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Characterisation by LSI-MS and ^{1}H NMR spectroscopy of tetra-, hexa-, and octa-saccharides of porcine intestinal heparin

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Abstract

The characterisation of oligosaccharide fragments isolated from enzymatically depolymerised porcine intestinal heparin is required in order to probe structure/function relationships of heparin in anticoagulation, antiangiogenesis and antiviral activity. We have used both LSI-MS and 600-MHz ^{1}H NMR with chemical shift assignment by comprehensive ^{1}H–^{1}H TOCSY experiments to fully characterise the major oligosaccharide components including 4 tetrasaccharides, 3 hexasaccharides, and 2 octasaccharides. One of the octasaccharides has not been identified previously and has the structure: ΔUA(2S)-GlcNS(6S)-IdoA(2S)-GlcNS(6S)-IdoA(2S)-GlcNS(6S)-GlcA-GlcNS(6S), where ΔUA is 4,5-unsaturated uronic acid (4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid), GlcN is → 4)-α-D-glucosamine, IdoA is → 4)-α-L-iduronic acid, GlcA is → 4)-β-D-glucuronic acid, and 2-*O*-, 6-*O*-, and 2-*N*-sulfate are abbreviated to 2S, 6S, and NS, respectively. Nearly complete NMR proton chemical shifts are reported for this data set. In addition a novel approach involving oxymercuration–lipid conjugation was used to independently assign sulfate substitution on the ΔUA residues.

Keywords: Glycosaminoglycans; Oligosaccharides; Oxymercuration; Neoglycolipids

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1. Introduction

The glycosaminoglycan (GAG) [1] polysaccharides of heparin are biosynthesised in mammalian tissues from *N*-acetylglucosamine and glucuronic acid to form a chain of alternating (1 → 4)-linked disaccharide units. As biosynthesis proceeds, regions of the chains are variously modified by *N*-deacetylation, epimerisation of glucuronic to iduronic acid, and addition of sulfate groups, resulting in considerable heterogeneity of these regions of the polysaccharide. It has also been shown that the GAG sequences of heparan sulfate proteoglycans vary in cell-type-specific ways [1,2]. The potential biological significance of highly sulfated regions has been highlighted by elucidation of the molecular requirement for a distinct pentasaccharide sequence in heparin GAGs for the activation of antithrombin III in the anticoagulation cascade [3,4]. However, the extent to which such molecular specificity extends to other heparin-binding proteins remains to be determined. Nevertheless, evidence is beginning to accumulate on protein–GAG interactions which implicate binding of distinct structural motifs on heparin, heparan sulfate, and other GAG chains of proteoglycans [5–9]. There is a clear need to devise improved approaches to the sequence determination of GAG chains in order to investigate specific activities and also for preparing well-defined materials for structure/function studies.

The difficulty in characterising intact heparin chains has encouraged development of methods to partially depolymerise heparin by either enzymatic or chemical means [10] to give fragments which can be submitted to detailed characterisation. Studies in the early 1980s utilised combined approaches of chemical manipulation and heparinase digestion of materials prepared by paper and column chromatography, and analysis by NMR spectroscopy [10]. More recently, structural investigations have relied mainly on spectroscopic methods (^{1}H and ^{13}C NMR, and mass spectrometry), while isolation and purification of depolymerised fragments have been carried out primarily by high-performance liquid chromatography (HPLC). Good separations by anion-exchange (SAX) HPLC have been achieved of oligosaccharide fragments from different sources of heparin at various stages of enzyme digestion. Characterised structures include at least 20 tetrasaccharides [11–18], six hexasaccharides [13–16,19,20], and one octasaccharide [15,16].

We now report the isolation and comprehensive structural characterisation of a series of extensively sulfated oligosaccharide fragments from porcine intestinal heparin, including four tetra-, three hexa-, and two octa-saccharides, achieved by strong anion-exchange (SAX) HPLC, liquid secondary ion mass spectrometry (LSI-MS), and ^{1}H NMR spectroscopy. One of the octasaccharides has not been identified before and more

[1] Abbreviations used are: HexA, hexuronic acid; HexN, hexosamine; GlcN, → 4)-α-D-glucosamine; GlcNS, *N*-sulfated → 4)-α-D-glucosamine; GlcNAc, → 4)-*N*-acetyl-α-D-glucosamine; GlcA, → 4)-β-D-glucuronic acid; IdoA, → 4)-α-L-iduronic acid; ΔUA, 4,5-unsaturated hexuronic acid (4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid); GAG, glycosaminoglycan; DHPE, L-1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine; LSI-MS, liquid secondary ion mass spectrometry; SAX-HPLC, strong anion-exchange–high performance liquid chromatography; DQCOSY, double-quantum-filtered correlation spectroscopy; TOCSY, total correlation spectroscopy; and ROESY, rotating frame nuclear Overhauser enhancement spectroscopy.

complete ^{1}H NMR assignments are made than previously on several of the remaining structures by extensive use of 2-dimensional experiments. This data set will assist in confirming the identity of these oligosaccharides from various sources in future and contribute to the structure elucidation of other fragments. In contrast to most types of glycoprotein oligosaccharides it indicates a greater variation in chemical shift values of heavily sulfated fragments from heparin and a greater requirement for attention to sample preparation procedures.

2. Experimental

Materials.—Heparin sodium salt from porcine intestinal mucosa, heparin disaccharides II-A (ΔUA-GlcNS) and I-S [ΔUA(2S)-GlcNS(6S)], and heparinase I (EC 4.2.2.7) were purchased from Sigma Chemical Company (Dorset, United Kingdom). Bio-Gel P-4 (200–400 mesh) was obtained from Bio-Rad Laboratories Ltd. (Hemel Hempstead, United Kingdom) and Sephadex G-10 from Pharmacia Biotech (Milton Keynes, United Kingdom). L-1,2-Dihexadecyl-*sn*-glycero-3-phosphoethanolamine (DHPE) was from Fluka Chemicals Ltd. (Glossop, Derbyshire, United Kingdom). Mercuric acetate and sodium cyanoborohydride ($NaBH_3CN$) were purchased from Aldrich (Gillingham, Dorset, United Kingdom), and primulin and $NaBD_4$ from Sigma. High-performance TLC (HP-TLC) plates (5 μm silica gel, aluminium-backed) were from Merck (Poole, Dorset, UK). All other reagents used were of analytical grade.

Preparation of heparin oligosaccharides.—Heparin was depolymerised using the enzyme heparinase I essentially as described by Linhardt et al. [14]. Typically, 100 mg of heparin sodium salt was incubated with 50 units of heparinase I in 6.25 mL of 0.2 M NaCl–5 mM sodium phosphate buffer (pH 7.0) at 30°C for 70 h. The reaction was stopped by inactivating the enzyme thermally. The reaction solution was heated in a water bath at 100°C for 1 min and then freeze-dried.

