Analytical Chemistry Research 3 (2015) 46–53

Contents lists available at ScienceDirect

Analytical Chemistry Research

journal homepage: www.elsevier.com/locate/ancr

Quantitative compositional analysis of heparin using exhaustive heparinase digestion and strong anion exchange chromatography

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ARTICLE INFO

Keywords:
Heparin
Heparinase digestion
3-O sulfated oligosaccharides
Building block quantification

ABSTRACT

Heparin is a linear sulfated polysaccharide widely used therapeutically as an anticoagulant. It is also the starting material for manufacturing low-molecular-weight heparins (LMWH). Quality control of heparin and LMWH is critical to ensure the safety and therapeutic activity of the final product. However due to their complex and heterogeneous structure, orthogonal analytical techniques are needed to characterize the building blocks of heparin. One of the state-of-the-art methods for heparin analysis is based on complete enzymatic digestion using a mixture of heparinases I, II, and III, followed by the separation of the resulting oligosaccharides by liquid chromatography. The European Pharmacopoeia strong anion-exchange chromatographic method, used to quantify 1,6-anhydro derivatives in enoxaparin, is here applied to the analysis of the heparin building blocks. Their quantification, namely the determination of their average w/w percentage in the heparin chain, is obtained after identification of all components including glycoserine derivatives and 3-O sulfated di- and tetrasaccharides. This work therefore provides a comprehensive overview of the building blocks of unfractionated heparin, including those chemically modified by the manufacturing process, either within the polysaccharide chain or at its reducing end.

1. Introduction

Heparin is a highly sulfated polymeric linear chain with repeating disaccharide units comprised of an uronic acid (glucuronic or iduronic) and a glucosamine. Heparin is widely used as an anticoagulant in the treatment of thromboembolic diseases either as unfractionated heparin, or as the starting material for the synthesis of low-molecular-weight heparins (LMWH).

Heparin can be extracted from various tissues in pigs, cattle, and sheep, including the lung, intestine, or skin. Although the European Pharmacopoeia (Ph. Eur) [1] and U.S. Pharmacopeia (USP) [2] specify that only heparin derived from porcine intestinal mucosa can be used as the starting material for manufacturing LMWH, bovine or ovine heparins can be found in industrial quantities necessitating the use of appropriate analytical methods to detect a potential contamination. At the end of 2007, the adulteration of heparin by oversulfated chondroitin sulfate (OSCS) resulted in several fatal issues during in its clinical use [3], further highlighting the importance of the quality control of heparin. The objectives of this quality control should address several issues: it must first assess the anticoagulant activities, enable discriminant structural characterization of the product, and detect potential contamination. However the analysis of heparin is very complex because heparin is a mixture of polydisperse heteropolymers with a high molecular weight (10,000–20,000 Da). No single method has sufficiently high-resolving power to characterize its structure, making the use of a set of orthogonal methods necessary; this is also true in the case of LMWH. The Ph. Eur. and USP monographs require ^{1}H NMR spectroscopy, strong anion exchange (SAX) chromatography, and molecular weight characterization by gel permeation chromatography; yet, these methods do not allow a precise structural assessment of the heteropolymer composition.

The use of enzyme lyases such as heparinases to depolymerize the glycan into building blocks that can be fully characterized by chromatography, is a technique of major interest as it is simple and informative. Although it remains far from being able to illustrate the true complexity of heparin or LMWH, this technique can provide a first insight into the heparin source (crude or pure heparin), its composition consistency, the animal species of origin, and potential contamination. Moreover, it can detect structural modifications to heparin and can therefore be used to monitor the chemical transformations that occur during the heparin manufacturing process. Currently, although this method indirectly appears in the USP and Ph. Eur. enoxaparin monographs for the control of 1,6-anhydro derivatives [5,6,14], it is not included in the current heparin monographs, nor is it recommended in the FDA guideline [4]. Previous publications have referred to this

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http://dx.doi.org/10.1016/j.ancr.2014.12.001
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Abbreviations for saccharide units and chemical modifications

U	uronic acid
IdoA	L-iduronic acid
GlcA	D-glucuronic acid
ΔU	4,5-unsaturated uronic acid, e.g. ΔGlcA
GlcN	D-glucosamine
NS	*N*-sulfate
NAc	*N*-acetyl
2S	2-O-sulfate
6S	6-O-sulfate
GalA	D-galacturonic acid

