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Carbohydrate Research 342 (2007) 835–842
Carbohydrate RESEARCH

Characterization of di- and monosulfated, unsaturated heparin disaccharides with terminal N-sulfated 1,6-anhydro-β-D-glucosamine or N-sulfated 1,6-anhydro-β-D-mannosamine residues

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Received 20 June 2006; received in revised form 5 December 2006; accepted 13 December 2006
Available online 21 December 2006

Abstract—Modified heparin disaccharides were obtained by the alkaline treatment of a solution containing the disulfated heparin disaccharide ΔHexA-α-(1→4)-D-GlcNSO$_3$,6SO$_3$. Their structures were characterized by one- and two-dimensional NMR spectroscopy: ΔHexA-α-(1→4)-1,6-anhydro-GlcNSO$_3$, ΔHexA-α-(1→4)-1,6-anhydro-ManNSO$_3$ and ΔHexA-α-(1→4)-ManNSO$_3$,6OSO$_3$. NMR spectroscopy, in combination with HPLC, provided the composition of the mixture. Characteristic NMR signals of the disaccharides were identified, even at low levels, in a high field of ^{1}H–^{13}C correlation NMR spectra (HSQC) of a low molecular weight heparin (LMWH) obtained by β-elimination (alkaline hydrolysis) of heparin benzyl ester, providing a more complete structural profile of this class of compounds.

Keywords: Low molecular weight heparin (LMWH); C-2 Epimerization; 1,6-Anhydro ring; Heparin; Disaccharides; NMR spectroscopy

1. Introduction

Heparin is defined as a family of polysaccharide species, whose chains are made up of alternating, 1→4 linked and variously sulfated residues of a uronic acid and D-glucosamine. Together with the major trisulfated disaccharide sequences L-IdoA2SO$_3$-α-(1→4)-α-D-GlcNSO$_3$,6SO$_3$, undersulfated sequences containing non-sulfated β-D-glucuronic acid (GlcA) and α-L-iduronic acid (IdoA), and mixed sulfated and non-sulfated sequences such as D-GlcA-β-(1→4)-α-D-GlcNSO$_3$ or L-IdoA2SO$_3$-α-(1→4)-α-D-GlcNSO$_3$,6OH are present in both heparin and the corresponding low molecular weight heparins (LMWHs). In addition, a minor sequence, a pentasaccharide containing a GlcA residue followed by a unique trisulfated GlcN residue (α-D-GlcNSO$_3$,3,6SO$_3$), is present in about one-third of the heparin chains. This pentasaccharide corresponds to the active site for antithrombin (AT) and is responsible for the widely exploited anticoagulant and antithrombotic properties of heparin.[1] Besides the antithrombotic and anticoagulant activities,[2] a wide range of other biological activities of heparin such as cell proliferation, differentiation and migration,[3] and regulation of angiogenesis,[4] result from its ability to interact with fibroblast growth factors, cytokines and with other proteins causing their activation, deactivation or stabilization.[3]

Abbreviations: 2D, bidimensional; DQF-COSY, double quantum filtered correlation spectroscopy; TOCSY, total correlation spectroscopy; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple bond coherence; ROESY, rotating frame overhouser effect spectroscopy; TPPI, time proportional phase incrementation; GlcNSO$_3$, A$_{NS}$, A, 2-deoxy-2-sulfoamino-D-glucose; IdoA, iduronic acid; ΔU, ΔHexA, 4-deoxy-α-L-threo-hex-4-enopyranosil uronic acid; ManNSO$_3$, M$_{NS}$, M, 2-deoxy-2-sulfamino-D-mannoso; ΔUA-GlcNS,6S, 2-deoxy-4-*O*-(4-deoxy-α-L-threo-hex-4-enopyranosuil uronic acid)-6-*O*-sulfo-2-sulfamino-D-glucose; ΔIIs^{epi}, ΔUA-ManNSO$_3$

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doi:10.1016/j.carres.2006.12.010

LMWHs are obtained from heparin by enzymatic or chemical depolymerization. The structure of LMWHs reflects the microheterogeneity of the corresponding parent heparins.[1,5] It is widely accepted that various LMWHs exhibit specific molecular and structural attributes, which are peculiar to the type of manufacturing process.[6] For example, the LMWH prepared by the benzylation of unfractionated heparin, followed by alkaline hydrolysis of benzyl ester, has a complex structure and its chains are characterized by a double bond at their non-reducing ends.[7]

Together with the major reducing N-sulfated glucosamine residues, two unique bicyclic structures, namely 1,6-anhydro glucose and mannose, can be present at its reducing ends.[6] Typically only 15–25% of the chains exhibit 1,6-anhydro ring sugars at their reducing end, that is only 1.4–2.3% in weight of the whole LMWH.[8]

