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# Heparin sodium compliance to the new proposed USP monograph: Elucidation of a minor structural modification responsible for a process dependent 2.10 ppm NMR signal

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ABSTRACT

Heparin is a highly sulfated hetero polysaccharide mixture found and extracted from mammalian tissues. It has been widely used as an anticoagulant drug during the past decades.

In the new proposed USP heparin monograph, the $^{1}$H NMR acceptance criteria to prevent contamination by over sulfated chondroitin sulfate (OSCS), or other persulfated glycosaminoglycans, specifies that no unidentified signals greater than 4% of the mean of signal height of 1 and 2 [1] should be present in the following ranges: 0.10–2.00, 2.10–3.20, and 5.70–8.00 ppm. However, those criteria do not take into account the impact of potential structural modifications generated by the heparin manufacturing processes. In fact, starting from pig mucosa, heparin purification involves oxidizing reagents such as sodium peroxide, potassium permanganate and peracetic acid. In the present work, we demonstrate that potassium permanganate treated heparins show a small but characteristic extra signal at 2.10 ppm. Controlled heparinase I depolymerisation is used to target and excise the oligosaccharide responsible for this extra signal from the polysaccharide backbone. By using orthogonal chromatographic techniques, the fingerprint oligosaccharide was isolated and its structure elucidated. Without the identification of this structural moiety, such purified heparins may have been considered as non-compliant drug substance and not suitable for pharmaceutical use.



## 1. Introduction

Heparin is a well known anticoagulant drug and has been widely used for several decades in prevention and treatment of thromboembolic diseases. Heparin is extracted from various mammalian tissues and more specifically from pig intestinal mucosa. This complex hetero polysaccharide mixture is also used as starting material for the synthesis of low molecular weight heparins (LMWH) such as Enoxaparin. At the end of 2007, heparin sodium contaminated by oversulfated chondroitin sulfate [2] was administered to patients with several fatal issues triggering tremendous wondering about the level of characterization of those complex molecules. All around the world, health authorities institutions and companies have been working together to define the best characterization methods to insure the highest heparin quality standard for patient's safety. Subsequently, a new USP heparin monograph has been proposed in a very challenging timeframe with new, highly stringent, analytical methods to avoid any contamination issue [1]. Since October 2009, this new USP monograph is applicable for all US heparin sodium market. More specifically, in the $^{1}$H NMR acceptance criteria for heparin, no unidentified signals greater than 4% of the mean of signal height of 1 (5.42 ppm; H1 of GlcNAc/GlcNS,6S) and 2 (5.21 ppm; H1 of IdoA 2S) [1] are present in the following ranges: 0.10–2.00, 2.10–3.20, and 5.70–8.00 ppm. No signals greater than 200% signal height of the mean of the signal height of 1 and 2 [1] are present in the 3.35–4.55 ppm for porcine heparin. As a consequence of those new analytical criteria, some pure heparins could become not compliant to the USP proposed monograph. From nine distinct heparin sources available on the market place, seven are showing an extra signal at 2.10 ppm in their $^{1}$H NMR spectra. For all sources possessing this extra signal, we found that potassium permanganate is used in their manufacturing process (examples: heparin sources EDE and EV). *A contrario*, heparin sodium manufactured without potassium permanganate (heparin sources ES and EDUS) does not exhibit this extra signal. For the heparin source ES, the manufacturing process uses both peracetic acid and hydrogen peroxide whereas EDUS involves only hydrogen peroxide as oxidative reagent. Therefore, it is suggested that a particular side reaction was occurring in the presence of potassium permanganate. 2D NMR $^{1}$H–$^{13}$C spectra show that this signal is due to an acetyl group located on the

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heparin backbone. Heparin batches that display the most intense 2.10 ppm extra signal were selected and depolymerised with heparinase I. The same enzymatic digestion was done on "ES" heparin sodium giving no NMR signal at 2.10 ppm, used here as a negative reference. Fractionation by size exclusion chromatography (SEC) of the above depolymerised heparins was performed. Oligosaccharide fractions of homogeneous size were simultaneously analysed by chromatography and NMR in order to point out which one exhibits the most intense extra signal. After several purification steps of the targeted oligosaccharide mixture, a pure tetrasaccharide containing the fingerprint structural alteration was obtained and the structure determination was achieved using 1D and 2D $^1$H NMR spectroscopy.

## 2. Experimental

### 2.1. Materials and chemicals

Two heparin batches from distinct suppliers displaying the 2.10 ppm extra signal were selected: batch EDE31432 and batch EV3031. Heparin batches ES1037-2555 and ES1037-2502 from a single supplier were used as negative reference.

All enzyme lyases from *Flavobacterium heparinum* (heparinase I (EC 4.2.2.7), heparinase II (no EC number) and heparinase III (EC 4.2.2.8)) were obtained from Grampian Enzymes (Aberdeen). All other reagents and chemicals were of the highest quality available. Water was purified with a Millipore Milli-Q purification system.

### 2.2. Enzymatic digestion and size exclusion chromatography

Exhaustive digestion of heparin or oligosaccharide (0.1 mg) was performed at room temperature for 48 h with a mixture of 2.5 mIU of heparinase I, 2.5 mIU of heparinase II and 2.5 mIU of heparinase III in a total volume of 30 μl of 100 mM sodium acetate buffer (pH 7.0) containing 2 mM $Ca(OAc)_2$ and 0.5 mg/ml BSA.

