore (USA) was employed for data collection and processing. The hexasaccharide mixture (150 mg) was loaded onto a semipreparative SAX column (Spherisorb 5-μm particle size 2 × 25 cm, Phase Separations, Norwalk, USA) pre-equilibrated with 0.64 M NaCl at pH 3.5 and the flow rate was set at 2 mL/min. The elution was carried out using a complex elution profile consisting of several steps. For the first 70 min a linear 0.64 → 0.85 M NaCl gradient was applied, followed by isocratic elution at 0.85 M NaCl for 60 min and a linear 0.85 → 1.15 M NaCl gradient over a 90-min time period. Finally the column was washed with 1.15 M NaCl for 30 min. The elution profile was monitored by recording the absorbance at 235 nm and 4-mL fractions were collected. Fractions corresponding to single peaks were pooled. Several runs were performed and like fractions from each run were combined, concentrated by ultrafiltration, and recovered from ethanol precipitation.

Samples corresponding to the major hexasaccharide components were rechromatographed on a Mono Q 16/10 column (1.6 × 10 cm, Pharmacia) using linear gradients and a centre cut of each component was taken. Identical fractions were pooled, concentrated by ultrafiltration with a PLAC filter (M_r cutoff 1000, Millipore), freeze-dried, desalted on a Sephadex PD 10 column (Pharmacia), pooled, and freeze-dried.

Analytical SAX HPLC of hexasaccharides.—SAX-HPLC was performed on a Mono Q HR 5/5 (0.5 × 5-cm, Pharmacia) pre-equilibrated with 0.66 M NaCl at pH 3.5 and a flow rate of 1 mL/min. The compound (500 μg) dissolved in 50 μL was injected and eluted using a complex NaCl gradient. For the first 35 min a linear 0.66 → 0.89 M NaCl gradient was applied, followed by a linear 0.89 → 0.94 M NaCl gradient over a 45 min time period and a linear 0.94 → 1.18 M NaCl gradient over a 30 min time period. Finally the column was washed with 1.5 M NaCl for 10 min. The elution was monitored by recording the absorbance at 235 nm. The area under each peak was measured by computer assisted integration of the chromatogram for evaluating the percentage of the resulting components of the hexasaccharide mixture.

^{1}H NMR spectroscopy.—Each sample was freeze-dried twice from D_2O (99.8%, Merck, Germany). One- and two-dimensional ^{1}H NMR spectra were acquired at 600 MHz (Bruker AMX spectrometer). The experiments were performed at 298 or 300 K on 1 mL samples with a concentration of 5–10 mM. Standard parameters were applied in the one- and two-dimensional ^{1}H NMR experiments. The residual HDO-signal in the spectra was suppressed through presaturation. All chemical shifts are reported relative to the signal from the unsaturated 4-proton, which was assigned to 6.00 ppm relative to tetramethylsilane.

3. Results and discussion

Isolation and HPLC characterization of the hexasaccharide components.—LMW heparin (tinzaparin sodium) was prepared by depolymerization with heparinase (EC 4.2.2.7) which acts on the linkages with the respective structures [→ 4)-α-D-GlcN*p*2S6S-(1 → 4)-α-L-IdoA*p*2S-(1 →], [→ 4)-α-D-GlcN*p*2S-(1 → 4)-α-L-IdoA*p*2S-(1 →]; and [–4)-α-D-GlcN*p*2S3S6S-(1 → 4)-α-L-IdoA*p*2S-(1 →]. A 4-deoxy-2-sulfo-α-L-threo-hex-4-enopyranosyluronic acid is formed at the nonreducing end [25,34–35]. A side fraction from the production of tinzaparin sodium was fractioned by precipitation of its sodium salt with ethanol resulting in fractions with a more narrow molecular weight distribution profile. The fractions with hexasaccharide as the principal oligosaccharide component were separated by low pressure gel filtration into tetra-, hexa-, octa-, and decasaccharides. The average molecular weights of the fractions were determined by GPC–HPLC essentially as described by Kristensen et al. [36]. Analyses and separation of the components of the mixture of hexasaccharides were carried out by SAX-HPLC. The use of a linear gradient of sodium chloride for the elution of heparin-derived oligossaccharides, as previously reported [25,30,31,37], did not result in optimal separation neither for preparative nor analytical SAX chromatography. The gradient elution was optimized resulting in separation with an elution gradient consisting of several steps, which implied prolongation of the run time. An improved resolution of the bunched peaks was obtained, and the elution profile was reproducible although still complex (Fig. 1). At least 20 compounds are present within the hexasaccharide mixture. However, only major components were obtained in adequate amounts and of satisfactory purity. Separation on the preparative Spherisorb SAX column required correction of the gradient to reproduce the optimized resolutions owing to aging of the column. On the