These small attributes, or fingerprints, of LMWHs are peculiar to the alkaline hydrolysis of benzyl ester of heparin, and other side reactions occur, which are known to affect heparin, when it is submitted to alkaline treatment. Among these reactions, the inversion of configuration of the α-L-iduronic-2-sulfate-acid ($IdoA2SO_3$) into α-L-galacturonic acid through the formation and opening of a C-2/C-3 epoxide was described.[9] The base-catalyzed C-2 epimerization of reducing glucosamine *N*,6-disulfate ($GlcNSO_3,6SO_3$), when it is in hemiacetalic form, with a partial conversion to the diastereoisomer 2-deoxy-6-*O*-sulfo-2-sulfamino-D-mannose, could also expected to occur according to previous disclosures on the prevalent unsaturated heparin disaccharide,[10] and on *N*-acetylglucosamine.[11] Possible formation of a bicyclic acetal between positions C-1 and C-6 of reducing glucosamine *N*,6-disulfate ($GlcNSO_3,6SO_3$), or mannosamine *N*,6-disulfate, can also be predicted.[8]

A complete resolution of signals in high field 2D NMR spectra of preparations of the LMWH obtained by β-elimination showed signals not associated with intrinsic heparin structures and assignment of some of these signals could not be directly performed because of a severe overlap.[12]

As a contribution to identification of process-related structures in LMWHs obtained by β-elimination, in the present work, a disaccharide mixture obtained by the alkaline treatment of a solution containing the heparin disulfated disaccharide ΔHexA-α-(1→4)-$GlcNSO_3,6SO_3$ (ΔII-S) was fully characterized by NMR spectroscopy. This disulfated disaccharide has been chosen for this study because its derivatives can be characterized more easily than the derivatives of the major trisulfated disaccharide. It can also be easily obtained as a reference compound avoiding side reactions such as the possible rearrangement of 4,5-unsaturated-2-sulfated uronic acid, as a consequence of the nucleophilic removal of the sulfate group from C-2, when the trisulfated disaccharide is subjected to the alkaline treatment.[9]

Together with the unmodified parent disaccharide and a disaccharide, corresponding to the ΔHexA-α-(1→4)-$ManNSO_3,6SO_3$ previously described,[10] two novel disaccharides with a reducing 1,6-anhydro-glucosamine and 1,6-anhydro-mannosamine were identified. The qualitative and quantitative assessment of the composition of the mixture of the four disaccharides has been obtained by both NMR signal integration and HPLC analysis. Most of the representative signals of the identified reducing residues have also been detected in the high field spectrum of a LMWH prepared by alkaline hydrolysis of heparin benzyl ester.

2. Results and discussion

2.1. NMR characterization

The proton spectrum of the sample obtained after dissolution in deuterium oxide (not shown) shows line broadening caused by the complexation of paramagnetic ions with the negatively charged groups. The addition of deuterated ethylene-diamine tetra-acetic acid (EDTA) provides a better resolved spectrum (Fig. 1).[13] The proton spectrum shows three groups of signals: the first one between 5.8 and 5.95 ppm is compatible with H-4 of α,β-unsaturated uronate residues (ΔU), the other two groups of signals at 5.4–5.65 and 5.1–5.3 ppm, respectively, agree with anomeric signals (Fig. 1).[14] The assignment of the proton and carbon signal patterns of each constituent monosaccharide was made through 2D DQF-COSY (data not shown), TOCSY (Fig. 2) and HSQC (Fig. 3) experiments and the relevant ^{1}H and ^{13}C chemical shifts values are summarized in Table 1. The H-4 signals of ΔU residues between 5.8 and 5.95 ppm correlate with the anomeric signals between 5.1 and 5.3 ppm. The position of the corresponding H-2/C-2 signals for all disaccharides (3.84–3.90/72–73 ppm) indicates the absence of the sulfate group in this position.[14] The signal pattern of the residue having anomeric proton at 5.47 ppm agrees with the presence of $GlcNSO_3,6SO_3$ (Fig. 2).[12] The anomeric signal at 5.42 ppm correlates with H-2 and H-3 at 3.65 and 4.11 ppm, respectively, in agreement with an α-N-sulfated mannosamine residue. The configuration of this hexosamine residue is also confirmed by the $^{3}J_{H\text{-}1,H\text{-}2}$ and $^{3}J_{H\text{-}2,H\text{-}3}$ values of 1.5 and 4.5 Hz, respectively, and is compatible with the equatorial position of H-1 and H-2, typical of the *manno* configuration.[10]