Partial digestion of heparin with heparinase I was performed in $NaH_2PO_4$ 5 mM (pH 7.0), containing 200 mM NaCl and 0.5 mg/ml BSA at 16 °C for 1–10 days. Heparin digests were directly injected on a size exclusion chromatography (SEC) column packed with polyacrylamide gel (Bio Gel P-30, Fine, Bio Rad), circulated with $NaClO_4$ 0.2 M using UV detection at 232 nm. For analytical scale heparin digests, 4–6 mg of heparin sodium sample was mixed with 50 mIU of heparinase I in 1 ml of the pH 7 buffer. SEC Bio Gel P-30 column (100 cm × 1 cm; I.D.) was circulated with $NaClO_4$ 0.2 M at 7 ml/h.

For preparative scale heparin digests, 200 mg of heparin were depolymerized with 0.5 IU of heparinase I in 2 ml of pH 7.0 buffer. After 5–10 days, the digest was injected on a 100 cm × 2.6 cm (I.D.) SEC Bio Gel P-30 column circulated with $NaClO_4$ 0.2 M (flow rate 36 ml/h). Our largest heparinase I depolymerisation was performed on 1 g of heparin batch EDE31432. At such scale, kinetic issues appeared on the digestion rate and 5 IU of heparinase I have been finally added to enable the desired depolymerisation degree. Total digest mixture (20 ml) was injected in SEC on a system with two 100 cm × 5 cm (I.D.) columns connected in series and circulated with $NaClO_4$ 0.2 M (flow rate: 85 ml/h). Selected fractions were first concentrated, desalted on Sephadex G10 columns (100 cm × 7 cm) and then lyophilized.

### 2.3. Oligosaccharide isolation and purification

After heparinase exhaustive digestion, CTA-SAX (cetyl trimethyl ammonium strong anion exchange) chromatograms of heparin sodium digests, that display the 2.10 ppm NMR extra signal, exhibit a common unknown oligosaccharide. CTA-SAX chromatography was performed as previously described [3].

SEC chromatograms of heparinase I heparin sodium digests, displaying 2.10 ppm signal, possess a small extra fraction compatible with odd numbered oligosaccharides (pentasaccharides). The specific unknown oligosaccharide was the major component of the SEC "pentasaccharide" fraction. This fraction was first desalted, lyophilized and then dissolved in 2 ml of 100 mM sodium acetate buffer (pH 7.0) containing 2 mM $Ca(OAc)_2$ and 0.5 mg/ml BSA. The oligosaccharide mixture was then depolymerised with 0.1 IU of heparinase II (no EC number). After 48 h, the digest was injected on a Carbopack AS11 column (25 cm × 2.1 cm) (Dionex) at a flow rate of 20 ml/min. Bound oligosaccharides were eluted with a linear gradient of $NaClO_4$ (up to 0.6 M). Double UV detection was monitored at 232 nm and at 202–230 nm. N-acetylated oligosaccharide selective signal (202–230 nm) is the result of the subtraction of the 202 nm wavelength UV signal from the 230 nm reference signal, as previously described [3]. Digested heparin disaccharides were first eluted and then, the targeted heparinase resistant oligosaccharide was eluted in the last part of the gradient. The purified oligosaccharide was desalted on Sephadex G10 columns (30 cm × 5 cm) and then lyophilized.

### 2.4. LC/MS conditions

Selected chromatographic conditions were developed starting from those described by Thanawiroon et al. [4]. HPLC separations were performed on a 5-μm Hypersil Elite $C_{18}$ column (2.1 mm × 250 mm, from Thermo Finnigan). Mobile phase A was water/acetonitrile (95:5) and mobile phase B was water/acetonitrile (30:70). Octylamine (20 mM) and ammonium acetate (45 mM) were added to both A and B. The mobile phase pH was adjusted to 6.0 with acetic acid. A linear gradient (from 40 to 100% of B in 40 min) at a flow rate of 0.22 ml/min was used for elution. Column temperature was adjusted to 40 °C. UV detection was set at 232 nm. 5 μl of exhaustively depolymerised EDE31432 heparin were injected.

### 2.5. Mass spectrometry conditions

ESI mass spectra were obtained using a Waters Q-Tof2 mass spectrometer. The electrospray interface was set in negative ion mode with a capillary voltage of 3000 V and a cone voltage of 37 V. The source and the desolvation temperatures were respectively 120 and 350 °C. Nitrogen was used as desolvation (450 l/min) and cone gas (25 l/min). The mass range used was 200–2000 Da (scan rate 1 s.)

### 2.6. NMR spectroscopy

All the NMR experiments were run on a 600 MHz Bruker Avance instrument (Bruker Biospin) equipped with a 5 mm inverse cryoprobe. One milligram of the purified oligosaccharide was dissolved in a 3 mm tube containing 150 μl of 99.9% $D_2O$. The temperature was set at 30 °C and pre-saturation of the residual water signals was used to improve signal to noise ratio. Spectral parameters for $^1$H spectra include 32 transients, approximately 30° pulse width, acquisition time of at least one second, time between transients of two seconds and a spectral window of 9600 Hz. Reference was set at 0.00 ppm for the TSP-d4 signal. 2D TOCSY was carried out in the phase-sensitive mode using TPPI with 32 scans for each 256 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 [5]. For 2D ROESY experiments, 32 transients were collected for each free-induction decay and mixing time of 250 ms. Both HSQC and ROESY experiments (matrix 1024 × 256 points) were zero-filled to 2 K × 1 K before Fourier transforma-