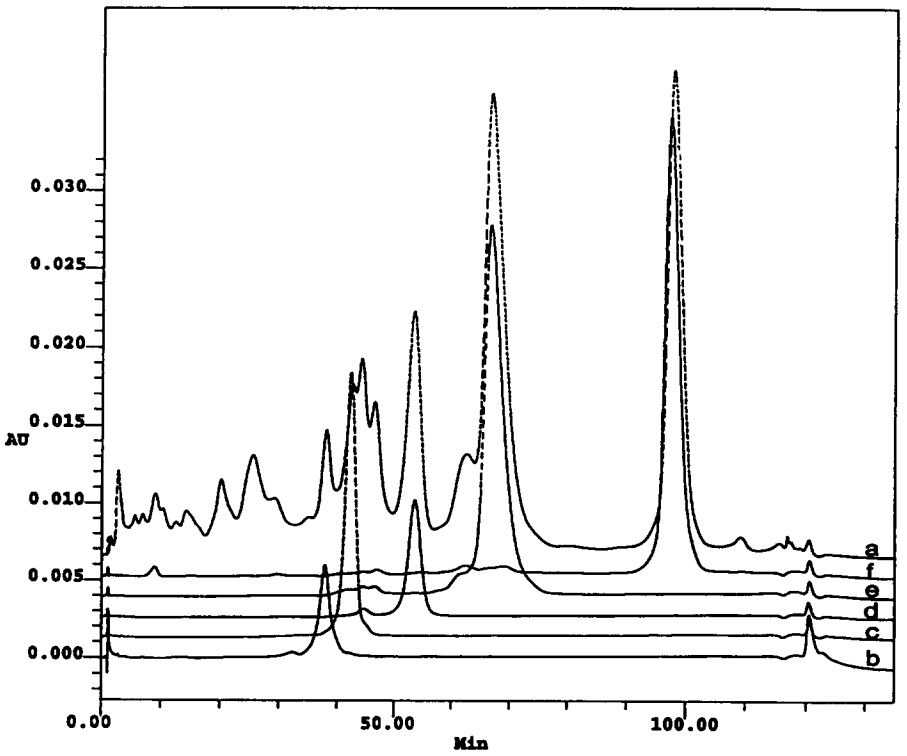

Fig. 1. Overlay plot of analytical SAX-HPLC of the hexasaccharide mixture obtained after preparative GPC (a), and of the hexasaccharide components resulting from preparative SAX-HPLC: Hexa1 (b), Hexa2 (c), Hexa3 (d), Hexa4 (e), and Hexa5 (f), using sodium chloride gradient elution as defined in the text.

other hand, the Mono Q column was very stable. Fractions, corresponding to the major peaks Hexa1, Hexa2, Hexa3, Hexa4, and Hexa5, were obtained from repetitive preparative SAX-HPLC and they were isolated and analyzed by analytical SAX-HPLC. The last eluting hexasaccharide components Hexa4 and Hexa5 were the major compounds accounting for 32 and 24 mol% of the hexasaccharide mixture, respectively. They were isolated at very high purity (> 95%) estimated by analytical SAX-HPLC. The hexasaccharides Hexa1, Hexa2, and Hexa3 were more difficult to isolate in sufficient amounts. Analytical SAX-HPLC revealed that they accounted for 1, 5 and 11 mol% of the hexasaccharide mixture and they were evaluated to be 94, > 95, and 94% pure by analytical SAX-HPLC.

^{1}H NMR analyses of the hexasaccharides.—The structures of the isolated hexasaccharides were established by application of highfield ^{1}H NMR spectroscopy. The structures for hexasaccharides Hexa2-Hexa5 are depicted in Fig. 2 and the ^{1}H NMR spectra for the hexasaccharides Hexa1-Hexa3 are given in Fig. 3. The structures are arranged so the 4,5-unsaturated sugar residue is always designated as residue A and the adjacent D-glucosamine as residue B. Assignments of the ^{1}H NMR resonances for the hexasaccharides Hexa2-Hexa5 were made from analyses of two-dimensional ^{1}H NMR

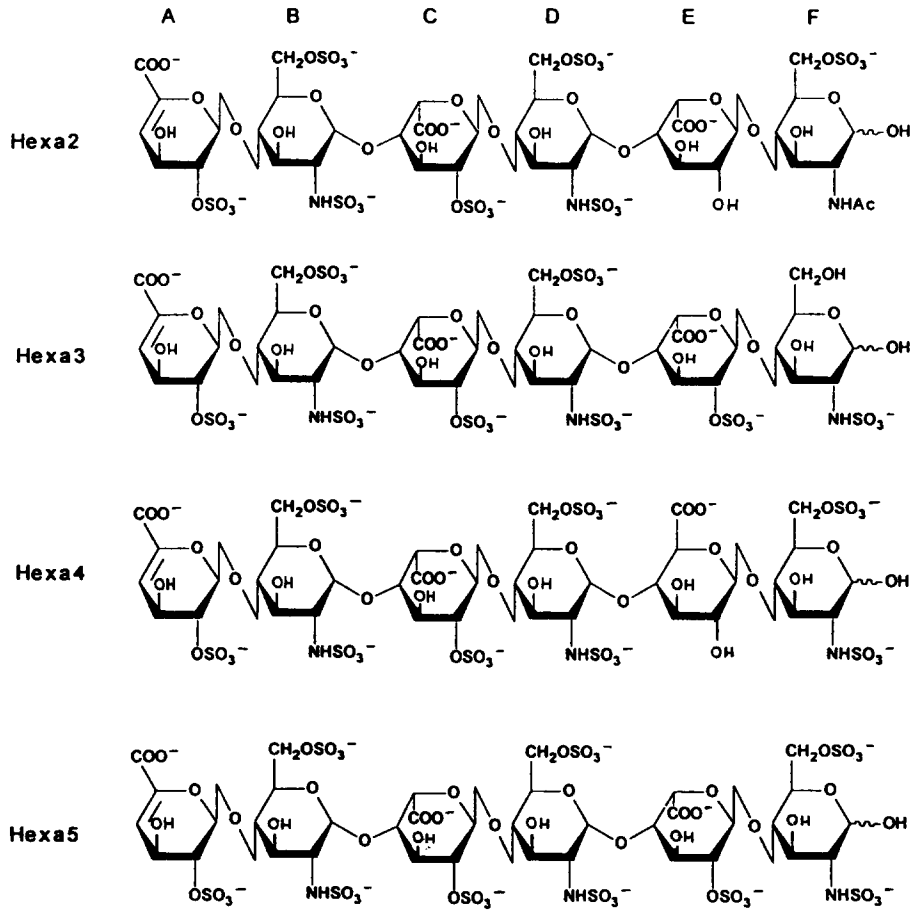

Fig. 2. Structures of the four hexasaccharide components Hexa2-5. Monosaccharide residues are designated by letters from A to F, starting with the nonreducing unit as residue A.