After desalting on a Sephadex G-10 column (1.6 × 36 cm) and eluting with water, the depolymerised material was freeze-dried before further gel filtration. A column of Bio-Gel P-4 (1.6 × 90 cm) was used to fractionate the resulting heparin oligosaccharides (Fig. 1) with elution by 0.2 M NH_4OAc at a flow rate of 15 mL/h. A refractive index detector was used to monitor the eluted oligosaccharides continuously and each fraction (1.5 mL) was also measured by UV at 232 nm. The pooled fractions (Fig. 1) were freeze-dried.

HPLC separation.—Fractions F2–F4 were chromatographed by strong anion-exchange HPLC on an analytical column S5-SAX (4.6 × 25 cm, Phase Separations Ltd., Clwyd, Wales) using a titanium-lined Gilson liquid chromatography system. An NaCl solution was used to elute the oligosaccharides [21] with a linear gradient of 0.2 M NaCl (solvent *A*) to 1.5 M NaCl (solvent *B*) at pH 3.5 at a flow rate of 1.5 mL/min (for F2 and F3a, 0 to 80% *B* in 45 min; for F3b, 10 to 90% *B* in 45 min; for F3a and F4, 30 to 100% *B* in 40 min). The eluate was monitored by UV detection at 232 nm. Each HPLC fraction (Fig. 2) was desalted on a Sephadex G-10 column, as described above, before further analysis.

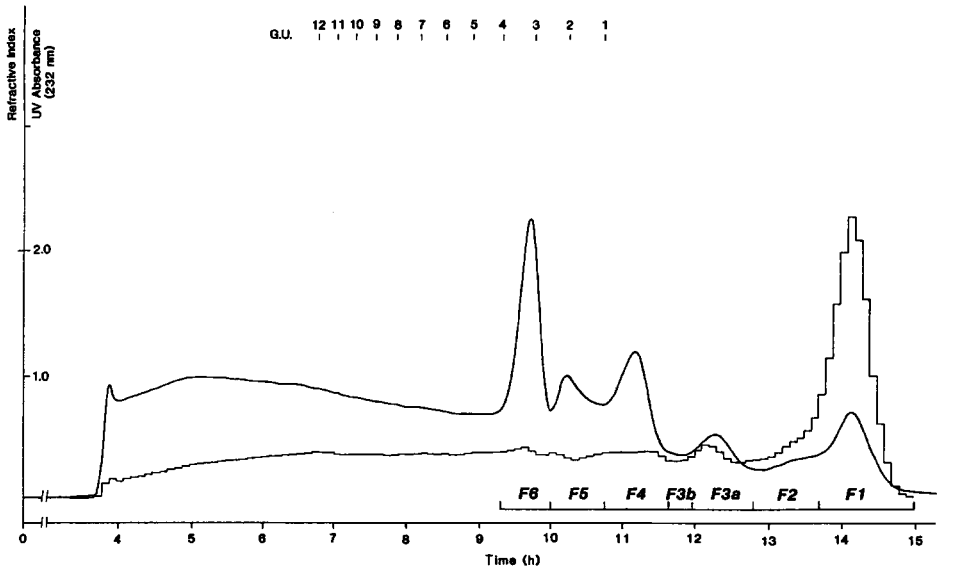

Fig. 1. Elution profile and fractionation of oligosaccharide fragments from depolymerisation of porcine intestinal heparin on a Bio-Gel P-4 column (stepped line is UV response).

Oxymercuration and DHPE conjugation.—Oxymercuration and DHPE conjugation were carried out essentially as described [22]. Briefly, to an aqueous solution of heparin oligosaccharide (10 μg in 20 μL H_2O) in an Eppendorf vial was added 20 μL of 20 mM $Hg(OAc)_2$ in 130 mM NaOAc (pH 5.0). The mixture was kept at room temperature for 30 min and then immediately desalted by a small column of AG50W-X8 (H^+ form) resin. To the lyophilised product were added H_2O (5 μL) and DHPE reagent (5 mg/mL $CHCl_3$–MeOH; 25 μL), and the solution was dried under a stream of N_2. The mixture was resuspended in H_2O (5 μL), 1:1 $CHCl_3$–MeOH (45 μL), and freshly prepared methanolic $NaBH_3CN$ (10 mg/mL; 1.5 μL), and incubated at 60°C for 18 h. The solution was dried under a stream of N_2 and the residue redissolved in 25:25:8 $CHCl_3$–MeOH–H_2O for TLC.

The DHPE conjugation mixture was applied as a 5-mm band to an aluminium-backed HP-TLC plate and developed in 95:45:9 $CHCl_3$–MeOH–H_2O for 8 cm. The bands were located under UV light after spraying with primulin reagent (0.001% primulin in 4:1 acetone–H_2O).

Mass spectrometry.—LSI-MS analysis was carried out on a VG ZAB-2E mass spectrometer equipped with a Cs ion gun operated at 25 keV with an emission current of 0.5 μA. Full mass spectra were acquired at 30 s/decade using the VG Analytical 11-250J data system in 'continuum' acquisition mode.

Sample solutions of the purified heparin oligosaccharides were prepared by dissolving the solid sample in distilled water at a concentration of 1–3 μg/μL. For LSI-MS analysis 1 μL of sample solution (1–3 μg) was typically applied on a standard VG stainless-steel probe tip coated with thioglycerol (1–2 μL).