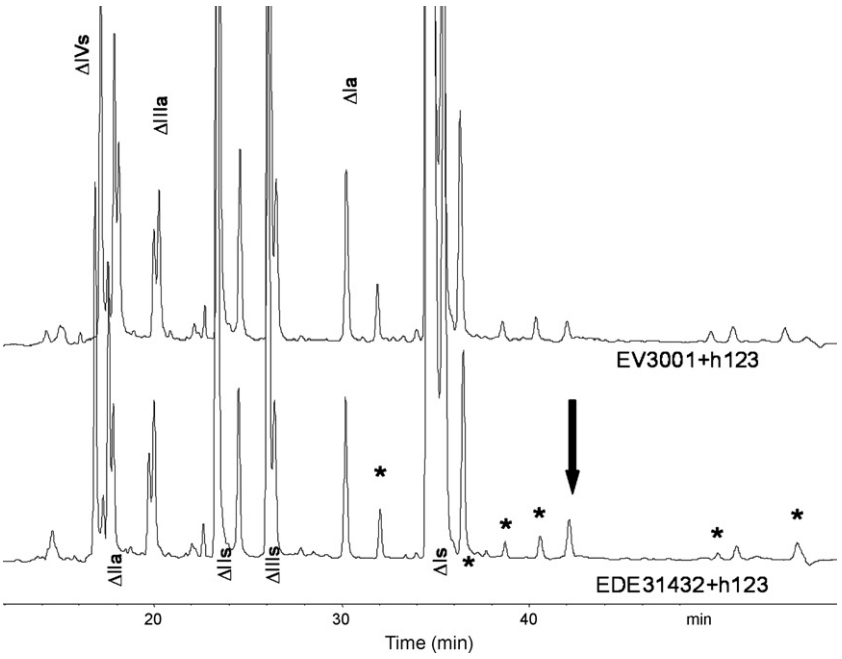


**Fig. 1.** Chromatograms by CTA-SAX of exhaustively digested heparins. Detection UV at 232 nm Peaks with * are 3-0 sulfated tetrasaccharide (ΔIs = ΔU2S-GlcNS,6S; ΔIa = ΔU2S-GlcNAc,6S; ΔIIs = ΔU-GlcNS,6S; ΔIIIs = ΔU2S-GlcNS; ΔIIIa = ΔU2S-GlcNAc; ΔIIa = ΔU-GlcNAc,6S; ΔIVs = ΔU-GlcNS).

tion. HMBC spectra were acquired by using 32 scans per series in 1 K × 512 data points [6]. The spectra were optimized with an $^{n}J_{C-H}$ of 10 Hz and $n$ = 2–3. The experiments were zero-filled and multiplied with sine-bell prior to Fourier transformation.

## 3. Results and discussion

### 3.1. Identification and purification of an oligosaccharide containing the 2.10 ppm extra signal

In the heparin purification process, potassium permanganate is mainly used as oxidizing agent in order to remove the glycoserine linkage sequence from the polysaccharide chain [7]. In preliminary experiments, heparins were digested with heparinase III and separated by SEC chromatography in order to investigate if degraded glycoserine sequences could be accountable for the extra NMR signal. After heparinase III digestion [8], oxidized glycoserine residues were found mainly in hexa and tetrasaccharide fractions but none of them show the 2.10 ppm NMR signal. In fact, this signal appeared in fractions of higher molecular mass (data not shown). At this point, it was clear that the structural moiety underlying behind 2.10 ppm was not due to the main oxidative action of potassium permanganate but implies side reactions on other regions of the polysaccharide backbone.

Therefore, exhaustive heparin digestion by mixture of heparinase I, II and III were conducted in order to try to identify unusual short oligosaccharides in the digest mixtures. Interestingly, it was observed that the digests from batch EDE31432 and batch EV3001 contained a specific oligosaccharide (arrowed in Fig. 1) which is absent from the digest of our negative control heparin (ES1037-2555). According to its retention time in CTA-SAX chromatography, this oligosaccharide likely contains 4 or 5 sulfates. Moreover, its retention time does not fit to any of the known heparinase resistant 3-0 sulfated tetrasaccharides (starry peaks in Fig. 1) [3].

After those first insights, the same heparin batches (EDE31432 and batch EV3001) as well as our negative control (ES1037-2555) were digested by heparinase I and then chromatographied by CTA-SAX. In Fig. 2, it appears that the same specific arrowed oligosaccharide, as in Fig. 1, is also present on the heparinase I digests. As a matter of fact, it became clear that $KMnO_4$ treated heparins (EDE31432 and batch EV3001) exhibit an unknown heparinase resistant oligosaccharide which is absent in the negative control batch digest. (it has to be noted that the collections of oligosaccharides common to all heparinase digested heparins were already identified in a previous work [3]).

SEC fractionation of the above depolymerised heparins was performed on 200 mg scale (Fig. 3). For EDE31432 digest, a small extra fraction pointed by an arrow appears on the chromatogram between tetrasaccharide and hexasaccharide fractions. *A contrario*, no extra fraction is present for ES1037-2555 in this chromatographic region. This extra fraction was collected and analysed by $^{1}$H NMR. The spectra show a strong increase of the 2.10 ppm signal height. The main component of this fraction is the same one than the unknown oligosaccharide pointed in the exhaustively digested heparins shown in Fig. 1. At this stage, the amount was not sufficient to allow further purification of the oligosaccharide bearing the targeted 2.10 ppm acetyl moiety. Therefore, heparinase I enzymatic digestion of heparin sodium batch EDE31432 was performed on 1 g scale exactly in the same manner than on 200 mg scale (Fig. 4). The SEC extra fraction was isolated and further depolymerized by heparinase II in order to facilitate the final purification, as described in the experimental section. The mixture was separated by preparative AS11 chromatography and the targeted oligosaccharide was collected in the last part of the gradient. The compound was desalted and then lyophilized to yield 1.9 mg of pure oligosaccharide. The purification sequence is shown in Fig. 5.