spectra. Analysis of total correlated (TOCSY) spectra assigned all signals belonging to each sugar residue and the spin connectivities within the rings were provided by examination of the correlated (COSY) spectra. For sequential assignment of the sugar residues rotating frame nuclear Overhauser enhancement spectra (ROESY) were recorded. Although the hexasaccharide fraction Hexa1 gave a single peak, when analyzed by SAX-HPLC, inspection of the ^{1}H NMR spectrum evidently revealed that Hexa1 was a mixture of at least two hexasaccharides. This demonstrates the chromatographic difficulties in purification of heparin-derived oligosaccharides. It is most likely that Hexa1 is a mixture of two hexasaccharides with the same sugar residue components (same charge) but with different sequences.

^{1}H NMR assignments for the hexasaccharides Hexa2-5 are given in Tables 1–4. The ^{1}H NMR spectrum of hexasaccharide Hexa2 showed six well-resolved single proton signals in the anomeric region. The resonances at 5.23 and 5.02 ppm revealed that both

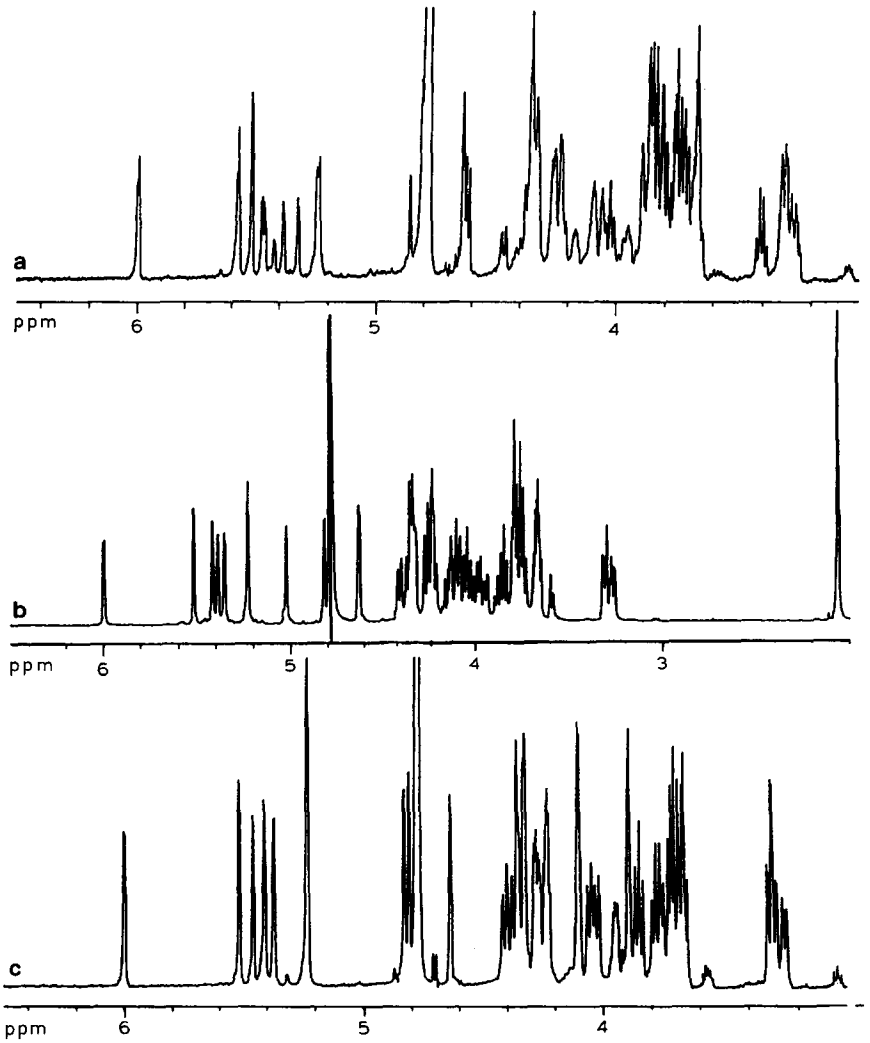

Fig. 3. 600-MHz ^1H NMR spectra of the Hexa1 (a), Hexa2 (b), and Hexa3 (c) recorded in D_2O at room temperature.

Table 1
^1H NMR assignments for the protons of the 4,5 unsaturated residue in heparin-derived hexasaccharides. Chemical shift in ppm from Me_4Si at 25°C

Residue	H-1	H-2	H-3	H-4
Hexa2	5.52	4.63	4.34	6.00
Hexa3	5.53	4.65	4.34	6.00
Hexa4	5.50	4.61	4.32	6.00
Hexa5	5.51	4.61	4.31	6.00

Table 2
^{1}H NMR assignments for the protons of the internal D-glucosamine residues in heparin-derived hexasaccharides. Chemical shift in ppm relative to Me_4Si 25°C

Residue	H-1	H-2	H-3	H-4	H-5	H-6a, H-6b
Hexa2 [a]	5.42	3.31	3.68	3.87	4.05	4.29, 4.38
[b]	5.37	3.28	3.69	3.79	3.99	4.25, 4.35
Hexa3 [a]	5.42	3.32	3.68	3.85	4.07	4.28, 4,38
[b]	5.38	3.30	3.71	3.78	4.03	4.28, 4.41
Hexa4 [a]	5.37	3.31	3.65	3.84	4.02	4.25, 4.37
[b]	5.58	3.29	3.67	3.77	3.98	4.26, 4.37
Hexa5 [a]	5.38	3.30	3.68	3.82	4.05	4.26, 4.36
[b]	5.39	3.29	3.66	3.77	4.04	4.29, 4.40

[a] Assignments for residue B within the given hexasaccharide.
[b] Assignments for residue D within the given hexasaccharide.