The conformation of the unsaturated uronate residues is normally influenced by 2-O-sulfation. The measured values of $^{3}J_{H\text{-}1,H\text{-}2}$, $^{3}J_{H\text{-}3,H\text{-}4}$ of 5.0–5.6 and 3.8–3.9 Hz, respectively, are consistent with a preferred $^{2}H_{1}$ half-chair conformation for the ΔU residue of all the disaccharides.[14] In contrast, the structural assignment to each of the two residues having the anomeric signal at 5.61

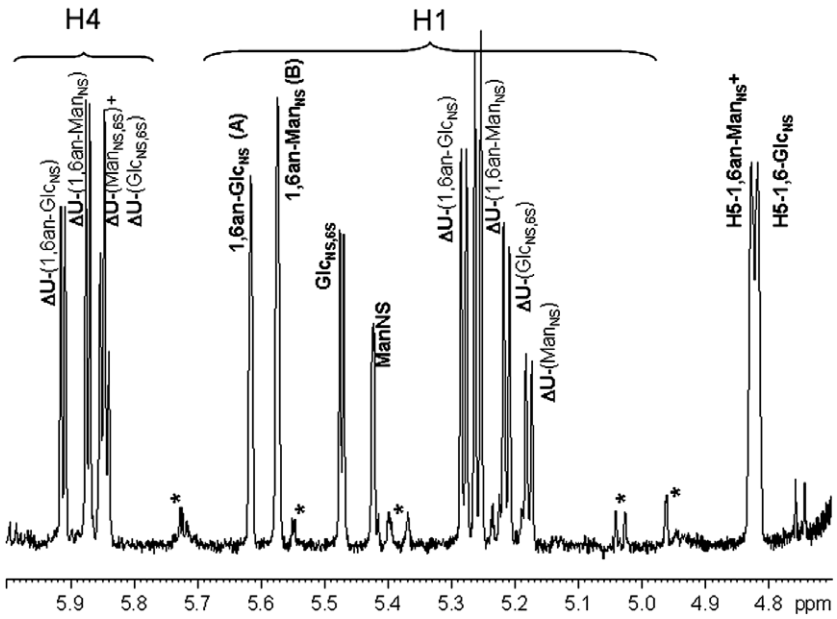

Figure 1. Anomeric region of the 500 MHz ^{1}H NMR spectrum of the disaccharide mixture. Asterisk indicates the minor unassigned structures.

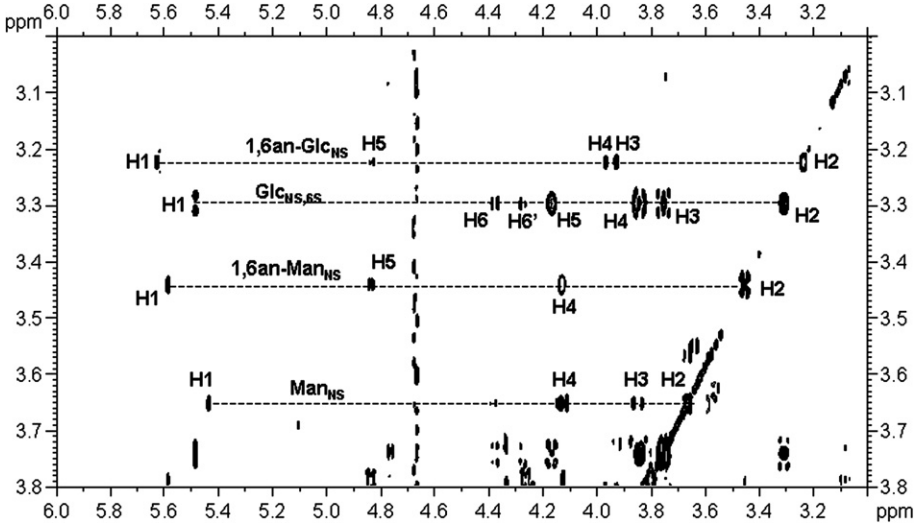

Figure 2. A portion of 2D-TOCSY spectrum. Signal patterns of the reducing hexosamine residues are shown.

(**A**) and 5.57 (**B**), respectively, is not straightforward. The chemical shift values of the anomeric signal of these reducing residues do not correspond to the typical values of the reducing anomeric signals. The proton shifts can be attributed to an α-linked *N*-sulfamino-pyranose residue, even though the corresponding carbon shifts are typical of β configuration, indicating the presence of an unusual heparin residue. The H-6/C-6 chemical shift values (3.8–4.2/68.0 ppm) suggest a possible substitution at this position. The ^{1}H–^{13}C long range correlation spectrum (HMBC, Fig. 4) shows an intra-residue long range correlation between the H-6 of residue **B** and the corresponding C-1 atom and indicates the presence of 1,6-anhydro structures. This correlation was observed also for the other amino-pyranose residue **A** (data not shown). This evidence also explains the unusual ^{1}H and ^{13}C chemical shifts found for the anomeric signals and the large difference in the chemical shifts of the two H6a and H6b protons due to a strong diastereotopic effect. Moreover, the 2D-ROESY spectrum (data not shown) indicates a dipolar interaction between H-4 and H-6 of 1,6-anhydro residues, which indicates that 1→6 link is located above the pyranose ring.