### 3.2. LC–MS characterization of the oligosaccharide

The mass spectrum of the isolated compound is shown in Fig. 6: the doubly charged parent ion $[M-2H]^{2-}$ at $m/z$ = 525 gives a molecular weight of 1052 Da.

### 3.3. NMR characterization of the targeted oligosaccharide

The methyl group signal, visible on proton spectrum at 2.10 ppm, is characteristic of the tetrasaccharide shown in Fig. 7. Thus, the signals integration on 1D proton spectrum indicates that

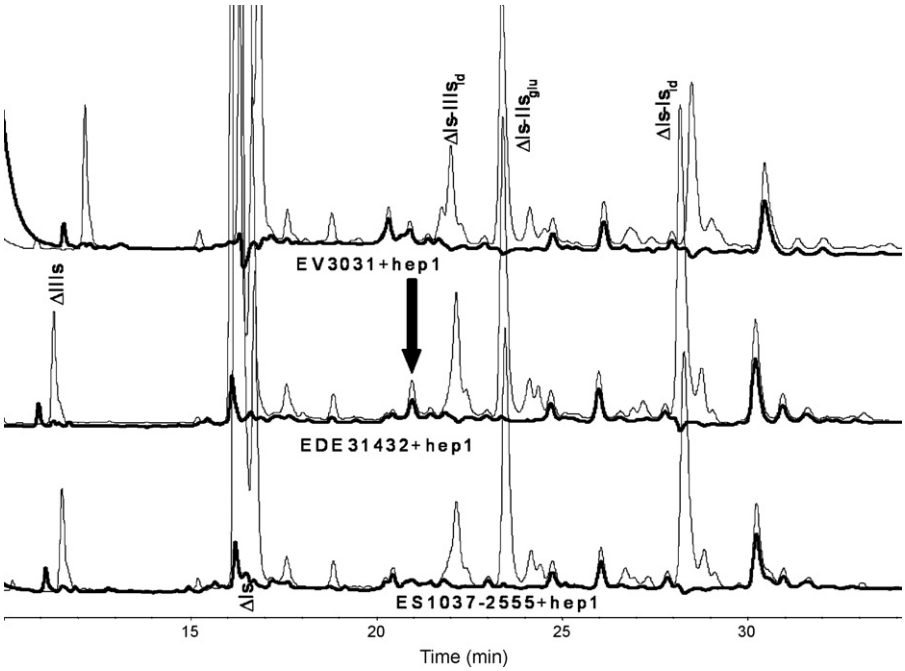


**Fig. 2.** Chromatograms by CTA-SAX of heparinase 1 digested heparin ((—) 232 m; (▬) 202–242 nm) (ΔIs-IIIs$_{id}$ = ΔU2S-GlcNS,6S-IdoA2S-GlcNS; ΔIs-IIs$_{glu}$ = ΔU2S-GlcNS, 6S-GlcA-GlcNS,6S; ΔIs-Is$_{id}$ = ΔU2S-GlcNS,6S-IdoA2S-GlcNS,6S).

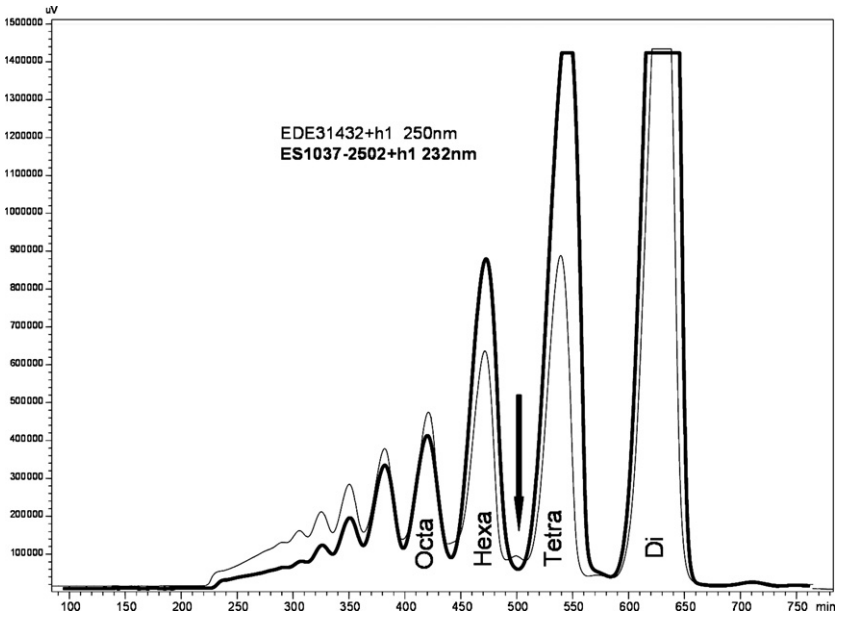


**Fig. 3.** SEC of heparinase 1 digest of heparin (detection UV 232 nm) (200 mg heparin + 0.5 IU heparinase 1).

**Table 1**
Proton and carbon chemical shifts (in ppm) of purified tetrasaccharide.

| | Tetrasaccharide residue | | | |
|---|---|---|---|---|
| | ΔGlcA 2S | GlcNS,6S | Ido Ac | Ox-GlcNAc,6S |
| 1 | 5.50 (99.9) | 5.37 (98.4) | 5.17 (104.0) | – (178.6) |
| 2 | 4.62 (77.2) | 3.28 (60.2) | 3.79 (71.5) | 4.39 (58.9) |
| 3 | 4.32 (65.6) | 3.65 (72.3) | 4.13 (71.2) | 4.22 (73.5) |
| 4 | 5.99 (108.7) | 3.84 (80.8) | 4.09 (77.9) | 3.88 (81.9) |
| 5 | –(147.2) | 3.98 (71.4) | 4.72 (71.6) | 4.14 (72.7) |
| 6 | –(171.8) | 4.21–4.36 (69.0) | –(177.4) | 4.16–4.27 (71.6) |
| CH3 | – | – | | 2.10 (24.7 and 176.7) |