Table 3
^{1}H NMR assignments for the protons of the internal hexuronic acid in heparin-derived hexasaccharides. Chemical shift in ppm relative to Me_4Si at 25°C

Residue	H-1	H-2	H-3	H-4	H-5
Hexa2 [a]	5.23	4.37	4.23	4.11	4.82
[b]	5.02	3.79	4.15	4.10	4.80
Hexa3 [a]	5.23	4.34	4.23	4.10	4.82
[b]	5.23	4.32	4.22	4.10	4.81
Hexa4 [a]	5.22	4.34	4.23	4.07	4.83
[b]	4.62	3.40	3.85	3.81	3.83
Hexa5 [a]	5.11	4.33	4.25	4.11	4.81
[b]	5.11	4.32	4.21	4.10	4.79

[a] Assignments for residue C within the given hexasaccharide.
[b] Assignments for residue E within the given hexasaccharide.

Table 4
^{1}H NMR assignments for the protons of the reducing end D-glucosamine residue in heparin-derived hexasaccharides. Chemical shift in ppm relative to Me_4Si at 25°C

Residue	H-1	H-2	H-3	H-4	H-5	H-6a, H-6b
Hexa2	5.40	3.94	3.78	3.74	4.03	4.28, 4.35
Hexa3	5.46	3.26	3.72	3.74	3.95	3.90, 3.95
Hexa4	5.45	3.29	3.74	3.73	4.15	4.31, 4.37
Hexa5	5.45	3.24	3.71	3.75	4.15	4.35, 4.39

internal hexuronic acids were iduronic acids of which only one was 2-*O*-sulfated. Sulfation results in a downfield shift of the anomeric proton resonance by 0.2 ppm. The signal at 2.08 ppm corresponded to the chemical shift of the methyl group of an *N*-acetylated glucosamine unit. The other proton chemical shifts were assigned by analyses of two-dimensional TOCSY and COSY spectra (Fig. 4). Starting from the characteristic downfield resonance at 6.0 ppm for the H-4 proton of the 4,5-unsaturated

nonreducing-end residue (A), a cross-peak showed connectivity to the AH-3 proton at 4.34 ppm, which in turn showed connectivity the AH-2 proton at 4.62 ppm. This is consistent with *O*-sulfation at this position [23,24,26,27,31]. Further connectivity to the AH-1 proton at 5.52 ppm established the chemical shifts of the A-ring (Fig. 4a). In a similar fashion by starting from the anomeric signals all proton resonances of the other residues (B–F) were assigned. All three D-glucosamine residues (B, D, and F) were 6-*O*-sulfated as revealed by the chemical shifts of the 6a and 6b protons (4.22 and 4.38 ppm) [31]. The chemical shift of the FH-2 proton (3.94 ppm) was in accordance with *N*-acetylation of this residue. *N*-Sulfation results in an upfield shift by ca. 0.65 ppm of the H-2 proton as observed for the BH-2 and DH-2 protons. Upfield shifts were also noticed for the chemical shift of the EH-1 (0.2 ppm), EH-2 (0.58 ppm), and EH-3 (0.08 ppm) protons due to the absence of 2-*O*-sulfate in the E residue [31]. The sequence was