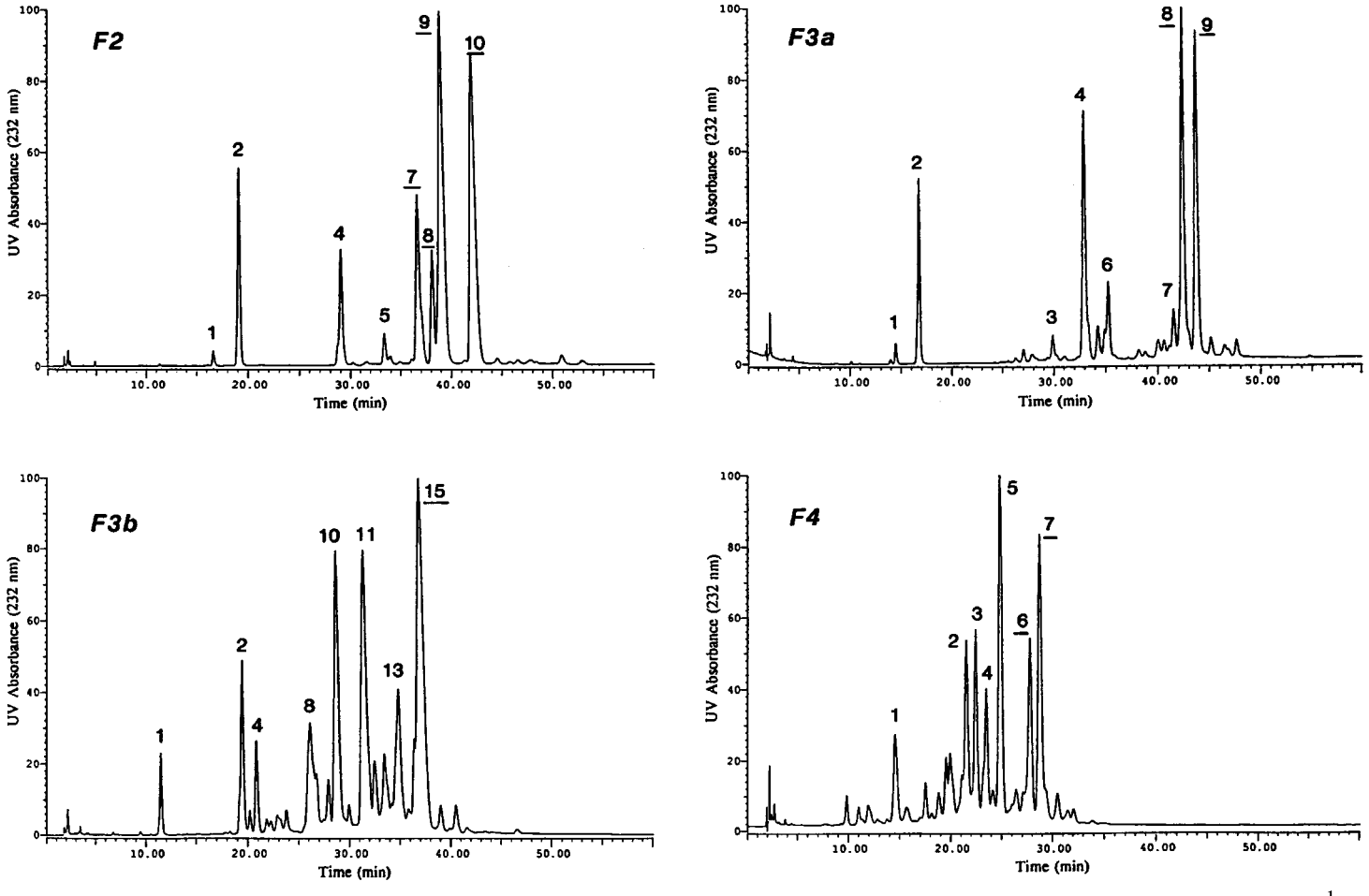

Fig. 2. HPLC chromatograms of Bio-Gel P-4 fractions, F2, F3a, F3b, and F4 on an S5-SAX column. The underlined fractions were collected for LSI-MS and ^{1}H NMR analyses.

NMR spectroscopy.—Samples evaporated three times from D_2O (Sigma; 99.90%) were dissolved in D_2O (500 μL; Sigma; 100%) containing acetone (0.1 μL) and transferred to 5-mm NMR tubes (Wilmad Glass Co, Ltd., Buena, NJ, USA). 1-Dimensional spectra, 2-dimensional homonuclear double-quantum-filtered correlation spectroscopy (DQCOSY), total correlation spectroscopy (TOCSY), and rotating frame nuclear Overhauser enhancement spectroscopy (ROESY) experiments were carried out at 22°C and pD 7 on a Varian Unity 600-MHz spectrometer. TOCSY data [23] were acquired using an MLEV-17 [24] mixing sequence of 100-ms duration and field strength of ca. 7 kHz. ROESY experiments [25–27] were carried out with a mixing time of 300 ms. Suppression of the water signal was achieved by selective presaturation in all NMR experiments. The chemical shift scale was derived from an internal reference signal of acetone set to resonate at 2.225 ppm at 22°C (295 K).

3. Results

Fig. 1 shows the fractionation by gel filtration on Bio-Gel P-4 of the oligosaccharide fragments from enzyme digestion of porcine intestinal heparin. The interaction between the strongly ionic sulfate groups and the gel-filtration medium leads to a retardation of oligosaccharide peaks relative to neutral dextran hydrolysate oligomers. The NH_4OAc (0.2 M) buffer used was insufficient to suppress this interaction. The pooled fractions F1 to F4 were fractionated further by HPLC (Fig. 2) and major subfractions were characterised by direct LSI-MS analysis. Negative ion mass spectra (Table 1, see Fig. 3 for selected spectra), acquired from thioglycerol matrix at low μg level, were dominated by clusters of sodiated anions. Generally, quasimolecular ions with the formula $[M - nH + (n - 1)Na]^-$, where n = the number of sulfate groups present, were most abundant from which the HexA/HexN composition and the total number of *O*- and *N*-sulfates, together with the presence of acetyl groups, could be deduced. Fragment ions due to consecutive desulfation contributed further to the complexity of the spectra (Fig. 3). No ions were found that could be confidently assigned as diagnostic of sequence, even after

Table 1
Major quasimolecular LSI-MS ions of oligosaccharide fractions and deduced compositions

Fraction	Major ions observed	Molecular mass	Compositions				
			ΔUA	HexA	HexN	Ac	SO_3
F2-7	1161 $[M-5H+4Na]^-$	1074	1	1	2	0	5
F2-8	1161 $[M-5H+4Na]^-$	1074	1	1	2	0	5
F2-9	1161 $[M-5H+4Na]^-$	1074	1	1	2	0	5
F2-10	1263 $[M-6H+5Na]^-$	1154	1	1	2	0	6
F3-15	1744 $[M-7H+6Na]^-$	1613	1	2	3	1	7
F3-8	1804 $[M-8H+7Na]^-$	1651	1	2	3	0	8
F3-9	1906 $[M-9H+8Na]^-$	1731	1	2	3	0	9
F4-6	2447 $[M-11H+10Na]^-$	2228	1	3	4	0	11
F4-7	2549 $[M-12H+11Na]^-$	2308	1	3	4	0	12