Structural symbols

ΔIVa	ΔU-GlcNAc
ΔIVs	ΔU-GlcNS
ΔIIa	ΔU-GlcNAc,6S
ΔIIIa	ΔU2S-GlcNAc
ΔIIs	ΔU-GlcNS,6S
ΔIIIs	ΔU2S-GlcNS
ΔIa	ΔU2S-GlcNAc,6S
ΔIs	ΔU2S-GlcNS,6S
ΔIVs_{gal}	ΔGalA-GlcNS
ΔIIs_{gal}	ΔGalA-GlcNS,6S
IIs_{glu}	GlcA-GlcNS,6S
$IIIs_{id}$	IdoA2S-GlcNS
IVs_{glu}	GlcA-GlcNS
IIa_{id}	IdoA-GlcNAc,6S
$IIIa_{id}$	IdoA2S-GlcNAc
Ia_{id}	IdoA2S-GlcNAc,6S
Is_{id}	IdoA 2S-GlcNS,6S

The iduronic (id) or glucuronic (glu) structure of uronic acids is indicated for oligosaccharides, e.g. ΔIs–$IIIs_{id}$. Underlined disaccharides have a 3-O-sulfated glucosamine, e.g. $\underline{IIs}_{glu}$ (GlcA-GlcNS,3S,6S).

method [7] but lack precision. It therefore appears important to provide precise and comprehensive description of this important and rather simple analytical tool.

The cleavage of the heparin glycosidic linkages by heparinases generates, after β-elimination, a new oligosaccharide with a Δ-4-5 unsaturated uronic acid at its non-reducing side. When the 3 heparinases are applied together, 8 major unsaturated disaccharides [22,23] are formed, with varying sulfation patterns in position C-2 and C-6 and on the glucosamine either *N*-sulfated or *N*-acetylated. In addition, longer oligosaccharides resistant to heparinases, such as 3-O sulfated tetrasaccharides, are generated [9–11]. Similarly, glycoserine tetrasaccharides, residues of the heparin–glycoprotein linkage and also heparinase-resistant are observed [12,13]. Some methods for the quantitative analysis of heparin and LMWH digests have already been developed [15–19]. However these have important limitations such as insufficient resolution, incomplete elution of the components mixture, or fragmentary achievement of the quantitative aspect. They also include fluorescent tagging on the saccharide reducing-end to increase detectability, which is justified for low-concentration biological samples but is inappropriate and a source of potential error for heparin samples where the UV absorbance of unsaturated acids at 232 nm is sufficient for detection. Moreover, the labeling efficiency of substrates is often variable (e.g. null for 1,6-anhydro derivatives) and side reactions can occur, further increasing the complexity of the sample analysis.

Quantification methodology using external standards is hard to implement due to the number of components, the time necessary to purify standards, and the expected precision associated with the purity of these standards. The most suitable alternative for quantification is internal standardization. Two previously published quantitative methods use ΔIp (ΔU2S-GlcNCOEt,6S) as internal standard and fluorescent tagging by 2-aminoacridone [16,18]. In the first one [16], capillary electrophoresis is used, but the selectivity is insufficient to resolve all components of heparin digests. In the second one [18], the separation is performed by reversed-phase chromatography, identification by mass spectrometry (MS) and quantification by double UV detection. A method of quantitation by LC–MS [19] using isotopic internal standards chemo-enzymatically prepared is also described, but this method cannot be applied in a control laboratory.

In the method described in this paper, the quantification is based on the data driven consensus assumption that molar extinction coefficient at 232 nm for Δ-4-5 unsaturated oligosaccharides are constant [8]. However, to apply this rule to heparin digests, it is necessary to identify every components of the digest to assess their molecular weights. We thus describe here a comprehensive method to identify and quantify the building blocks of heparin generated by heparinase digestion. All natural components (disaccharides and tetrasaccharides) and other major features generated by the heparin purification process are reported.

2. Material and methods

2.1. Materials

All heparin samples used in this study were extracted from porcine mucosa. Purified heparin samples were obtained from different sources available on the industrial market. 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, UK). All other reagents and chemicals were of the highest quality available. Water was purified using a Millipore Milli-Q purification system.

2.2. Enzymatic digestion

Heparin digestion (20 μL of a 20 mg/mL solution in water) was performed at room temperature for 48 h, in a total volume of 160 μL containing 20 μL heparinase I, II, and III mixture (0.5 IU/mL of each heparinase in a potassium phosphate buffer pH 7.0 [10 mM KH_2PO_4 and 0.2 mg/mL of bovine serum albumin(BSA)]) and 120 μL of 100 mM sodium acetate buffer (pH 7.0) containing 2 mM of $Ca(OAc)_2$ and 0.1 mg/mL BSA.