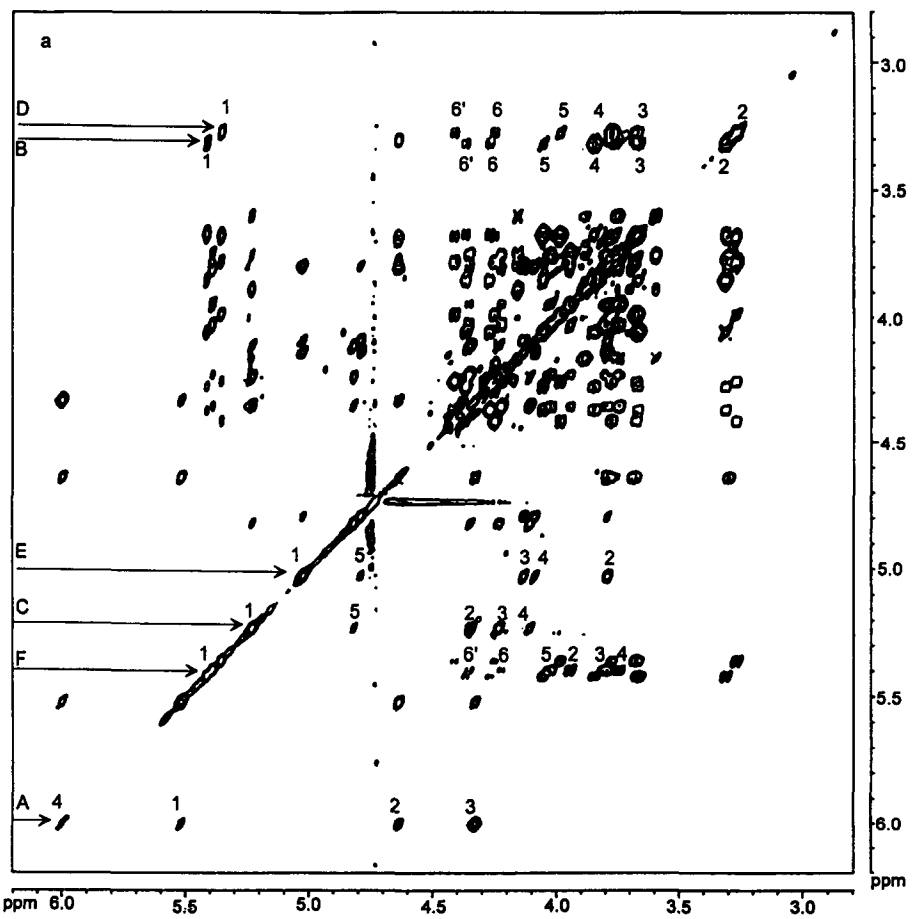

Fig. 4. 600-MHz ^{1}H NMR TOCSY (a) and COSY (b) spectra of hexasaccharide Hexa2 recorded in D_2O at room temperature. (a) Assignment of cross-peaks for each residue is shown. (b) The spin connectivity of H-1, H-2, H-3, and H-4 protons in the A-residue is shown.

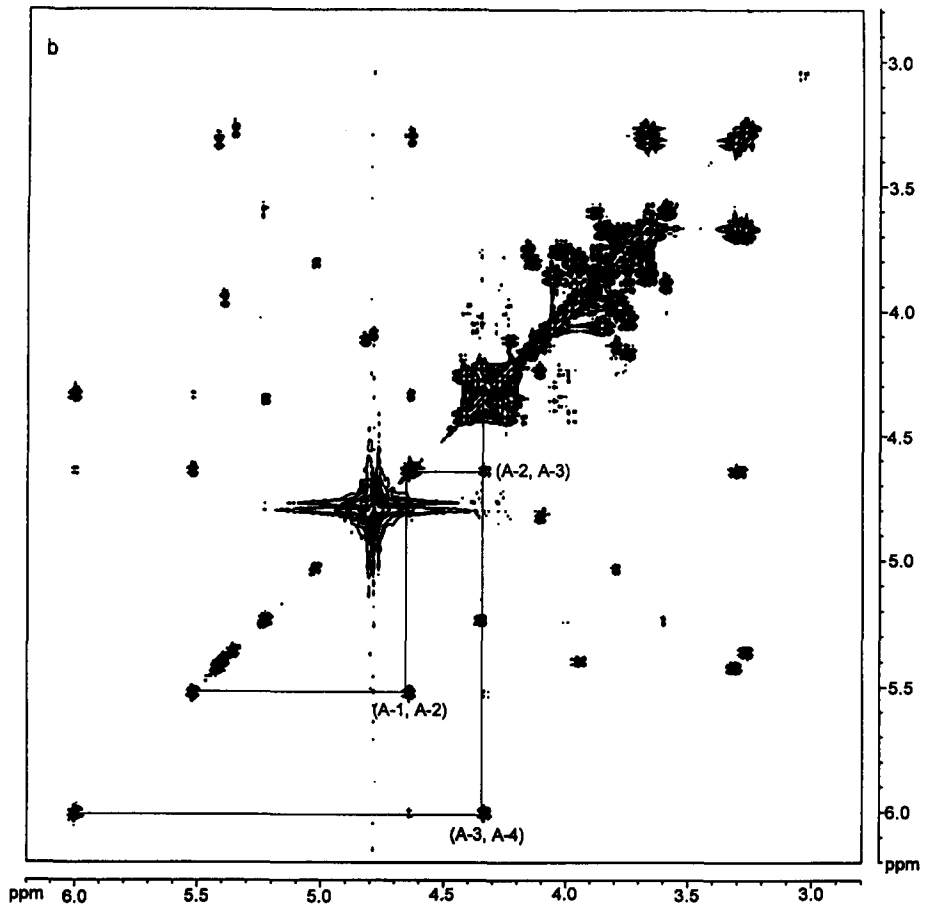

Fig. 4 (continued).

established from analysis of the ROESY spectrum (Fig. 5). For all hexasaccharides the cross-peaks between the H-1 proton of the D-glucosamines and the 4-linked hexuronic acid and H-1 proton of the hexuronic acids and the adjacent D-glucosamine are given in Table 5 and 6, respectively, and the relative intensities are evaluated. The sequence was thus easily determined.

The reducing end *N*-acetylated D-glucosamine residue is inconsistent with the specificity for heparinase. The enzyme-preparation could be contaminated with small amounts of heparitinases as Hexa2 accounted for only 5 mol% of the hexasaccharide fraction. Heparitinase is capable of cleaving a glycosidic linkage containing a *N*-acetylated D-glucosamine residue. Alternatively, hexasaccharide Hexa2 might represent a terminal saccharide sequence from the original heparin chain. This is in accordance the previous finding where *N*-acetylated D-glucosamine has been identified as a reducing end-residue of heparin chains [38].