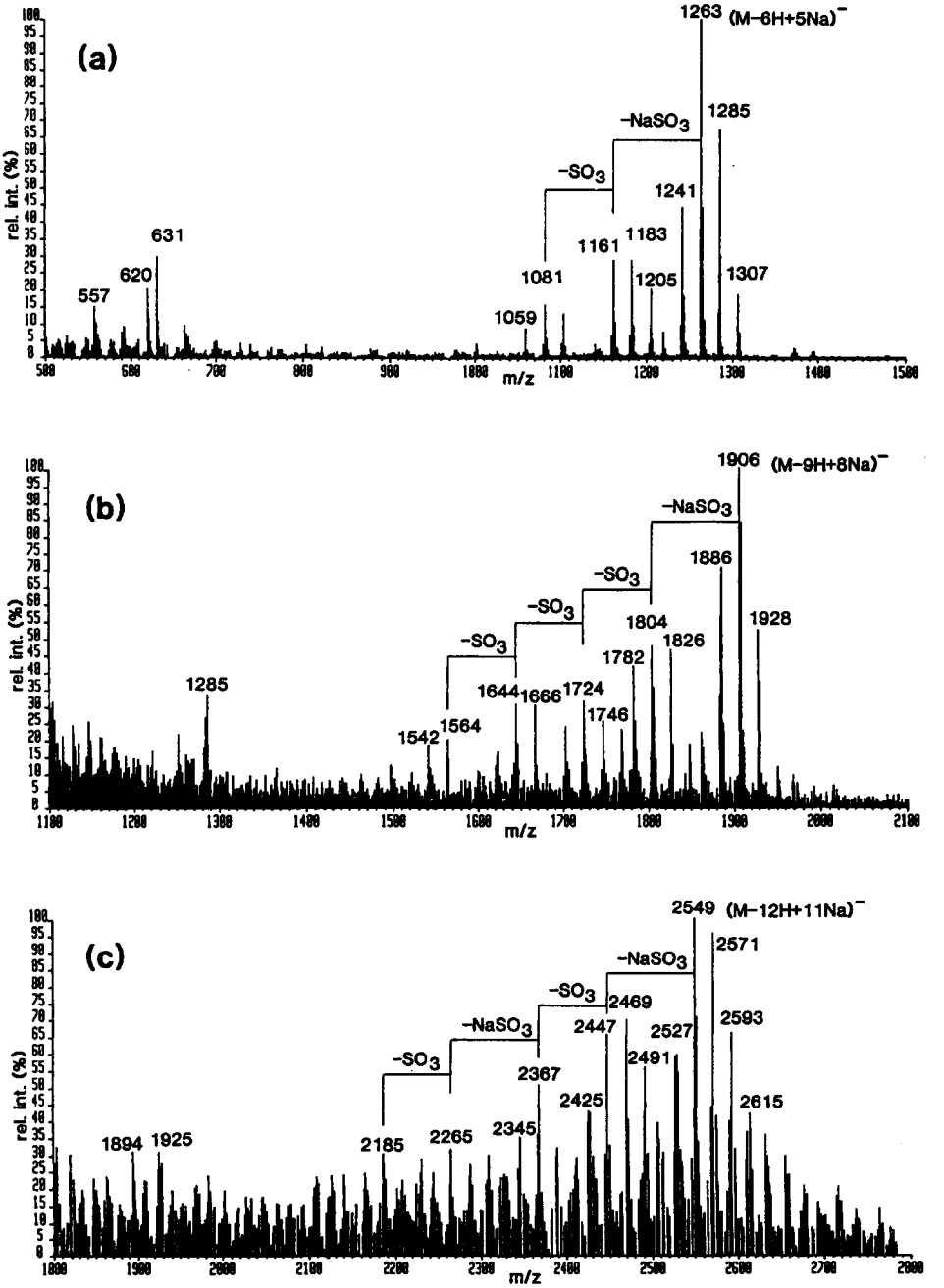

Fig. 3. Negative ion LSI-MS spectra of SAX-HPLC fractions: (a) F2-10; (b) F3-9; and (c) F4-7.

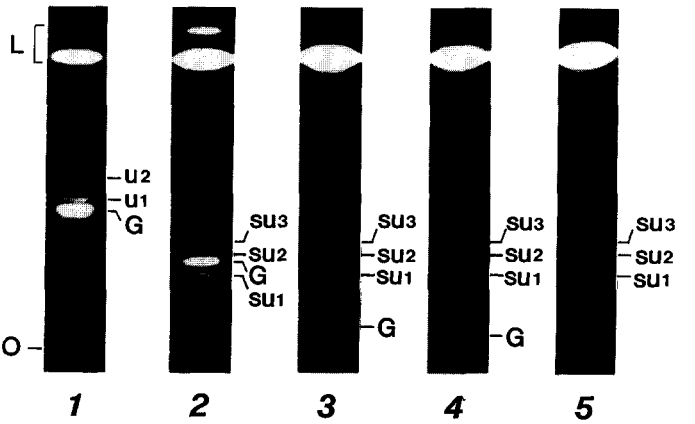

Fig. 4. Thin layer chromatograms of neoglycolipids (DHPE) prepared from products of oxymercuration cleavage of heparin fragments from: lane 1, ΔUA-GlcNAc(6S); lane 2, ΔUA(2S)-GlcNS(6S); lane 3, F2-7; lane 4, F2-10; and lane 5, F3-9. TLC bands marked G indicate the DHPE derivatives of the respective oligosaccharides after removal of the ΔUA or ΔUA(2S) residues. Bands U1 and U2 are the neoglycolipids of products of a ΔUA residue, and SU1, SU2, and SU3 those from a ΔUA(2S) residue.

increasing the sample amounts and using a liquid matrix of triethanolamine as suggested previously [13].

A microscale analysis was carried as an alternative approach to define the terminal ΔUA and its sulfate content. This convenient and very sensitive method [28] entails the cleavage of ΔUA from the oligosaccharide by oxymercuration and lipid DHPE conjugation of the reaction products. The TLC patterns of the DHPE derivatives of the cleaved products from the oligosaccharide fractions F2-7, F2-10, and F3-9 were examined (Fig. 4) and compared with those produced from standard heparin disaccharides II-A and I-S which contained ΔUA and ΔUA(2S), respectively. Each product band was analysed by TLC–LSI-MS (Table 2 and see discussion of individual fractions).

The detailed structural assignment of oligosaccharides was made from NMR chemical shifts (Tables 3–5) and correlated with the mass spectral data as follows.

Fraction F2-7.—The composition of F2-7 was deduced, from the quasimolecular ion m/z 1161 $[M - 5H + 4Na]^-$ in the LSI spectrum (Table 1), to comprise one ΔUA, one

Table 2
Mass spectral data from DHPE derivatives of oxymercuration products

Sample	$[M-H]^-$ of TLC bands of DHPE derivatives					
	U1	U2	SU1	SU2	SU3	G
II-A	806	806				947
I-S			904	886	886	985
F2-7			904	886	886	1516 [a]
F2-10			904	886	886	1618 [a]
F3-9			904	886	886	

[a] Observed as Na and boron adduct ions: m/z 1516 $[M-H+Na+B]^-$ and m/z 1618 $[M-2H+2Na+B]^-$.

Table 3
The ^{1}H NMR chemical shifts in ppm from DSS at 22°C and coupling constants for four heparin sulfate tetrasaccharides