Small $^{3}J_{\mathrm{H\text{-}1,H\text{-}2}}$ coupling constant values (<2 Hz) were found for both reducing residues **A** and **B**. The different values of $^{3}J_{\mathrm{H\text{-}2,H\text{-}3}}$ (1.0 and 4.4 Hz for residues **A** and **B**, respectively) indicate a different geometry of dihedral angle between H-2 and H-3 in the two structures,

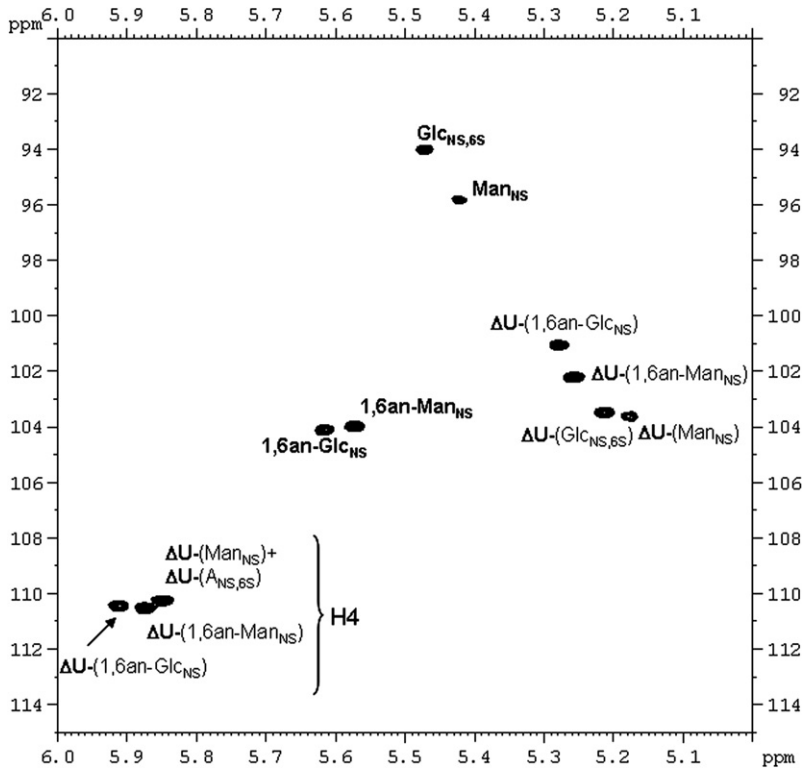

Figure 3. Anomeric region of the ^{1}H–^{13}C-HSQC spectrum.

Table 1a. ^{1}H chemical shifts (ppm) of the constituent monosaccharides of the four disaccharides

Residue	^{1}H chemical shifts (ppm)						
	H-1	H-2	H-3	H-4	H-5	H-6	H-6′
ΔU	5.213	3.856	4.224	5.856			
A_{NS}	5.471	3.294	3.738	3.837	4.156	4.359	4.270
ΔU	5.178	3.838	4.237	5.849			
M_{NS}	5.420	3.654	4.112	3.840	4.126	4.373	4.270
ΔU	5.258	3.850	4.238	5.879			
1,6an-M_{NS}	5.571	3.438	4.114	4.109	4.820	4.247	3.787
ΔU	5.280	3.904	4.215	5.921			
1,6an-A_{NS}	5.614	3.219	3.912	3.953	4.820	4.234	3.806

Chemical shift values were measured downfield from trimethylsilyl propionate sodium salt (TSP) as the standard at 40 °C.

Table 1b. ^{13}C chemical shifts (ppm) of the constituent monosaccharides of the four disaccharides

Residue	^{13}C chemical shifts (ppm)					
	C-1	C-2	C-3	C-4	C-5	C-6
ΔU	103.47	72.86–73.20	69.19–69.72	110.23		
$A_{NS,6S}$	94.00	60.59	72.25	81.98	70.98	69.52
ΔU	103.47	72.86–73.20	69.19–69.72	110.23		
M	95.80	60.20	70.08	79.37	71.18–71.65	69.52
ΔU	102.18	72.86–73.20	69.19–69.72	110.52		
1,6an-M_{NS}	103.95	55.07	71.19	80.59	76.16	67.41
ΔU	101.03	72.49	69.19–69.72	76.16		
1,6an-A_{NS}	104.08	58.40	72.98	67.41	76.16	67.93

Chemical shift values were measured downfield from trimethylsilyl propionate sodium salt (TSP) as the standard at 40 °C.