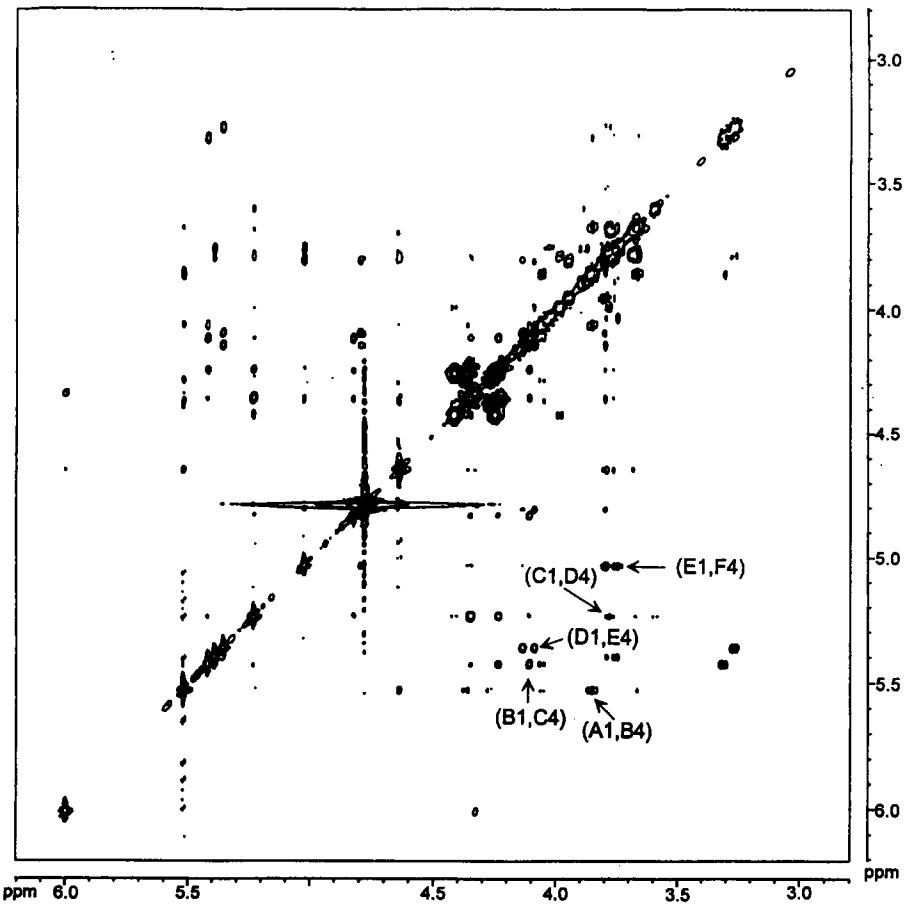

Fig. 5. 600-MHz ^{1}H NMR ROESY spectrum of hexasaccharide Hexa2 recorded in D_2O at room temperature. Assignments of the ROE cross-peaks that establish the sequence are shown.

Table 5
Cross-peaks [a] observed in ROESY spectra between the H-1 proton of the glucosamine residue and the protons of the adjacent hexuronic acid residue

GlcN*p* → Ido/GlcA *p*	Hexa5	Hexa4		Hexa3	Hexa2 [b]	
	(B, C);(D, E)	(B, C)	(D, E)	(B, C);(D, E)	(B, C)	(D, E)
(H-1,H-2)	M	M		M	W	
(H-1,H-3)	S	S	S [c]	S	M	S
(H-1,H-4)	vS	vS	S [c]	S	S	S
(H-1,H-5)			S [c]			

[a] vS, very strong; S, strong; M, medium strong; W, weak.
[b] Spectrum of general low intensity.
[c] Difficult to evaluate due to overlap.

Table 6
Cross-peaks [a] observed in ROESY spectra between the H-1 proton of the hexuronic acid residue and the protons of the adjacent glucosamine residue

Ido/GlcA*p* → GlcN*p*	Hexa5	Hexa4		Hexa3		Hexa2 [b]
	(C, D);(E, F)	(C, D)	(E, F)	(C, D)	(E, F)	(C, D);(E, F)
(H-1,H-3)	S	S	S	M	M	M
(H-1,H-4)	S	S	S	S	S	M
(H-1,H-6a,6b)	M [c]	M [c]		M [c]	M	W

[a] vS, very strong; S, strong; M, medium strong; W, weak.
[b] Spectrum of general low intensity.
[c] Difficult to evaluate due to overlap.

The ^{1}H NMR spectrum of hexasaccharide Hexa3 showed four well-resolved single proton signals and one two-proton signal at 5.23 ppm in the anomeric region, indicating that both internal hexuronic acid residues were L-iduronate 2-sulfate residues. The absence of any signal around 2.08 ppm demonstrated that none of the D-glucosamines was *N*-acetylated. The chemical shift of the protons were assigned using TOSCY and COSY methods as described for hexasaccharide Hexa2. The chemical shifts of the H-2 protons of the D-glucosamines at 3.26–3.32 ppm were consistent with *N*-sulfation of the D-glucosamines. The reducing end D-glucosamine residue was lacking a sulfate group on C-6, as revealed by the upfield shifts of H-5, H-6a, and H-6b protons by 0.20, 0.53, and 0.48 ppm, respectively. The identity of the internal hexuronic acids as L-iduronic acid 2-sulfate was confirmed by the chemical shift of the anomeric protons (5.23 ppm) and the H-2 protons (4.32 and 4.34 ppm). The assignments are depicted on the TOCSY spectra (Fig. 6). The sequence was established from the assignment of the ROE cross peaks shown in Fig. 7.