2.3. Analysis of heparin digests by SAX chromatography

Exhaustively-digested heparin (4–10 μL) was injected onto a Spherisorb-SAX chromatography column (250 × 3.2 mm, 5 μm, column temperature 50 °C, Waters, France). Mobile phase A was 1.8 mM NaH_2PO_4 at pH 3.0 and mobile phase B was an aqueous solution of 1.8 mM NaH_2PO_4 with 1 M $NaClO_4$ adjusted to pH 3.0. A linear gradient of mobile phase B ($t_{0\ min}$ 3%; $t_{20\ min}$ 35%; $t_{50\ min}$ 100%) was applied with a flow rate of 0.45 mL/min. Double UV detection was performed at 232 nm and 202–247 nm. *N*-acetylated oligosaccharide selective signal (202–242 nm) is the result of the subtraction of the 202 nm wavelength signal from the 247 nm reference signal, as previously described [10].

2.4. Analysis of heparin digests by LC/MS

Heparin digests were injected on ion-pair LC/MS chromatography using experimental conditions derived from those described by Doneanu et al. [20]. Briefly, Acquity UPLC BEH C18 column

(2.1 × 150 mm, 1.7 μm, column temperature 30 °C, Waters) was used. Mobile phase A was water and mobile phase B was water:acetonitrile (25:75) and both mobile phases contained 15 mM of pentylamine or hexylamine (PTA or HXA, ion-pairing reagents) and 50 mM 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP; buffering agent) with a flow rate 0.25 mL/min.

Electrospray ionization mass spectra were obtained using a Xevo Q-Tof MS (Waters). The electrospray interface was set in positive-ion mode with a capillary voltage of 2000 V and a sampling cone voltage of 20 V. The source and the desolvation temperatures were 120 °C and 400 °C, respectively. Nitrogen was used as desolvation (750 L/min) and cone gas (25 L/min). The mass range was 200–2000 Da (scan rate 0.5 s). Under these conditions, molecular ion peaks were adducts of the ion-pairing agent (PTA or HXA). The number of adducts and their molecular weight was obtained, as described previously [21], by running a first injection with PTA and a second injection with HXA.

3. Results and discussion

Heparin is present in various animal tissues as a proteoglycan where the polysaccharide chain is linked to a ser-glycine core protein through the glycoserine sequence -GlcA β1-3 Gal β1-3 Gal β1-4 Xyl β1-0-Ser, which is the biosynthesis starting point. In the first step of the manufacturing process, the protein is eliminated by proteolysis in alkaline environment. Crude heparin is then obtained by treatment with anion exchange resins or precipitation with quaternary ammonium salts.

In the second step, the purification of crude heparin eliminates all by-products (protein, peptide, DNA and dermatan) co-extracted with heparin. Notably, this step involves treatments in strong alkaline media as well as the use of oxidizing agents such as potassium permanganate, peracetic acid and hydrogen peroxide. It is during this second phase that most of the structural modifications of the endogenous heparin backbone occur.

3.1. Identification of endogenous oligosaccharide building blocks

The most exact image of endogenous heparin can be found in some crude heparins, especially those with limited alkaline treatment. The main oligosaccharide components found in these heparin digests, excluding the 8 main unsaturated disaccharides will be described first.

The original glycoserine tetrasaccharide ΔGlcA β1-3 Gal β1-3 Gal β1-4 Xyl β1-0-Ser (ΔGlyser) [12,13] was present in almost all crude heparins. This compound is cleaved by heparinase III [12] and, as it contains no sulfate, has the lowest retention on SAX chromatograms. Although 85% of the heparin chains are depolymerized into disaccharides, more complex heparinase-digestion behavior is observed with 3-O-sulfated moieties. Depending on the saccharidic environment, 3-O-sulfated moieties can be digested into tetrasaccharides or, more rarely, into disaccharides (ΔIIs and ΔIs). The heparin-resistant tetrasaccharide (ΔIs–$\underline{\text{IIs}_{glu}}$) [24] was first isolated from a digest of bovine lung heparin by heparinase I, but has also been detected in porcine mucosa heparin. Three other 3-O-sulfated tetrasaccharides (ΔIIa–$\underline{\text{IIs}_{glu}}$, ΔIIa–$\underline{\text{IVs}_{glu}}$, and ΔIIs–$\underline{\text{IIs}_{glu}}$) were isolated from porcine mucosa heparin by enzymatic digestion with heparinase I and II [9]. A more exhaustive insight into the heparin digest by the heparinase mixture, as assessed by cetyltrimethylammonium-SAX chromatography, has been published [10]. However the porcine heparin used in this earlier study was purified and some unidentified tetrasaccharides observed, possibly mistaken for unknown 3-O sulfated derivatives, might in fact have been residues of heparin partially transformed by the purification process.