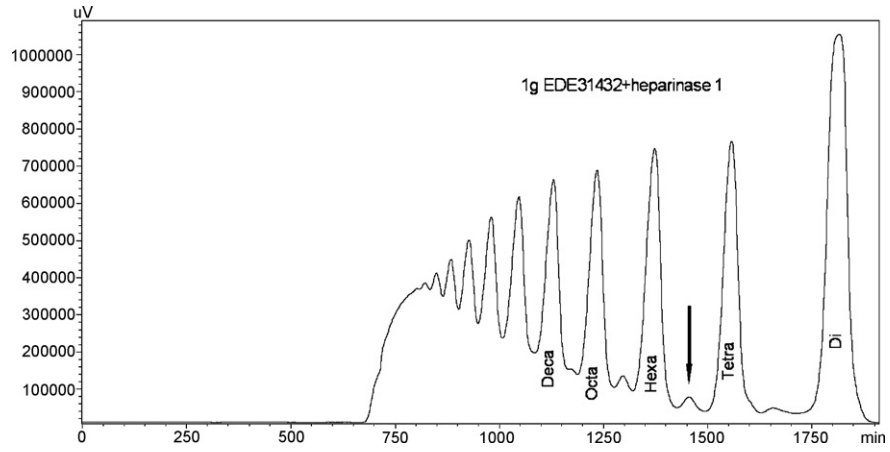


**Fig. 4.** SEC of heparinase 1 digest of heparin batch EDE31432 from Organon Europe (detection UV at 232 nm).

this compound complies with a tetrasaccharidic backbone. The structure determination of the tetrasaccharide was achieved using $^{1}H$ and $^{13}C$ NMR spectroscopy. The full $^{1}H$ assignment is reported in Table 1.

Analysis of COSY (spectrum not shown) together with TOCSY spectrum (Fig. 8) provides intra residue correlations for assignment of all protons. Three anomeric protons corresponding to three residues are clearly identified on TOCSY spectrum. The ethylenic proton H4 observed at 5.98 ppm is used to start assignment of the 4,5-unsaturated uronic acid (ΔUA 2S), at the non-reducing end. Similarly, the spin systems corresponding to the two other residues are also elucidated. *N*-sulfated, 6-sulfated glucosamine (GlcNS,6S), and iduronic acid (IdoA) are identified. Chemical shifts together with inter proton coupling constants for the residues ΔUA 2S, GlcNS,6S, and IdoA are in full agreement with data published [9].

However, an unexpected spin system is observed, composed of six protons with chemical shifts in the range 3.8–4.4 ppm. An expansion of this region is shown in Fig. 4. The number of signals observed, the unusual chemical shifts together with absence of anomeric H1 proton, suggest the opening of a glucosamine pyranose ring, together with chemical modifications.

In order to determine the sequence of the residues, a ROESY experiment was recorded with the tetrasaccharide (spectrum not shown). Unambiguous inter residue correlations are observed on 2D ROESY spectrum between protons H1 and H4 of adjacent saccharidic units. All these information indicate that tetrasaccharide sequence is ΔUA 2S-GlcNS,6S-IdoA-GlcNAc,6S.

In order to investigate the 2.10 ppm signal, additional 2D heteronuclear HSQC and HMBC spectra were also recorded with the same sample. Fig. 9 shows the 2D $^{1}H$–$^{13}C$ HSQC spectrum obtained in $D_2O$. Carbon chemical shifts are reported in Table 1. Signal at 2.10 ppm exhibits a correlation with carbon at 24.7 ppm, indicating that signal observed at 2.10 ppm is the resonance of acetyl group.

Fig. 10 shows the heteronuclear 2D $^{1}H$–$^{13}C$ HMBC region containing the correlations with carbonyl signals visible on the spectrum. A clear correlation is observed between methyl protons at 2.10 ppm and carbon at 176.7 ppm. This chemical shift is compatible with hypothesis of an acetyl group at 2.10 ppm. Moreover, long range heteronuclear correlation is observed between proton at 4.37 ppm and carbonyl at 176.7 ppm. This information indicates the position of the acetyl group in the molecule.

Another interesting heteronuclear correlation is observed on HMBC spectrum between proton at 4.37 ppm and a second car-