The most negatively charged hexasaccharide Hexa5 was identified to be derived from the trimer of the repeating disaccharide unit → 4)-α-L-idopyranosyluronic acid 2-sulfate-(1 → 4)-2-amino-2-deoxy-α-D-glucosamine 6,*N*-disulfate. Except for downfield shifts of the H-5, H-6a, and H-6b protons of the reducing end D-glucosamine residue (residue F), no significant differences were observed when compared with the data from hexasaccharide Hexa3. Hexasaccharide Hexa4 differed from hexasaccharide Hexa5 in substitution of the α-L-idopyranosyluronic acid adjacent to the reducing end with a β-D-glucopyranosyluronic residue. Both hexasaccharides have been isolated and characterized by ^{1}H NMR and ^{13}C NMR (refs. [27] and [28], respectively), and our work confirm the structures and the assignments of the two major hexasaccharide components. In addition, it is worth while noting that distinctive chemical shifts of internal L-iduronic acid residues of hexasaccharides containing two L-iduronic acid were observed (Hexa2, Hexa3, and Hexa5). The L-iduronic acid residue adjacent to the reducing end D-glucosamine showed slightly upfield chemical shifts for the H-2, H-3, and H-5 proton resonances, the difference in the H-5 proton resonance is especially characteristic. Also the D-glucosamine residue adjacent to the 4,5-unsaturated residue can be identified by the distinctive downfield chemical shift of the H-4 proton resonance at ~ 3.85 ppm compared to the H-4 chemical shift of other residues (3.74 ppm).

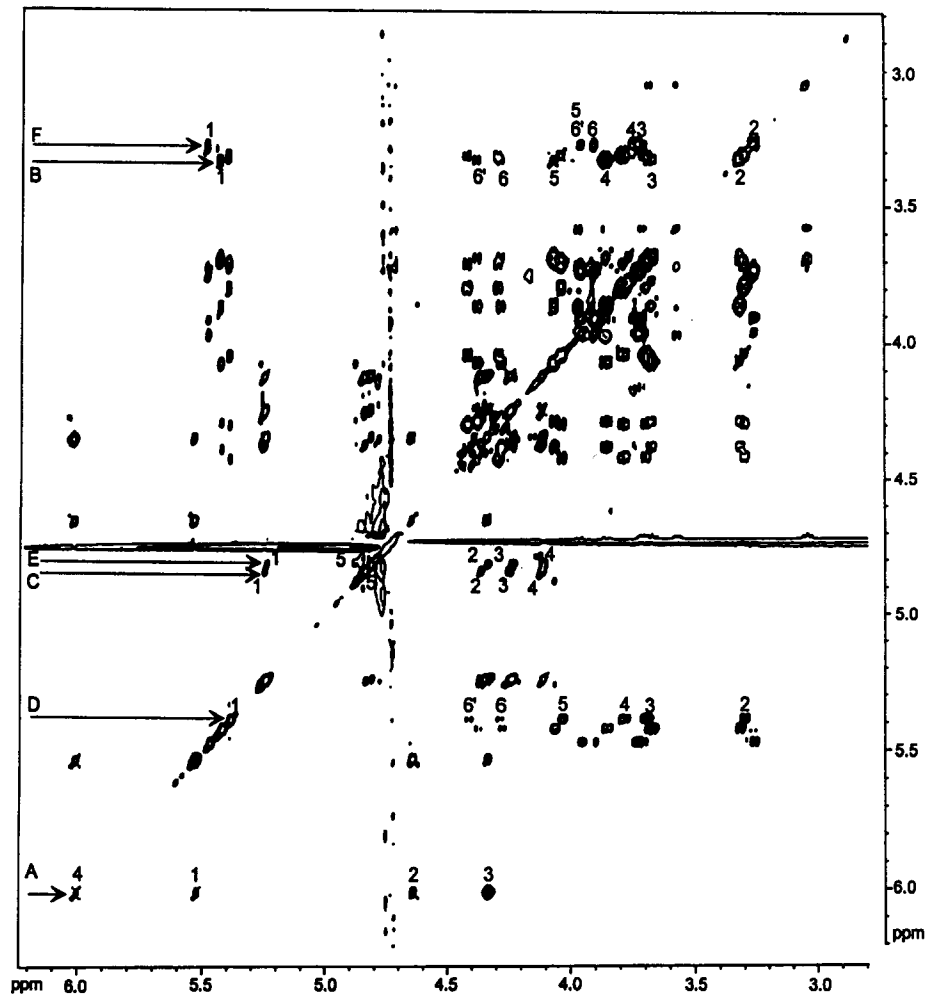

Fig. 6. 600-MHz ^{1}H NMR TOCSY spectra of hexasaccharide Hexa3 recorded in D_2O at room temperature. Assignment of cross-peaks for each residue is shown.

In all the one-dimensional ^{1}H NMR spectra it was possible to detect signals originating from the β anomer. For *N*-sulfated reducing end D-glucosamines (Hexa3, Hexa4, and Hexa5) the equilibrium greatly favors the α anomer [27,39], and only minor signals were observed for the β anomer. The resonances of the H-1 protons of the β anomers occurred in the same region as the water signal (4.8 ppm) so the resonances of the H-2 protons ($\sim$ 3.05 ppm) were the most distinct signal of the β anomers. For the *N*-acetylated reducing end D-glucosamine in Hexa2 the equilibrium also greatly favored the α anomer. The most distinctive resonance of the β anomer at 3.6 ppm was assigned to the H-2 proton. Less intense resonances for the H-1 proton of the α anomer of the reducing end D-glucosamine were observed for hexasaccharide Hexa4 and Hexa5 only