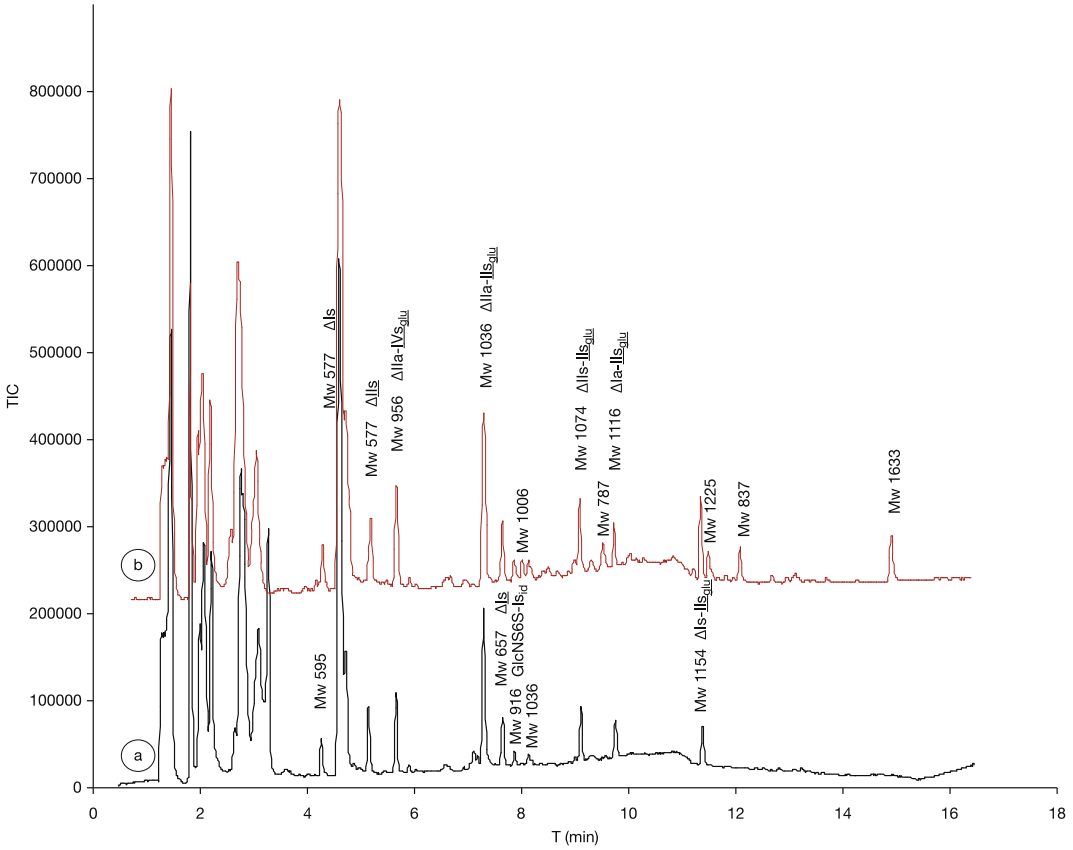


Fig. 1. LC–MS analysis of a crude mucosa heparin (a) compared with a purified heparin (b) depolymerized by the heparinase mixture.

In order to sum up these data and give a specific view of the 3-O-sulfated di and tetrasaccharides characteristic of porcine mucosa heparin, a liquid chromatography/mass spectrometry (LC–MS) chromatogram of a digested crude heparin is shown in Fig. 1a. In addition to the 3-O-sulfated tetrasaccharides previously isolated, we identified ΔIa–$\underline{\text{IIs}_{glu}}$ [10], as well as two 3-O-sulfated disaccharides (Δ$\underline{\text{IIs}}$ and Δ$\underline{\text{Is}}$). The tetrasulfated disaccharide Δ$\underline{\text{Is}}$ was first isolated from bovine intestinal heparin sulfate [26]. It was then isolated in an octasaccharide from semuloparin [27] an ultra-LMWH prepared from porcine mucosa heparin. The presence of ΔIa–$\underline{\text{IIs}_{glu}}$ is also in direct agreement with results already obtained by nitrous acid depolymerization [25]. Two fragments corresponding to Is_{id} (Mw 595) and GlcNS–Is_{id} (Mw 916) are residues of the original non-reducing end of the heparin chain. A similar saturated minor fragments (+18 Da) can be detected for major unsaturated disaccharides of the mixture, all originating from the non-reducing end. These compounds accounts globally for about 4% in the heparin polysaccharide chain (1 disaccharide out of 25; taking into account that the heparin mean molecular weight is about 15,000 Da and the average mean molecular weight of a disaccharide is 600 Da). The influence of this non-reducing sequence had a negligible effect on the percentage of each building block in the heparin chain.