		F2-7	F2-8	F2-9	F2-10
ΔUA	H-1	5.503 (2.74) [a]	5.489 (3.05)	5.523 (2.75)	5.523 (3.06)
	H-2	4.617 (2.44)	4.614 (2)	4.613 (n.d.) [c]	4.615 (4.0)
	H-3	4.302 (3.60)	4.312 (4)	4.336 (4.58)	4.317 (4.88)
	H-4	5.976	5.974	6.050	6.084
GlcNS	H-1	5.399 (3.36)	5.355 (3.66)	5.567 (3.67)	5.410 (3.66)
	H-2	3.287 (10.78)	3.265 (10.37)	3.305 (10.69)	3.293 (10.37)
	H-3	3.636 (9)	3.630 (9)	3.672 (8.85)	3.663 (9.0)
	H-4	3.826 (9.8)	3.820 (10)	3.841 (10)	3.841 (9.0)
	H-5	4.029 (2)	3.973 (2)	4.153 (1.83)	3.973 (2.44)
	H-6a	4.343 (11.6)	4.352 (11)	4.336 (11.3)	4.341 (11)
	H-6b	4.240 (2)	4.205 (2)	4.175 (2.75)	4.220 (2.44)
IdoA/GlcA [b]	H-1	5.186 (1.83)	4.997 (1.8)	4.613 [b] (7.8)	5.231 (3.05)
	H-2	4.293 (5.79)	3.755 (5)	3.385 (9.4)	4.301 (5.50)
	H-3	4.209 (3.66)	4.131 (3)	3.713 (10.1)	4.269 (3.1)
	H-4	4.083 (2.4)	4.004 (2.4)	3.841 (9.87)	4.116 (2.44)
	H-5	4.761	4.651	3.911	4.991
GlcNS	H-1	5.449 (3.35)	5.455 (3.64)	5.463 (3.66)	5.444 (3.66)
	H-2	3.228 (10.07)	3.265 (10.37)	3.263 (10.07)	3.256 (10.38)
	H-3	3.678 (9)	3.684 (11)	3.802 (9.16)	3.699 (9.0)
	H-4	3.715 (9)	3.678 (10)	3.861 (10.07)	3.718 (9.0)
	H-5	3.926 (3.66)	4.144 (4.17)	3.967 (n.d.)	4.146 (n.d.)
	H-6a	3.888 (12.21)	4.290 (n.d.)	4.31–4.32	4.30–4.31
	H-6b	3.858 (2.74)	4.260 (2.4)		

[a] coupling constants in order $J_{1,2}$, $J_{2,3}$, $J_{3,4}$, $J_{4,5}$, $J_{5,6a}$, $J_{6a,6b}$, $J_{5,6b}$.
[b] F2-9 has glucuronic acid.
[c] n.d., Not determined.

HexA, two HexN residues, and a total of five sulfate groups. The mass was consistent with both HexN amino groups being non-acetylated.

The chemical shifts for F2-7 were assigned by 1D ^{1}H NMR, DQCOSY, and TOCSY experiments. These indicated the presence of ΔUA, IdoA, and GlcNS in 1:1:2 ratios, consistent with the mass spectral data. Comparison of the chemical shift data (Table 3) with that of the 'tetrasaccharide 2' of Horne and Gettins [16], which has the same monosaccharide composition but differs in degree of sulfation, showed respective chemical shifts for H-1 to H-4 of 2-*O*-sulfated ΔUA that are 0.180, 0.180, 0.013, and 0.012 ppm downfield of those reported by these authors. Other studies of unsaturated hexuronic acid residues in heparin oligosaccharides have given chemical shifts of ca. 5.16, 3.81, 4.25, and 5.82 ppm for nonsulfated ΔUA [17,29], and ca. 5.50, 4.58, 4.34, and 5.97 ppm for the 2-sulfated derivative [12,29]. Hexasaccharides which differ only in the sulfation pattern at C-3 of reducing end GlcNS(6S) have been characterised with chemical shifts for the ΔUA H-1 to H-4, respectively, of 5.54, 4.61, 4.31, and 6.10 ppm [19], 5.52, 4.64, 4.33, and 6.00 ppm [20], and 5.50, 4.63, 4.33, and 5.98 ppm [14] for spectra obtained at 295 K. Our data for F2-7 are therefore consistent with those reported for 2-*O*-sulfated ΔUA. Microscale analysis, as described above, was carried out to

Table 4
Comparison of the chemical shifts for the tetrasaccharide, ΔUA(2S)-GlcNS(6S)-IdoA(2S)-GlcNS(6S), given by three independent studies

		Horne and Gettins (1992) [16]	Merchant et al. (1985) [12]	Present study (F2-10)
ΔUA(2S)	H-1	5.485	5.49	5.523
	H-2	4.599	4.58	4.615
	H-3	4.289	4.32	4.317
	H-4	5.964	5.99	6.084
GlcNS(6S)	H-1	5.414	5.37	5.410
	H-2	3.278	3.30	3.293
	H-3	3.618	3.67	3.663
	H-4	3.805	3.82	3.841
	H-5	4.025	4.09	3.973
	H-6a	4.320	4.35	4.341
	H-6b	4.233	4.28	4.222
IdoA(2S)	H-1	5.181	5.24	5.231
	H-2	4.293	4.32	4.306
	H-3	4.176	4.22	4.269
	H-4	4.089	4.01	4.116
	H-5	4.707	4.81	4.991
GlcNS(6S) reducing	H-1	5.423	5.43	5.444
	H-2	3.243	3.28	3.256
	H-3	3.667	3.73	3.699
	H-4	3.728	3.83	3.718
	H-5	4.111	4.12	4.146
	H-6a	4.327	4.34	4.3–4.31
	H-6b	4.280	4.22	

confirm the monosulfate substitution of ΔUA. The DHPE derivatives of the cleaved products were consistent with a ΔUA(S) as determined from their TLC pattern (Fig. 4, lane 3) and LSI-MS analysis. The latter showed $[M-H]^-$ at m/z 904 for band SU1, and m/z 886 for both SU2 and SU3 (Table 2). The TLC band marked G gave a spectrum with a quasimolecular ion at m/z 1516 (Table 2) consistent with the DHPE derivative of a trisaccharide having four sulfates following removal of the ΔUA residue. This clearly showed that the ΔUA was monosulfated.

Chemical shifts for the internal HexA were consistent with a 2-sulfated iduronic acid, those for H-1 to H-4 being 0.005, 0.0, −0.033, and −0.006 ppm from reported values for IdoA(2S) of the 'tetrasaccharide 2' [16]. The chemical shifts for the H-1 to H-3 of reducing end GlcN in our study differ by 0.026, −0.015, and 0.011 ppm from signals in the latter study, suggesting 2-*N*-sulfation and lack of 3-*O*-sulfation. However the chemical shifts for H-4, H-5, H-6a, and H-6b were 0.013, 0.185, 0.422, and 0.439 upfield, consistent with the absence of 6-*O*-sulfation [30]. The chemical shifts of internal GlcNS(6S) were within 0.02 ppm of the reported values [16]. The other studies quoted above gave similar chemical shifts for a GlcNS(6S) residue bounded by ΔUA(2S) and IdoA(2S). Taken together the data are unambiguous for the following assignment for F2-7:

ΔUA(2S) − GlcNS(6S) − IdoA(2S) − GlcNS

Previous reports of this structure have been made [11,14,18] but chemical shift data have been incomplete, in particular for the reducing end GlcNS without 3- or 6-sulfation.