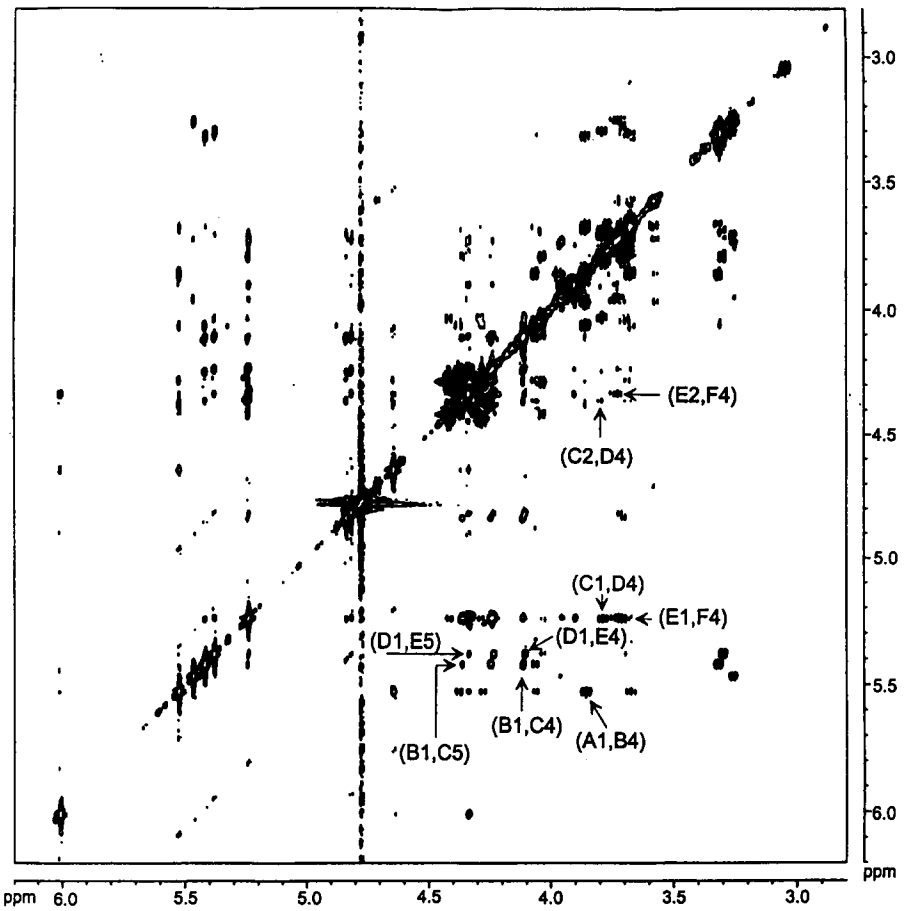

Fig. 7. 600-MHz ^{1}H NMR ROESY spectrum of hexasaccharide Hexa3 recorded in D_2O at room temperature. Assignments of the ROE cross-peaks that establish the sequence are shown.

and thus assignment of the terminal D-glucosamine by means of the intensity of the anomer signals as previously reported [27] could not be confirmed for all the hexasaccharides analyzed in this study. Nevertheless, analysis of ROESY spectra provide an additional way to assign the reducing end D-glucosamine residue. Whereas the anomeric protons of internal residues show intra- as well as inter-residue cross-peaks, the anomeric proton of the reducing end-residue gives only a cross-peak to the H-2 proton of the same residue.

Analysis of the conformation of the glycosidic linkages.—Besides that the ROE cross-peaks that established the sequence in the hexasaccharides additional information about the conformation of the glycosidic linkages can be derived from interresidue cross-peaks. Except for the spectrum of Hexa2, which had a lower signal-to-noise ratio, the same pattern was observed for the ROEs between the H-1 proton of a D-glucosamine and the protons in the adjacent L-iduronic acid unit. The most intense signals were to the

H-4 protons, but also ROE connectivities between the H-1 proton of D-glucosamine residues and the H-3 and H-2 protons of the L-iduronic acid residues were observed. This is essentially in accordance with NOE results made on whole heparin [40], indicating that the conformation of the glycosidic linkage [→ 4)-α-D-GlcN*p*-(1 → 4)-α-L-IdoA *p*-(1 →] basically is the same in shorter heparin fragments, i.e., hexasaccharides, as in whole heparin. The ROEs of the H-1 proton of a L-iduronic acid to protons in the adjacent D-glucosamine ring were in general less intense, the most intense signals were to the H-3 and H-4 signals. The ROEs to the H-6 protons were difficult to evaluate due to overlap with the signal from the H-2 proton of the iduronic acid residue. Only the most downfield H-6 proton from the reducing end D-glucosamine lacking a sulfate group on the C-6, was well resolved and exhibited a relatively strong ROE. Comparing these results with the NOE results from studies on longer heparin chains [40] some discrepancies in the NOE pattern are noticed. In those experiments the H-1 proton of the L-iduronic acid displayed strong NOEs to H-6a,6b protons and only a weak NOE to the H-4 proton of the D-glucosamine residue. The clear deviation demonstrates the different conformation of the [→ 4)-α-L-IdoA *p*-(1 → 4)-α-D-GlcN*p*-(1 →] linkage in hexasaccharides and longer heparin chains. The change in conformation of the glycosidic linkages might affect the biological properties of small heparin fragments, so the biological activities is influenced not only by the reduction in size of the heparin fragments but also by the altered conformation of the chains.

The H-1 proton of the D-glucosamine succeeded by a D- glucuronic acid resulted in ROEs to the H-3, H-4, and H-5 protons. Due to overlap of the signals it is difficult to evaluate the intensities of the individual signals. When the effect of the H-1 proton of a D-glucuronic acid on the adjacent D-glucosamine were studied, NOE to the H-3 and H-4 protons could be identified.

In summary this study has described the isolation and characterization of four highly-purified hexasaccharides differing in degree of sulfation and sugar residue composition. The study contributes to a greater insight into the structural variation of heparin composition. Comparison with reported data on whole heparin [40] indicated different conformation of the glycosidic linkages in hexasaccharides and longer heparin chains. Moreover, such fractions are valuable in future studies dealing with structure–activity relationships for heparin-derived oligosaccharides and protein interactions.

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