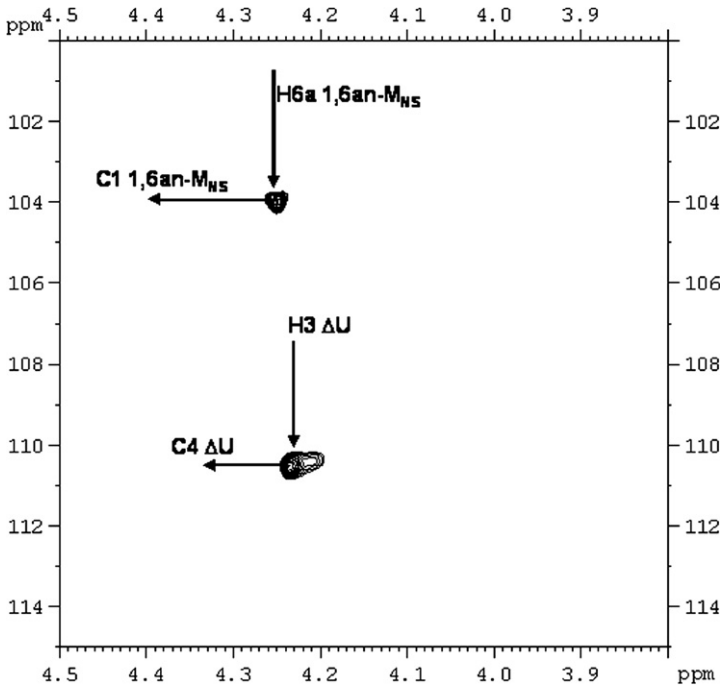

Figure 4. A portion of the ^{1}H–^{13}C long range correlation spectrum (HMBC) of the mixture. The correlation between H-6 and C-1 of the reducing 1,6-anhydro-*N*-sulfate-mannosamine and between H-3 and C-4 of the unsaturated uronic acid are shown.

compatible with a different configuration of C-2. Moreover, $^{3}J_{H\text{-}3,H\text{-}4}$ couplings of reducing residue of both **A** and **B** disaccharides are small (1–2 Hz), as a consequence they are not compatible with the trans-axial position of H-3 and H-4 of the typical α-D-hexosamine in $^{4}C_{1}$ chair conformation. These data, together with chemical shift values, are consistent with the presence, at the reducing end, of a 2-amino-1,6-anhydro-2-deoxy-β-D-glucopyranose for disaccharide **A** and a 2-amino-1,6-anhydro-2-deoxy-β-D-mannopyranose for disaccharide **B**, both in the $^{1}C_{4}$ conformation, in agreement with the data previously reported for monomeric models.[15]

Since the intensities of dipolar interactions of ROESY experiment are related to the corresponding proton distances, the stronger H-1/H-2 dipolar interaction measured for residue **A** (about ~3.2%), in comparison

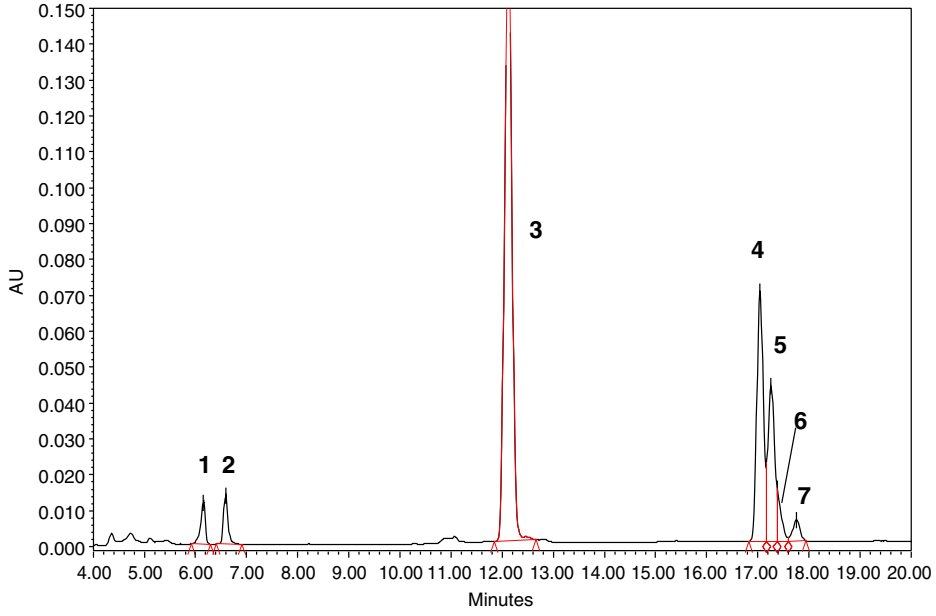

Figure 5. SAX-HPLC profile of the mixture of disaccharides, detector UV set at λ_{max} 234 nm. Peaks 1 and 2: unknown disaccharides, peak 3: ΔU-GlcNSO$_3$/ManNSO$_3$, 1,6-anhydro, peaks 4 and 6: ΔU-GlcNSO$_3$,6SO$_3$ (α and β anomers), peaks 5 and 7: ΔU-ManNSO$_3$,6SO$_3$ (α and β anomers).