3.2. Identification of fingerprint building blocks found in digests of purified heparin

In contrast with the compounds cited in the previous section, some oligosaccharides detected in purified heparin are not present in crude heparin and were specifically generated by side reactions that occur during the purification step. The increased complexity of the LC–MS chromatogram in Fig. 1b, compared with Fig. 1a, suggests that these oligosaccharides are numerous and very difficult to fully identify. When purified heparin digests are separated by the Spherisorb-SAX method (Fig. 2), new components were also detected (Fig. 2; arrows 1–5).

The processes during heparin purification that generate structural modifications are not just linked to an alkaline environment, but can also be linked to the oxidation reagents. Some of these modifications have already been studied and identified [28,33] as they give a ^{1}H NMR extra signal that could interfere with potential signals characteristics to the OSCS contamination [2].

We have previously demonstrated that peak 1 on Fig. 2 is the tetrasaccharide responsible for the 2.10 ppm NMR extra signal detected in $KMnO_4$-treated heparins [28]. Other labeled oligosaccharides in Fig. 2 have yet to be identified. Thus, in purified heparin, a significant proportion of the oligosaccharides eluted after ΔIs are not 3-O-sulfated tetrasaccharides, but process-modified oligosaccharides.

3.2.1. Glycoserine derivatives

The oxidizing step during the second phase of the heparin process is generally used to decrease the coloration of the drug product. Chemically, we observed that the glycoserine reducing-end is denatured into characteristic fingerprints that specifically reflect the oxidation conditions (reactant, stoichiometry, temperature) [13]. To investigate structural modifications, glycoserine fragments were isolated in the heparin digests and identified by MS and NMR