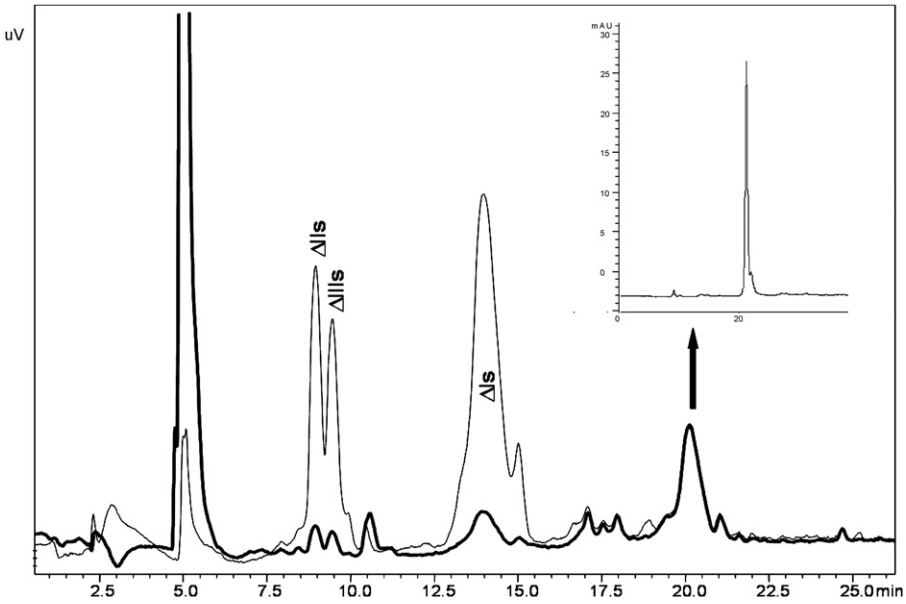


**Fig. 5.** Chromatographic purification on semi preparative AS11 column of the SEC pentasaccharide fraction ((—) 232 m; (▬) 202–230 nm).

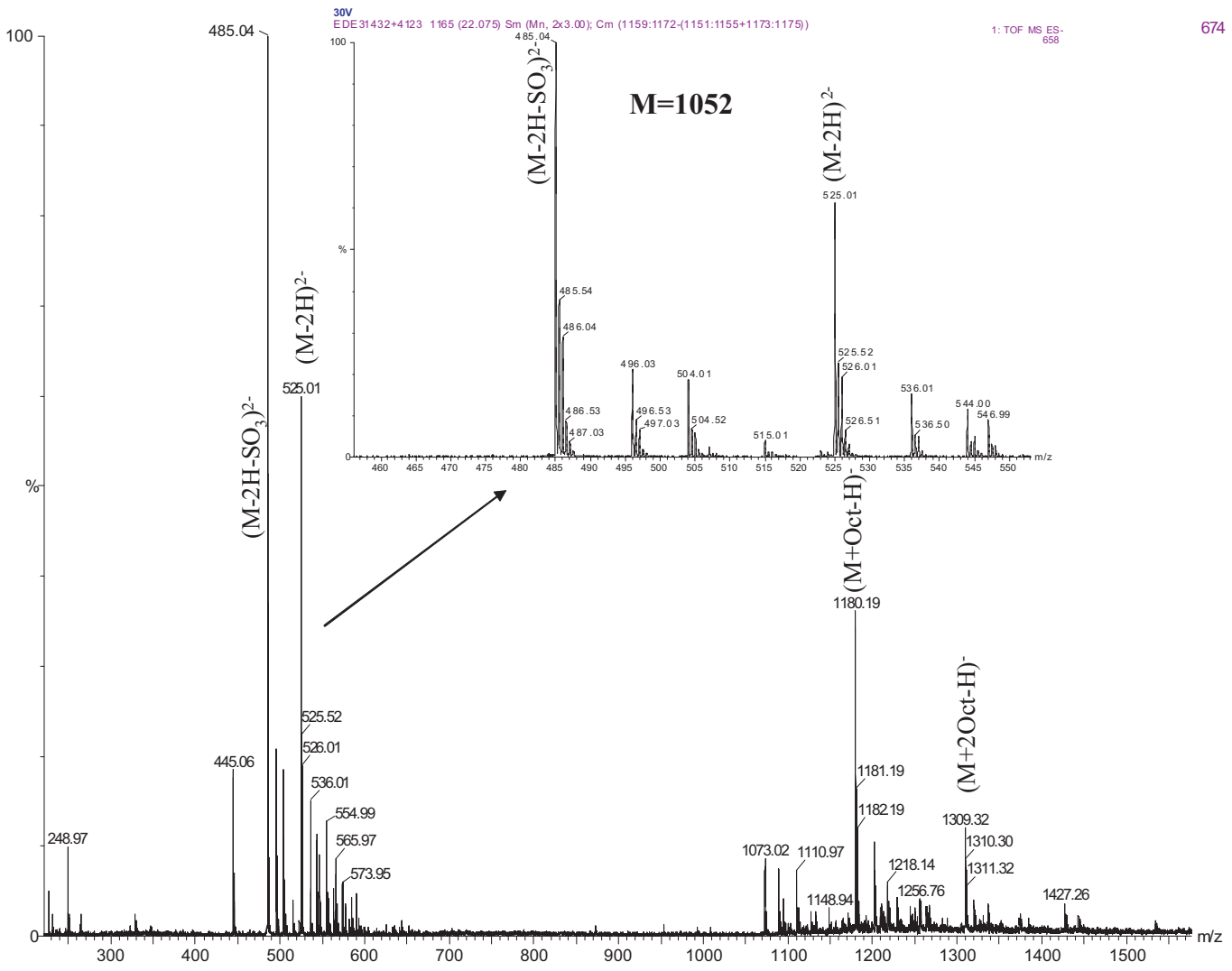


**Fig. 6.** Mass spectrum of the isolated oligosaccharide obtained by injection in LC/MS of exhaustively digested heparin (batch EDE31432) (Oct: octylamine adduct: 129 u.m.a.; $SO_3$: sulfate: 80 u.m.a.).