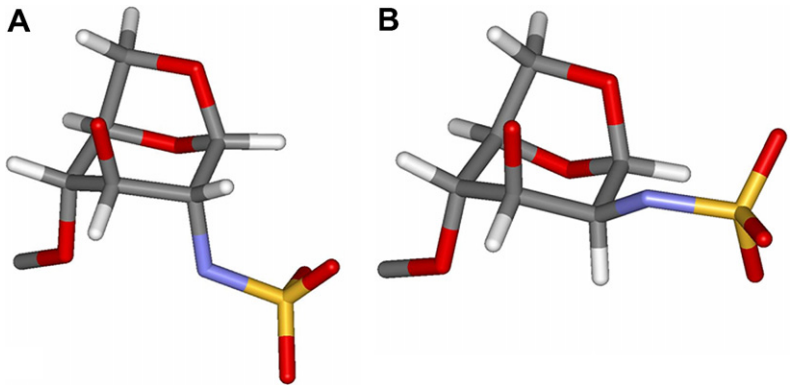

Figure 6. Proposed structure for the 1,6-anhydro *N*-sulfate-glucosamine (**A**) and 1,6-anhydro *N*-sulfate-mannosamine (**B**). The models were obtained using the MACROMODEL program (v. 7.1). Library carbohydrate structures were suitably modified and their energies were minimized in vacuo using the BATCHMIN program. It was used the force field AMBER*.[21]

Table 2. Recovery of the disaccharides from the mixture and quantitation by NMR and SAX-HPLC analyses

Disaccharide	NMR (%)	SAX-HPLC (%)
ΔU–1,6an-A_{NS}	27	58
ΔU–1,6an-M_{NS}	33	
ΔU–$A_{NS,6S}$	24	25
ΔU–$M_{NS,6S}$	16	15
Unknown disaccharides	—	5

with residue **B** (about ~2.2%), indicates a shorter H-1/H-2 distance of residue **A**, in agreement with the proposed structures (Fig. 6). The disaccharide composition was determined by the integration of the anomeric proton signals (Table 2).

These residues characterized by 1,6-anhydro rings in both *manno* and *gluco* configuration together with reducing N,6-disulfated mannosamine, generated by alkaline treatment and not present in heparin, were identified in the LMWH obtained by β-elimination (Fig. 7). This advance in the NMR characterization will be of help to improve the understanding of the structure of heparin and heparin-derived preparations