Fraction F2-8.—The LSI mass spectrum of fraction F2-8 (Table 1) indicated it was also a tetrasaccharide of the same overall composition as fraction F2-7 containing five sulfate groups, two of which were located on the HexN residues, as indicated by the absence of the appropriate mass for two acetyl groups. NMR analysis confirmed the monosaccharide composition of ΔUA, IdoA, and GlcNS in 1:1:2 ratios (Table 3). Chemical shifts assigned for the H-2–H-4 of ΔUA were within 0.01 ppm of those for F2-7, suggesting 2-*O*-sulfation as discussed above. Similar chemical shifts to those for F2-7 were also given for H-1 to H-6 of internal GlcNS(6S) and for H-1 to H-3 of reducing end GlcNS. Major differences in the shifts for the H-5, H-6a, and H-6b of the reducing end GlcNS of F2-8 compared to F2-7 showed that this residue is 6-*O*-sulfated (Table 3). Differences in chemical shifts for the internal IdoA suggest that this is lacking the 2-sulfate group. The H-5 and H-6 shifts of the internal GlcNS are consistent with previous data in the literature for a 6-*O*-sulfated GlcNS. In summary the structure is proposed as:

ΔUA(2S)-GlcNS(6S)-IdoA-GlcNS(6S)

This structure has been reported as a minor component from heparin lyase digestion of porcine heparin based on SAX chromatography, enzyme specificity, and chemical shifts of reporter groups [14]. Additional evidence for the assignment for F2-8 comes from the analysis of fractions F2-9 and F2-10 below.

Fraction F2-9.—The major tetrasaccharide species in fraction F2-9 contained glucuronic acid as evidenced by the H-1 signal at 4.613 ppm ($J_{1,2}$ 7.8 Hz) and the H-2 signal at 3.385 ppm ($J_{2,3}$ 9.4 Hz). In addition, the 5 sulfate groups indicated by mass spectrometry (Table 1) suggested that F2-9 was equivalent to the 'tetrasaccharide 1' recorded by Horne and Gettins [16] and the 'tetra 2' of Merchant et al. [12]. Comparison of the NMR data with their data identified the reducing end GlcNS(6S) with chemical shifts for H-1 to H-5 being within 0.02 ppm of the 'tetrasaccharide 1' [16] and 0.03 ppm of the 'tetra 2' [12]; shifts for H-1 of the internal GlcNS were within 0.04 ppm of the 'tetrasaccharide 1' [there is probably an error in the Merchant et al. study which gives H-1 of reducing GlcNS(6S) of 'tetra 2' as 5.34 ppm whilst the other chemical shifts agree with our data]. The chemical shift data for the ΔUA residue fall close to the downfield range for the 2-*O*-sulfated derivative as discussed above for F2-7, giving the proposed assignment for F2-9:

ΔUA(2S)-GlcNS(6S)-GlcA-GlcNS(6S)

The same oligosaccharide has also been reported with limited NMR data documentation [11,31].

Fraction F2-10.—LSI-MS (Table 1) and NMR analysis (Table 3) together indicated this to be a tetrasaccharide composed of ΔUA, IdoA, and GlcNS in 1:1:2 ratios plus six sulfate groups, and suggested that it was the previously reported 'tetrasaccharide 2' [16] and 'tetra 1' [12]. Oxymercuration cleavage, DHPE conjugation, and TLC and TLC–LSI-MS analyses (Fig. 4, lane 4 and Table 2) were used to confirm mono-*O*-sulfation of the terminal ΔUA. Hence F2-10 was assigned the structure:

ΔUA(2S)-GlcNS(6S)-IdoA(2S)-GlcNS(6S)

Table 5
The ^{1}H NMR chemical shifts (ppm from DSS at 22°C) of heparin sulfate hexa- and octa-saccharides

		F3-8	F3-9	F3-15	F4-6	F4-7
ΔUA	H-1	5.552	5.519	5.530	5.529	5.540
	H-2	4.643	4.612	4.612	4.623	4.631
	H-3	4.340	4.317	4.354	4.316	4.317
	H-4	6.260	6.053	6.243	6.116	6.175
GlcNS	H-1	5.387	5.402	5.337	5.393	5.387
	H-2	3.297	3.273	3.274	3.290	3.285
	H-3	3.636	3.661	3.642	3.625	3.632
	H-4	3.833	3.840	3.833	3.835	3.838
	H-5	3.925	3.976	4.310	3.923	3.906
	H-6a	4.33	n.d.	n.d.	n.d.	4.3
	H-6b	4.3	n.d.	n.d.	n.d.	4.29
IdoA	H-1	5.145	5.17	5.133	5.20	5.210
	H-2	n.d. [d]	4.33	3.184	4.34	4.1–4.5
	H-3	n.d.	4.33	4.078 [c]	4.22 [c]	
	H-4	4.121	4.10	4.196	4.12	
	H-5	5.218	5.17	5.030	n.d.	5.150
GlcNS	H-1				5.433	5.424
	H-2				3.285	3.285
	H-3				3.654	3.623
	H-4				3.838	3.718
	H-5				3.923	3.916
	H-6				n.d.	4.26, 4.30
IdoA	H-1				5.20	5.210
	H-2				4.34	4.1–4.5
	H-3				4.22 [c]	
	H-4				4.15	
	H-5				n.d.	5.150
GlcNS/GlcNAc [a]	H-1	5.584	5.439	5.305 [a]	5.588	5.436
	H-2	3.278	3.289	3.937	3.285	3.285
	H-3	3.645	3.645	3.725	3.625	3.623
	H-4	n.d.	3.715	3.761	3.876	3.838
	H-5	n.d.	3.956	3.905	3.948	3.916
	H-6a	4.30–4.33	n.d.	4.432	n.d.	4.26–4.30
	H-6b		n.d.	4.329	n.d.	
IdoA/GlcA [b]	H-1	4.654 [b]	5.23	4.625 [b]	4.632 [b]	5.243
	H-2	3.402	4.30	3.399	3.390	4.1–4.5
	H-3	3.737	4.27	3.714	3.715	
	H-4	3.850	4.14	3.865	3.749	
	H-5	4.096	5.12	4.049	3.995	5.085
GlcNS	H-1	5.461	5.446	5.461	5.464	5.447
	H-2	3.262	3.257	3.418	3.265	3.260
	H-3	3.718	3.735	4.464	3.715	3.718
	H-4	3.708	3.699	3.945	3.733	3.648
	H-5	4.155	4.15	4.207	4.216	4.155
	H-6a,6b	4.3–4.36	4.28			4.29–4.39

[a] F3-15 has *N*-acetyl group 2.037 ppm.
[b] F3-8, F3-15, and F4-6 have glucuronic acid.
[c] Signals may be interchanged
[d] n.d., Not determined.

Table 4 shows a comparison of the NMR data from three studies for this tetrasaccharide, indicating some discrepancy in the chemical shifts observed under neutral conditions. The temperature used in one of the studies (Ref. [16] at 315 K rather than 295 K) was responsible for some differences but the inconsistency in chemical shifts of some signals is more likely to be due to some other factor, or combination of factors, such as salt or sample concentrations. In our study the oxymercuration assay confirmed the mono-*O*-sulfation of ΔUA.