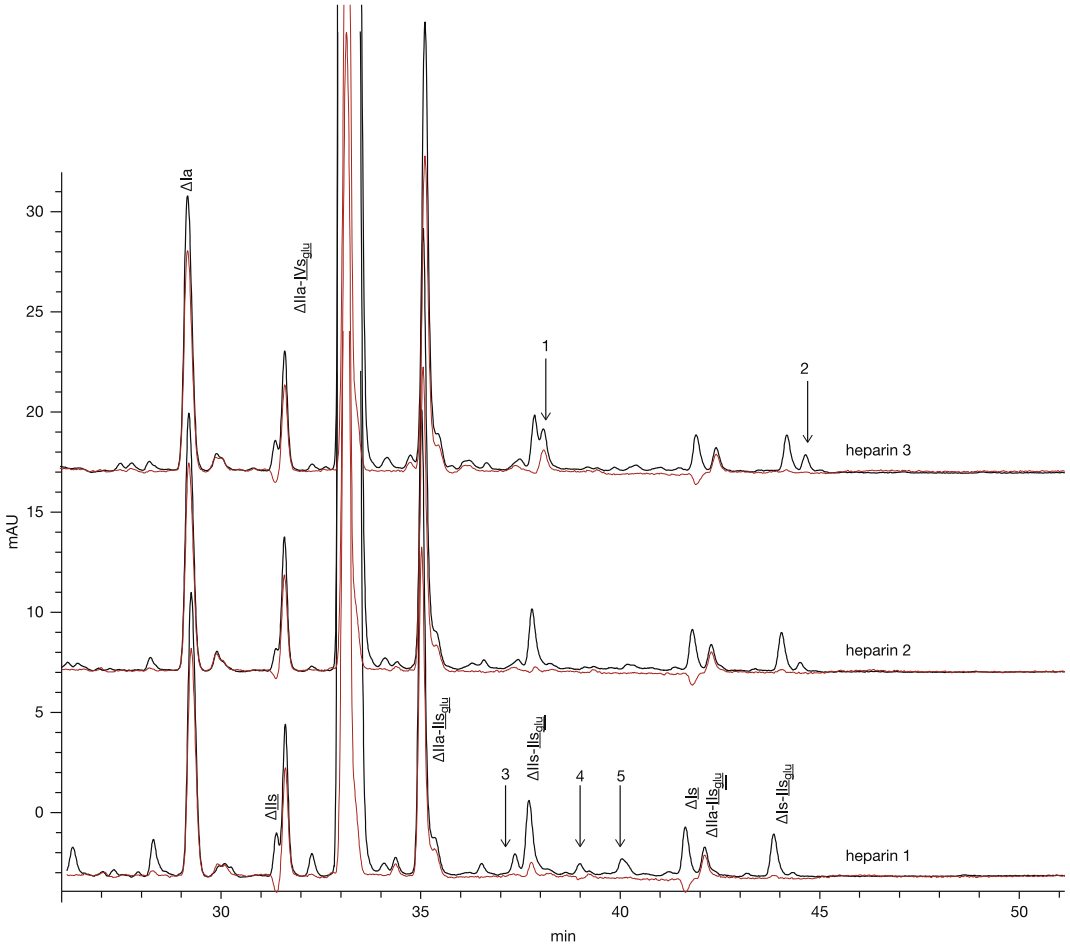
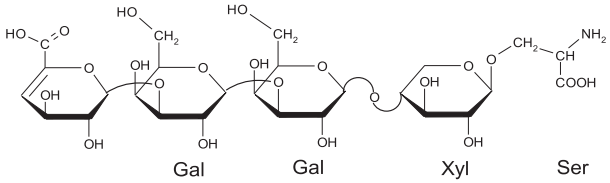
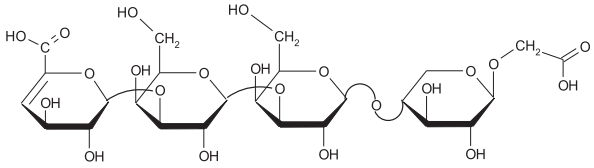
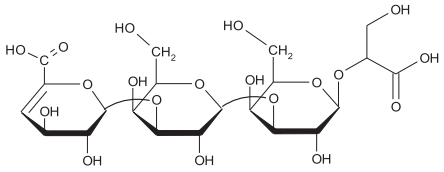
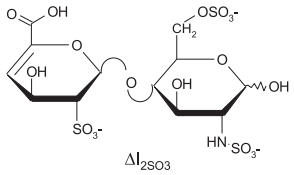
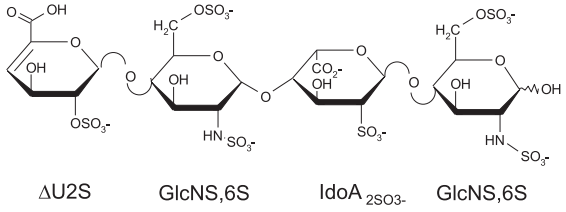
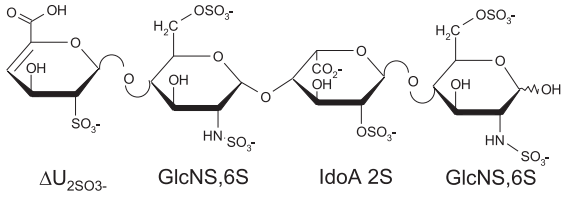


Fig. 2. SAX chromatograms of the 3 purified heparin batches depolymerized by the heparinase mixture (focus on the elution of tetrasaccharides). Black line: 232 nm; red line: 202–242 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Heparin derivatives identified.

Endogenous glycoserine ΔGlyser	
Non-endogenous glycoserine derivatives $\Delta Glyser_{ox1}$	
$\Delta Glyser_{ox2}$	
Other non-endogenous derivatives ΔI_{SO3} [29]	
Tetrasaccharide A $\Delta Is\text{-}I_{2SO3}$	
Tetrasaccharide B $\Delta I_{2SO3}\text{-}Is_{id}$	

(Table 1). NMR spectra of $\Delta Glyser_{ox1}$ and $\Delta Glyser_{ox2}$ are given in the Supporting Information.

3.2.2. Other derivatives

The second step of the heparin manufacturing process, namely purification in oxidative and alkaline media, is usually designed to have limited impact on the structure of the oligosaccharidic chain. However, alkaline media can cause side reactions, such as the partial 2-O-desulfation of iduronic acids detected in the digest [30] especially when conditions are not well controlled. The 2 main markers of 2-O-desulfation in heparin digests are derivatives of galacturonic acid: ΔIIs_{gal} and ΔIVs_{gal}.

A Δ4,5-uronic acid with a 2-sulfonic acid substitution has also been reported previously [29]. This compound was also identified in selected heparin batches in the present study and designated ΔI_{SO3} (Table 1). Two tetrasaccharide precursors of this sulfonic disaccharide were also identified in a rejected enoxaparin sample: one with the sulfonic disaccharide at the reducing-end (Tetrasaccharide A); the other with the sulfonic disaccharide at the non-reducing-end (Tetrasaccharide B) (Table 1). NMR spectra for these tetrasaccharides are provided in the Supporting Information. It is interesting to note that the NMR signal characteristic of sulfonic disaccharides inside the heparin chain has chemical shifts corresponding to Tetrasaccharide A.