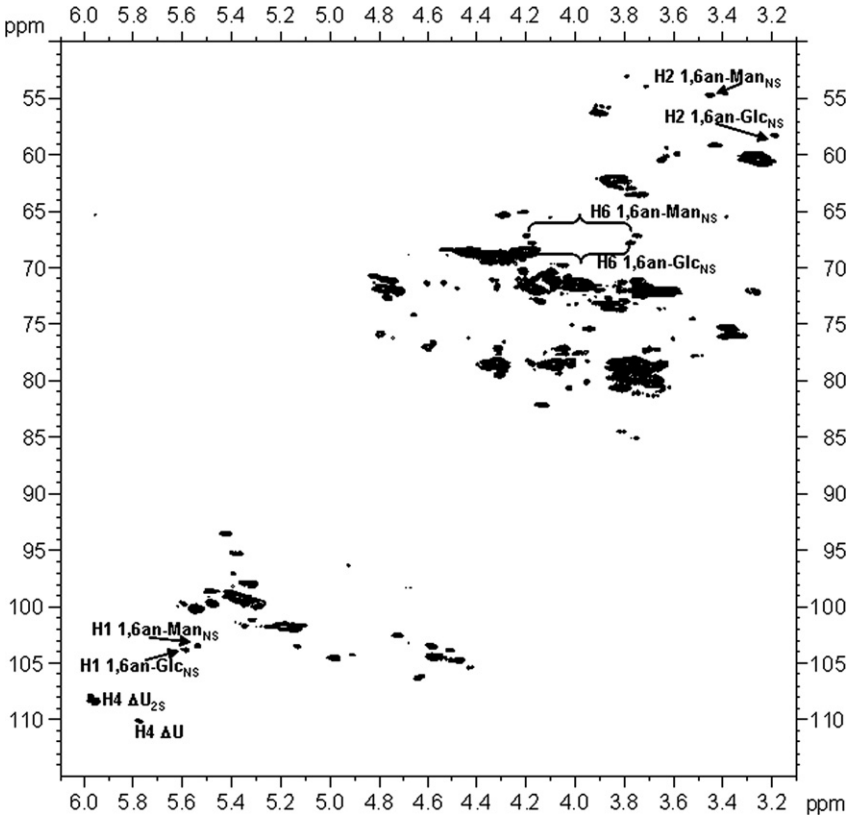

Figure 7. ^{1}H–^{13}C-HSQC spectrum of LMWH obtained by β-elimination carried out by the alkaline treatment of heparin benzyl ester. The signals corresponding to the reducing 1,6-anhydro residues and non-reducing uronate are shown.

as determined by different NMR methods described recently.[16,17]

2.2. Identification and quantification by HPLC

The chromatogram obtained by SAX-HPLC shows a group of four peaks with retention times between 17.0 and 18.0 min; they were assigned to the double signal of ΔU-GlcNSO$_3$,6SO$_3$ α and β anomers and to the double signal of ΔU-ManNSO$_3$,6SO$_3$ α and β anomers,[18] (Fig. 5). Peaks 4 and 6 were attributed to the unmodified profile of the disaccharide submitted to the alkaline treatment. The proportion and retention times of anomers α and β are in accordance with the separation elsewhere reported.[7]

Peak 3 at 12.1 min, the most represented, was assigned to the mixture of ΔU–1,6an-GlcNSO$_3$,6SO$_3$ and ΔU–1,6an-ManNSO$_3$,6SO$_3$ residues. Since the structures are very similar, the two species were coeluted. The confirmation of the assignment of the 1,6-anhydro structure corresponding to peak at 12.1 min was obtained from the chromatogram of the NaBH$_4$-reduced sample. The retention times of these 1,6-anhydro structures were not affected by the reduction carried out with sodium borohydride. In contrast, the retention times of the two species assigned to ΔU-GlcNSO$_3$,6SO$_3$ and ΔU-ManNSO$_3$,6SO$_3$ were modified by reduction reaction; they eluted after 15.4 and 16.0 min, respectively. Peaks 5 and 7, analogously with the 4 and 6 ones, represent the anomers α and β of mannosamine containing disaccharide ΔU-ManNS,6S (ΔIIs^{epi}).[7]

The disaccharide quantification was performed assuming that all disaccharides have the same molar absorbance. In this manner the quantity of each species can be estimated by its area percentage. Two unknown disaccharides were detected at a shorter retention time and at a very low concentration (Table 2). These data are consistent with the quantification performed by NMR.

3. Experimental

3.1. Preparation of the disaccharides

Ninety microlitres of a 2 mg/mL solution of disaccharide ΔII-S (GE-H1002, Grampian Enzymes, Scotland UK) in water was mixed with 10 μL of a 1 N solution of sodium hydroxide. The solution was incubated at 40 °C for 16 h, then cooled to room temperature and pH was adjusted to neutrality with about 10 μL of a 1 N solution of hydrochloric acid. In order to verify the effect of reduction on HPLC retention times of disaccharides, 10 μL of a 30 mg/mL solution of sodium borohydride was added to 50 μL of neutralized solution. Six hours after mixing, the reduced solution was ready for injection.

3.2. NMR spectroscopy

Due to the low amount of the sample (about 0.2 mg), carbon spectra were recorded at 600 MHz by using a Bruker Avance 600 spectrometer equipped with a TCI cryoprobe. Disaccharides were prepared from solutions obtained by dissolving 0.2 mg of sample in 0.6 mL of D_2O 99.99% (Isotec). To avoid signal broadening caused by the presence of paramagnetic ions, deuterated Na/EDTA (Aldrich) was added to the sample to remove these ions.[13] ^{1}H NMR spectra were recorded at 600 MHz at the temperature of 35 °C, with pre-saturation of the residual water signals and with recycle delay of 12 s. 2D DQF-COSY and TOCSY were carried out in the phase-sensitive mode using TPPI with 32 scans for each 384 FIDs, and a shifted square sine-bell function was applied before Fourier transformation. 2D HSQC spectra were obtained with sensitivity improvement and phase sensitive using Echo/Antiecho TPPI gradient selection.[19] For 2D ROESY experiments, 32 transients were collected for each free-induction decay and mixing time of 300 ms. Both HSQC and ROESY experiments (matrix 1024×320 points) were zero-filled to $2K \times 2K$ before Fourier transformation. HMBC spectra were acquired by using 128 scans per series in $1K \times 128W$ data points.[20] The spectra were optimized with a $^nJ_{C-H}$ of 6 Hz and $n = 2$–4. The experiments were zero-filled and multiplied with sine-bell prior to Fourier transformation.