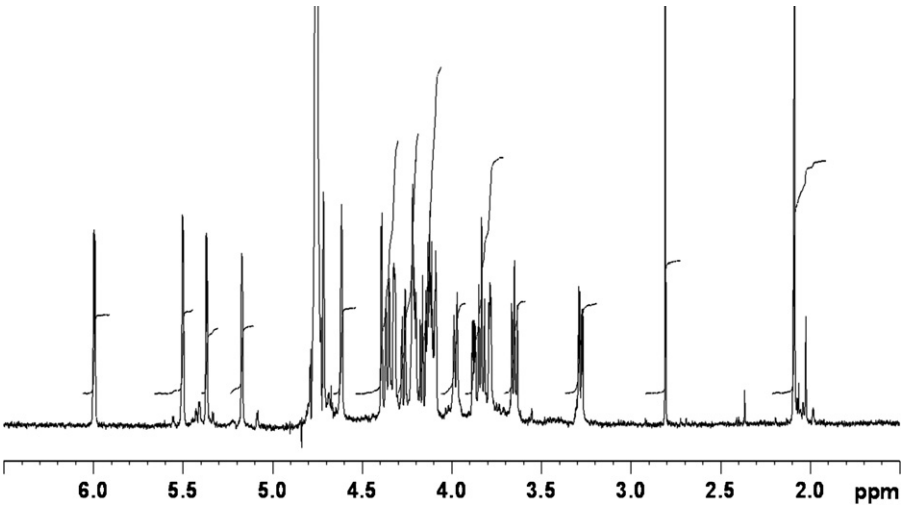


**Fig. 7.** 1D proton spectrum of the purified tetrasaccharide containing acetyl signal at 2.10 ppm. Signal at 2.8 ppm indicates the presence of methyl sufonate in the sample (used for tetrasaccharide purification) – spectrum in $D_2O$ – 30 °C – 600 MHz.

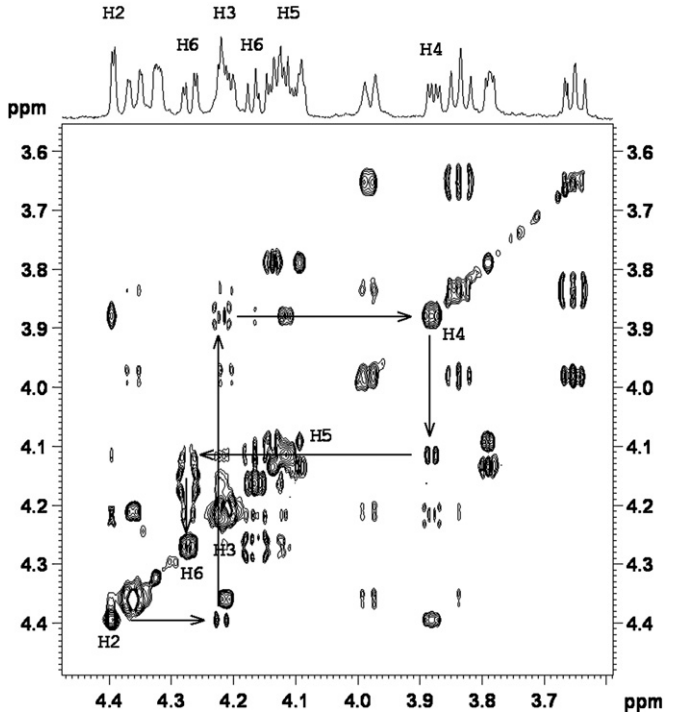


**Fig. 8.** Tetrasaccharide 1D proton (above) and 2D TOCSY spectra region containing unusual saccharidic correlations. Arrows on 2D spectrum indicate sequence of proton in the spin system – $D_2O$ – 30 °C – 600 MHz.

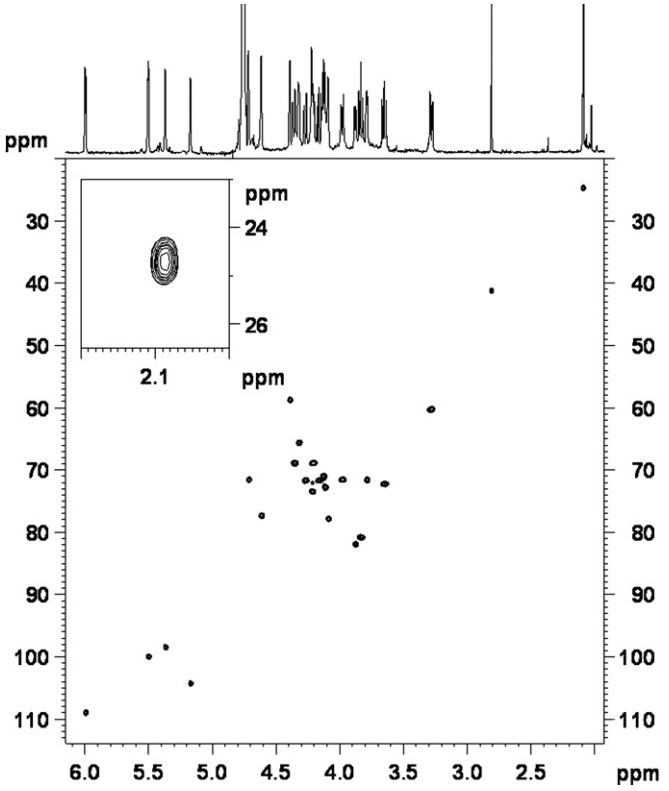


**Fig. 9.** 1D proton (above) and 2D HSQC spectra of tetrasaccharide and, (in the box) region of proton acetyl signal (2.10 ppm) with carbon correlation at chemical shift 24.7 ppm – $D_2O$: – 30 °C – 600 MHz.