3.3. Elution of oligosaccharide building blocks

Fig. 3 shows the molecular weights of peaks according to their identification on the chromatogram. Other examples of chromatograms of heparin depolymerized batches are given in Fig. 4. In contrast with the sample shown in Fig. 3, which still contains its native glycoserine linking region, the example in Fig. 4a included two denatured residues, namely $\Delta Glyser_{ox1}$ and $\Delta Glyser_{ox2}$, which result from potassium permanganate oxidation; ΔI_{SO3}, described

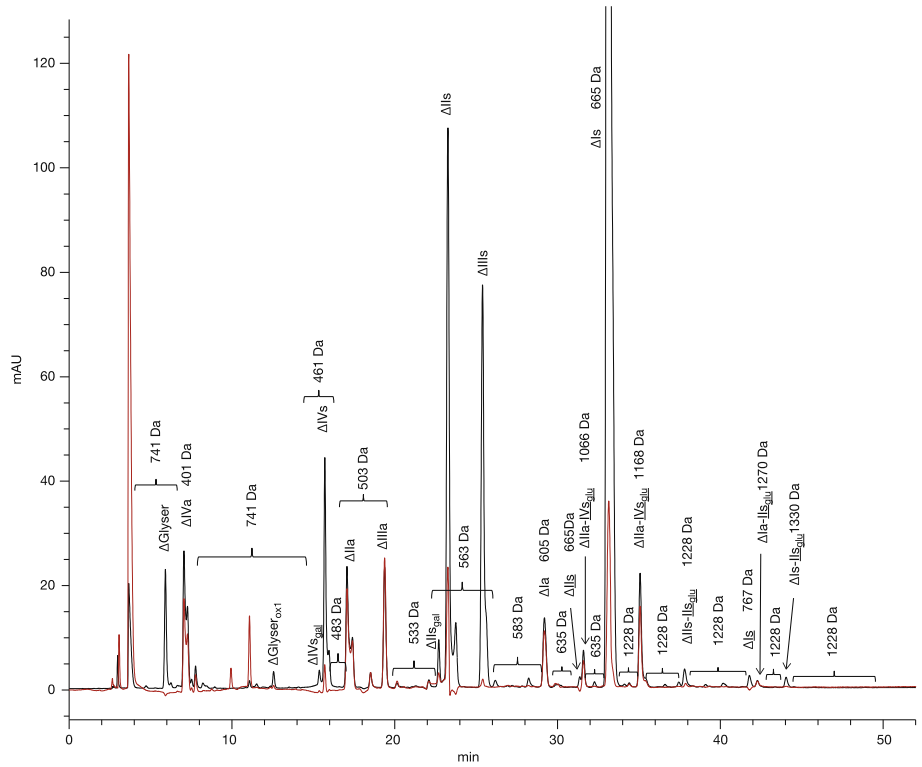


Fig. 3. Heparin batch depolymerized by heparinase mixture and molecular weights applied for the quantification. Black line: 232 nm; red line: 202–242 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