Fraction F3-8.—^{1}H NMR data for the larger oligosaccharides are shown in Table 5. These were interpreted with respect to the detailed coupling constant assignments of Table 3. No major differences in coupling constants were found for monosaccharides in similar sequences. Additional sequences found in larger oligosaccharides are discussed separately below.

Fraction F3-8 contained a hexasaccharide having eight sulfate groups (Table 1). ^{1}H NMR gave characteristic signals of appropriate intensity for one GlcA residue. This suggested that F3-8 might be the 'hexasaccharide 1' previously characterised by ^{1}H NMR [16]. Comparison of the ^{1}H NMR data for the reducing end glucosamine residue in F3-8 (Table 5) and in 'hexasaccharide 1' [16] was consistent with GlcNS(6S) adjacent to unsulfated GlcA, with signals being within 0.02 ppm. Comparison of the chemical shifts in the present series (Tables 3 and 5) for internal residues suggested that those for F3-8 were consistent with GlcNS(6S)-IdoA(2S)-GlcNS(6S). The chemical shifts for the non-reducing end ΔUA were more variable, as discussed above, despite in all cases this residue being adjacent to a GlcNS(6S). However, as the shifts for H-1–H-3 for fractions F3-8, F3-9, F3-15, F4-6, and F4-7 were comparable, oxymercuration cleavage was only carried out on F3-9 as representative (see below). The chemical shifts for H-1–H-3 for ΔUA of F3-8 ($\pm$0.03 ppm) most resembled those quoted by Lindhardt et al. [19], suggesting definite 2-*O*-sulfation. Comparison of the COSY spectrum reported previously [16] with the TOCSY spectrum recorded in the present study (not shown) was consistent with the equivalence of the two structures. Hexasaccharide F3-8 was therefore assigned as:

ΔUA(2S)-GlcNS(6S)-IdoA(2S)-GlcNS(6S)-GlcA-GlcNS(6S)

Fraction F3-9.—The major oligosaccharide of fraction F3-9 was a hexasaccharide having nine sulfate groups (Table 1). ^{1}H NMR analysis (Table 5) was consistent with a monosaccharide composition of ΔUA, IdoA, and GlcNS in 1:2:3 ratios. This suggested that F3-9 might be the 'hexasaccharide 2' described previously [16]. The oxymercuration assay of F3-9 confirmed mono-*O*-sulfation of the unsaturated hexuronic acid (Table 2, Fig. 4, lane 5) despite the unusual downfield shift for ΔUA H-4 (Table 5). Similarity of the chemical shifts for F2-9 and F2-10 of this non-reducing end residue with those of F3-9 indicated a ΔUA(2S)-GlcNS(6S) sequence. In addition, its reducing end sequence was suggested as IdoA(2S)-GlcNS(6S) by comparison with the values for F2-10. The other internal IdoA and GlcN residues had similar chemical shifts, respectively, to the GlcNS(6S) and IdoA(2S) of the non-reducing and reducing end disaccharides as shown by the overlapping cross-peaks in the TOCSY experiment. A ROESY experiment (results not shown) gave in addition single cross-peaks between H-1 of GlcNS(6S) and H-3, H-4 of IdoA(2S) suggesting the equivalence of two GlcNS(6S)-IdoA(2S) units. The

LSI-MS data (Table 1 and Fig. 3) and comparison of the TOCSY experiment with those for F2-7 and F2-10 lead to the conclusion that hexasaccharide F3-9 has the sequence:

ΔUA(2S)-GlcNS(6S)-IdoA(2S)-GlcNS(6S)-IdoA(2S)-GlcNS(6S)

The LSI-MS of this structure was reported by Linhardt et al. [15] together with limited NMR data.

Fraction F3-15.—The composition of F3-15 was deduced from the LSI-MS spectrum to contain ΔUA, HexA, and GlcN in 1:2:3 ratios plus 7 sulfate groups, together with an acetyl group which was assumed to be on one of the GlcN residues (Table 1). The ^{1}H (Table 5) and 2D ^{1}H–^{1}H TOCSY NMR spectra showed coupled signals at 4.625 (H-1; $J_{1,2}$ 7.9 Hz), 3.399 (H-2; $J_{2,3}$ 9.1 Hz), 3.714 (H-3), 3.865 (H-4), and 4.049 (H-5) ppm of appropriate chemical shifts and intensity for a single GlcA residue. This GlcA was assigned as a non-sulfated residue linked to reducing end GlcNS by

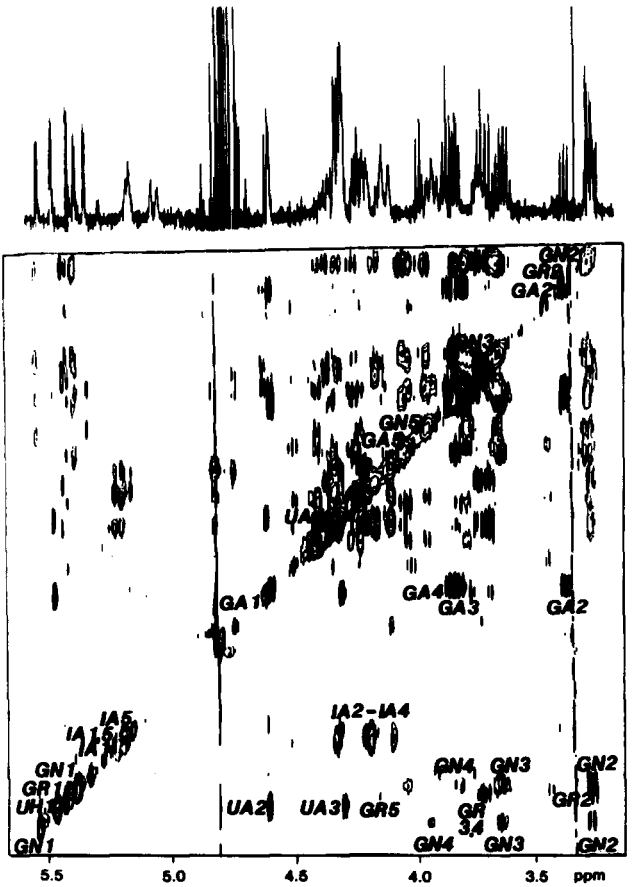

Fig. 5. 2D TOCSY spectrum of F4-6 upfield of the ΔUA H-4 signal at 5.529 ppm. GR indicates reducing end GlcN, GN internal GlcN, GA glucuronic acid, IA iduronic acid, and UA unsaturated hexuronic acid.

comparison of its NMR chemical shift data with those for F3-8 and F4-6 (Table 5). However, the chemical shifts for the terminal reducing GlcN residue (Table 4) suggested that this may be 3- and 6-*O*-sulfated because of the downfield shift of H-2 and H-3 relative to other oligosaccharides in the series. This agreed with chemical shifts for a hexasaccharide containing the reducing end sequence GlcNAc(6S)-GlcA-GlcNS(6S,3S) [19,20] and differed with those from a similar hexasaccharide lacking the 3-sulfate group on the reducing end GlcN [14]. Despite inconsistencies in some chemical shifts the structure of F3-15 was confirmed as the earlier reported hexasaccharide [19] as:

ΔUA(2S)-GlcNS(6S)-IdoA-GlcNAc(6S)-GlcA-GlcNS(6S,3S)

Fractions F4-6 and F4-7.—By LSI-MS the oligosaccharide components of fractions F4-6 and F4-7 were deduced to be octasaccharides having 11 and 12 sulfate groups, respectively (Fig. 1c and Table 1). The 1D and 2D TOCSY NMR spectra, shown in Figs. 5 and 6 with chemical shifts in Table 5, indicated that F4-7 is the octasaccharide

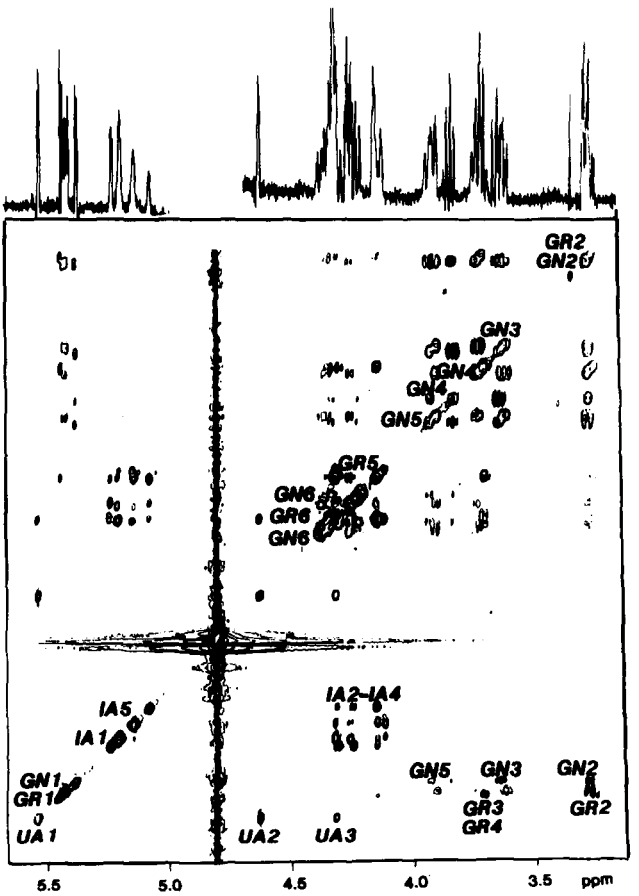

Fig. 6. 2D TOCSY spectrum of F4-7 upfield of the ΔUA H-4 signal at 5.540 ppm. Abbreviations used are as in Fig. 5.

reported by Horne and Gettins [16] while F4-6 is a previously unreported octasaccharide having an unsulfated GlcA replacing a 2-*O*-sulfated IdoA of F4-7. The position of GlcA linked to a reducing end GlcNS(6S) was suggested by the 1D ^{1}H and 2D ^{1}H–^{1}H TOCSY experiments from which comprehensive assignments of the chemical shifts were obtained (Fig. 5, Table 4), and by comparison with the spectrum of F4-7 (Fig. 6). In addition, a ROESY experiment gave cross-peaks for internal GlcNS(6S) H-1 to H-3, H-4 of IdoA (see above for discussion of F3-9), showing that at least one and probably both IdoA(2S) residues are linked to C-1 of a GlcNS(6S). The data are therefore consistent with the following respective assignments for F4-6 and F4-7:

ΔUA(2S)-GlcNS(6S)-IdoA(2S)-GlcNS(6S)-IdoA(2S)-GlcNS(6S)-GlcA-GlcNS(6S)

and

ΔUA(2S)-GlcNS(6S)-IdoA(2S)-GlcNS(6S)-IdoA(2S)-GlcNS(6S)-IdoA(2S)-GlcNS(6S).

The structure for F4-7 was also reported by Linhardt et al. [15] with limited NMR data, but F4-6 has not been documented previously.

4. Discussion

Almost complete assignments of ^{1}H NMR chemical shifts and coupling constants were made for four tetra-, three hexa-, and two octa-saccharide fragments which will contribute to reliable structural assignment of polysulfated oligosaccharides. These oligosaccharides represent the most abundant heavily sulfated fragments purified from the digestion of heparin. Several groups have characterised oligosaccharide fragments from the same source, using a combination of mass spectrometry and NMR spectroscopy, although complete ^{1}H NMR assignments have not always been documented and some variations in chemical shift values are apparent. LSI-MS analysis of some of the present fragments suggested that they may have been previously unreported while high-field 1D ^{1}H NMR characterisation revealed some anomalies in the chemical shifts of certain residues and those recorded before for structures deduced to be identical. In contrast to the well-established ^{1}H NMR chemical shifts of N- and O-linked glycoprotein oligosaccharides, where comparisons for the same oligosaccharides agree to within ± 0.005 ppm or even ± 0.002 ppm and vary little with small temperature changes [32,33], assigned chemical shifts of these GAG chains can vary considerably. Differences of 0.1 ppm or even greater have been reported in the literature. Comparison of the chemical shifts for individual protons in the same heptasulfated hexasaccharide by two groups [19,20] reveals differences, in one instance of 0.52 ppm. Similarly, disparities of ~ 0.1 ppm for both H-2 and H-5 of the internal GlcA residue of GAG structures [11,12,16] were observed. While some of these differences in chemical shifts are possibly misassignments or mistakes, as pointed out [16,20], others could be due to chemical shift variations. One prominent variation in chemical shift value of the above-mentioned heptasulfated hexasaccharide is of the H-4 of ΔUA (0.1 ppm) which from its low-field resonance is unlikely to have been wrongly assigned. In the present study of the same compound (F3-15) this was observed even further downfield than previously (6.243 ppm) [19,20]. Variation in analysis conditions, including temperature

and pH, should clearly be controlled during NMR experiments of GAG chains; however, other factors such as possible interaction between the strongly ionic chains may be influenced by concentration or by the nature and content of salt. In the present study the salt content of samples was minimised as much as possible. It was noted that when the concentration of sodium salt was increased in a sample of a trisulfated heparin disaccharide all signals shifted downfield together by the same degree (data not shown).

The analysis of fragments by LSI-MS is an essential adjunct to NMR spectroscopy particularly for establishing composition and sulfate content. Sequence ions were not obtained in the present study despite reports of such ions being produced when large amounts of GAG oligosaccharides (20 μg) are used in combination with a triethanolamine matrix [13,15]. Any potential sequence ions present were of low intensity and hence unhelpful for interpretation of spectra from unknown oligosaccharides. In the present study, variation in chemical shifts of protons in the unsaturated non-reducing uronic acid residue led us to confirm sulfate content in this monosaccharide. Irrespective of the size of the GAG fragment, the TLC pattern and LSI-MS analysis of the DHPE derivatives of products from oxymercuration cleavage of the ΔUA residue were highly sensitive in establishing its sulfate substitution.

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