3.3. HPLC analysis

The chromatography was performed by a Waters Alliance 2695 (Milford, MA, USA) equipped with a Photodiode Array Detector Waters PDA 2996. Ten microlitres of the sample was injected into a Waters Spherisorb S5 SAX column (250×4.6 mm) equipped with a guard column (10×4.6 mm). The flow rate was 1.4 mL/min and the detector was set to collect the UV spectra at 190–350 nm. The gradient system included two eluents: eluent A was 2.5 mM NaH_2PO_4, pH 2.9 and eluent B was 1 M $NaClO_4$ containing 2.5 mM NaH_2PO_4, pH 3.0.

The elution gradient was 0–40 min, linear gradient from 3% to 60% of eluent B; 40–60 min, linear gradient from 60% to 80% eluent B; 60–70 min, linear gradient from 80% to 3% eluent B; and 70–75 min, re-equilibration of the system to 3% eluent B.[18]

Acknowledgement

The authors thank Professor B. Casu for useful discussions.

References

1. Casu, B.; Lindahl, U. *Adv. Carbohydr. Chem. Biochem.* **2001**, *57*, 159–206.
2. Marcum, J. A.; Rosenberg, R. D. In *Heparin, Chemical and Biological Properties, Clinical Application*; Lane, D. A., Lindahl, U., Eds.; Edward Arnold: London, 1989; pp 275–294.
3. Casu, B. Structure and active domains of heparin. In *Chemistry and Biology of Heparin and Heparan Sufate*; Garg, H. G., Linhardt, R. J., Hales, C. A., Eds.; Elsevier Ltd, 2005; pp 1–28.
4. Folkman, J.; Ingber, D. E. In *Heparin, Chemical and Biological Properties, Clinical Application*; Lane, D. A., Lindahl, U., Eds.; Edward Arnold: London, 1989; pp 317–333.
5. Casu, B.; Torri, G. *Sem. Thromb. Hemost.* **1999**, *25*, 17–26.
6. Fareed, J.; Hoppensteadt, D.; Schultz, Q.; Ma, M. F.; Kujawski, M. F.; Neville, B.; Messmore, H. *Curr. Pharm. Des.* **2004**, *10*, 983–999.
7. Mourier, P. A. J.; Viskov, C. *Anal. Biochem.* **2004**, *332*, 299–313.
8. Bianchini, P.; Mascellani, G. *Pharmaeuropa Notes* **2005**, *1*, 1–3.
9. Rey, N. R.; Perlin, A. S. *Carbhoydr. Res.* **1990**, *200*, 437–447.
10. Yamada, S.; Watanabe, M.; Sugahara, K. *Carbohydr. Res.* **1998**, *309*, 261–268.
11. Toida, T. E.; Vlahov, I. R.; Smith, A. E.; Hileman, R. E.; Linhardt, R. J. *J. Carbohydr. Chem.* **1996**, *15*, 351–360.
12. Yates, E. A.; Santini, F.; Guerrini, M.; Naggi, A.; Torri, G.; Casu, B. *Carbohydr. Res.* **1996**, *294*, 15–27.
13. Neville, G. A.; Mori, F.; Holme, K. R.; Perlin, A. S. *J. Pharm. Sci.* **1989**, *78*, 101–104.
14. Ragazzi, M.; Ferro, D. R.; Provasoli, A.; Pumilia, P.; Tassinari, A.; Torri, G.; Guerrini, M.; Casu, B.; Nader, H. B.; Dietrich, C. P. *Carbohydr. Chem.* **1993**, *12*, 523–525.
15. Černy, I.; Buděšinsky, M.; Trnka, T.; Černy, M. *Carbohydr. Res.* **1984**, *130*, 115–124.
16. Guerrini, M.; Raman, R.; Venkataraman, G.; Torri, G.; Sasisekharan, R.; Casu, B. *Glycobiology* **2002**, *12*, 713–719.
17. Guerrini, M.; Naggi, A.; Guglieri, S.; Santarsiero, R.; Torri, G. *Anal. Biochem.* **2005**, *337*, 35–47.
18. Mourier, P.; Viskov, C. U.S. Patent 2005/0119477 A1; *Chem. Abstr.* **142**, 89363.
19. Schleucher, J.; Schwendinger, M.; Sattler, M.; Schmidt, P.; Schedletzky, O.; Glaser, S. J.; Sorenson, O. W.; Griesinger, C. *J. Biomol. NMR* **1994**, *4*, 301–306.
20. Summers, M. F.; Marzilli, L. G.; Bax, A. J. *J. Am. Chem. Soc.* **1986**, *108*, 4285–4294.
21. Homans, S. W. *Biochemistry* **1990**, *29*, 9110–9118.