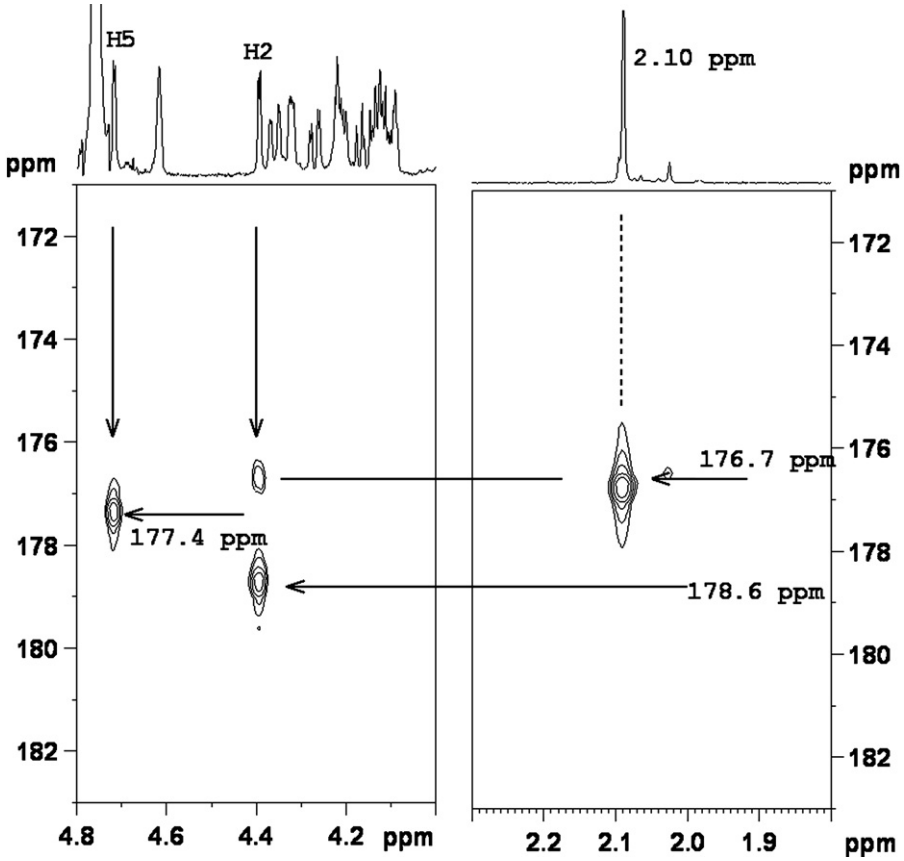


**Fig. 10.** 2D HMBC $^{1}H$–$^{13}C$ spectrum tetrasaccharide: region of the carbonyl resonances – $D_2O$ – 30 °C – 600 MHz.

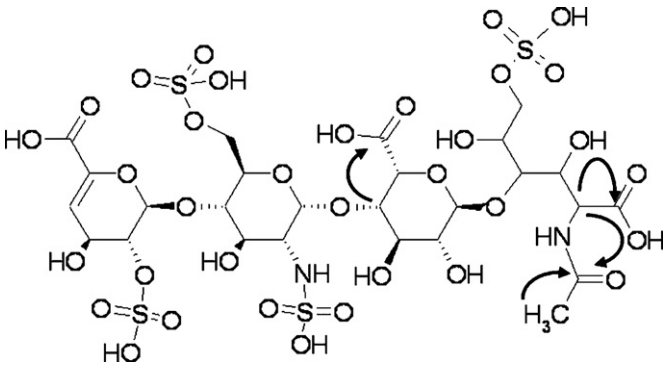

**Fig. 11.** Structure of the purified tetrasaccharide.

bonyl group at 178.6 ppm. This correlation indicates the presence of a carboxylic acid group at this position.

The following proposed structure (Fig. 11) is compatible with all NMR data, and complies with mass spectrometry information (MM = 1052 g/mol). Important heteronuclear long range correlations are reported on structure using arrows (correlations between the different carbonyl groups of terminal residue and their neighbour protons, including methyl at 2.10 ppm).

## 4. Conclusion

As heparin sodium is a complex hetero polysaccharidic mixture, its full characterization has never been performed. Each minor chemical modification of the macromolecules mixture can potentially generate extra $^{1}$H NMR signals in a spectrum region which could raise compliance issue regarding new USP heparin monograph. The signal at 2.10 ppm is an example of such issue. After extensive use of orthogonal analytical and separative techniques, the structural moiety responsible for this extra signal was isolated and characterized. In each case when this signal appears, we found out that potassium permanganate was used. When peracetic acid and hydrogen peroxide are used as oxidative reagent, such as in the negative control heparins, this side reaction does not place. Evidences have been provided that this structural moiety is process dependent and part of the polysaccharide backbone. The estimated amount of this moiety in heparin is respectively about 1.5% in EDE and 0.6% in EV heparin sources (based on the N-acetyl integration ratios between the extra and regular signals of heparin sodium, assuming that heparin sodium contains about 15–20% *N*-acetylated disaccharides building blocks). This side reaction is formally the oxidation of the aldehyde at the reducing end of the polysaccharide into the carboxylic acid derivative. This particular oxidation of the reducing end has always been present in potassium treated heparin but its characterization has been triggered by new analytical requirements of this complex biological drug. In conclusion, such regulatory stimulation promotes a better understanding of the structural peculiarities of heparin sodium in order to insure the highest analytical standards to the benefit of patient health and safety.

## References

[1] Heparin sodium monograph in pharmacopeial forum, vol. 35(5) [Sept–Oct 2009] p. 3. $^{1}$H NMR spectrum. Signal 1 is observed at 5.42 ppm (H1 of GlcNAc/GlcNS,6S) and signal 2 at 5.21 ppm (H1 of IdoA 2S). The ppm values of these signal should not differ by more than +0.03 ppm.
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