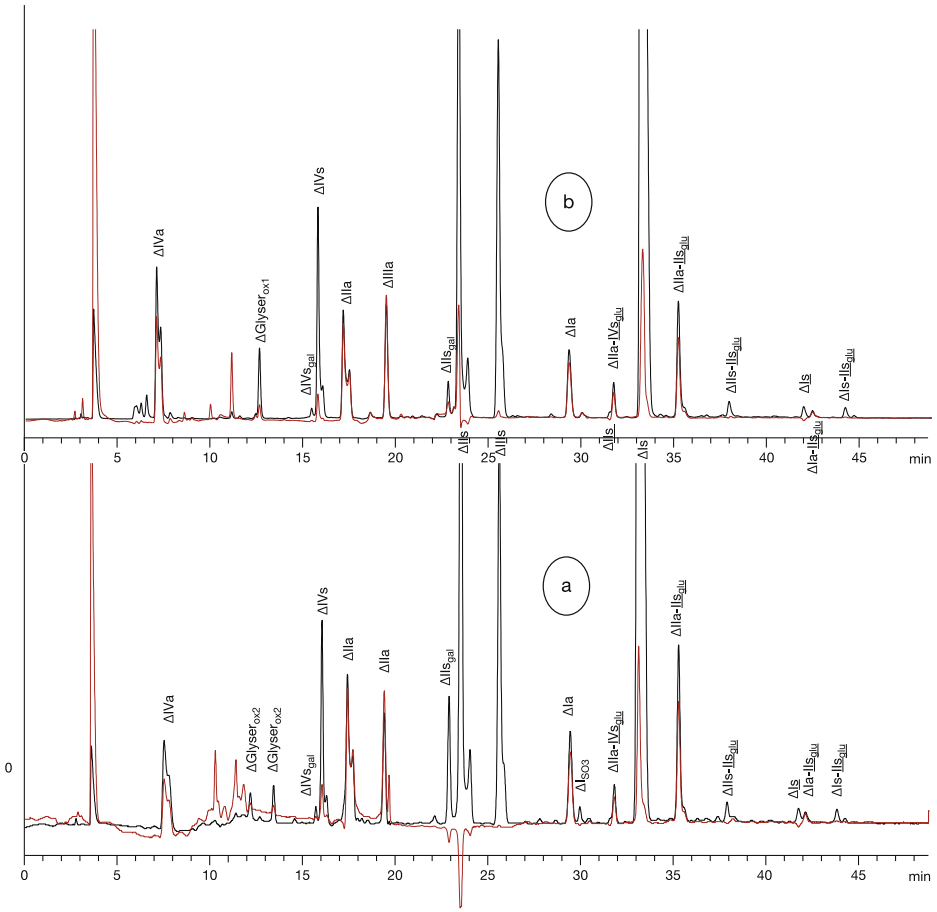


Fig. 4. Heparin batches a and b (porcine intestinal mucosa) depolymerized by heparinase mixture. Black line: 232 nm; red line: 202–242 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

previously, was also present. In the example in Fig. 4b, only ΔGlyser$_{ox1}$ is present, as a consequence of the use of peracetic acid as the oxidation reagent [33]. Other minor glycoserine derivatives are eluted before ΔIVa, with a similar retention time to ΔGlyser. ΔGlyser may be one of these peaks, present in a small quantity, however the other peaks detected are various glycoserine derivatives modified by the purification process. The large variety of minor components observed requires use of a system similar to that described in Fig. 3, where the molecular weight of each component is linked to a defined chromatographic time range. The values of Mw for unidentified compounds are given in relation to their retention times on the chromatogram. In first estimate, the retention on SAX chromatography for unsaturated disaccharides is governed by the sulfation pattern and more precisely, the number of sulfates. The retention times of known disaccharides (ΔIVa, ΔIVs, ΔIIa, ΔIIIa, ΔIIs, ΔIIIs, ΔIa and ΔIs) is partially correlated with their molecular weights (Fig. 3). In the time ranges on the chromatogram corresponding to the transition between two disaccharides with different Mw values (ΔIVs → ΔIIa , ΔIIIs → ΔIa, ΔIa → ΔIs), it was chosen to follow this rule and give the average value of the 2 bracketing disaccharide molecular weights. In addition, all unidentified saccharides eluted after ΔIs were assigned, for the sake of simplicity, with a molecular weight of 1228 Da. Fig. 5 shows chromatograms of heparin digests from porcine and bovine origin. Each example has a specific disaccharide distribution. ΔIIIs, ΔIa, and 3-O-sulfated residues from the 3 samples are very different. Although the large proportion of galacturonic acid observed in the heparin from bovine lung is related to the pronounced alkaline step in the purification process used, and not the species of origin, this method can still be used as a simple and informative control to check at a first glance what is the animal origin of a heparin sample [7]. Crude and pure heparin batches purchased are periodically analyzed by this technique to monitor product consistency against preset criteria.

3.4. Quantification of oligosaccharide building blocks

Quantification is based on the consensus assumption about the consistency of the molar response coefficients at 232 nm [8,31], and the maximum UV absorbance of unsaturated oligosaccharides obtained after heparinase digestion. The principle and the chromatographic methods are identical to those used in the USP for the quantification of 1,6-anhydro derivatives in enoxaparin [5,6,14,32].

The weight/weight (w/w) percentage for each component is given by the following formula:

$$\text{Component } i\ \%\ (w/w) = \frac{100 \times Mw_i \times A_i}{\Sigma_x Mw_x \times A_x} \quad (1)$$

Mw_i and A_i are the molecular weight and the chromatographic area at 232 nm of the assayed component i, respectively. Mw_x and A_x are the molecular weight and the chromatographic area, respectively, of either the peak X or the zone X specified by its retention time; the sum being related to all the eluted components.

The heparin batch from Fig. 3 was taken as an example for building blocks quantification in the Supplementary Data. The method was entirely validated in its use for the titration of 1,6-anhydro derivatives in enoxaparin. This validation has been provided to the USP and Ph. Eur. to support the introduction in the monographs.

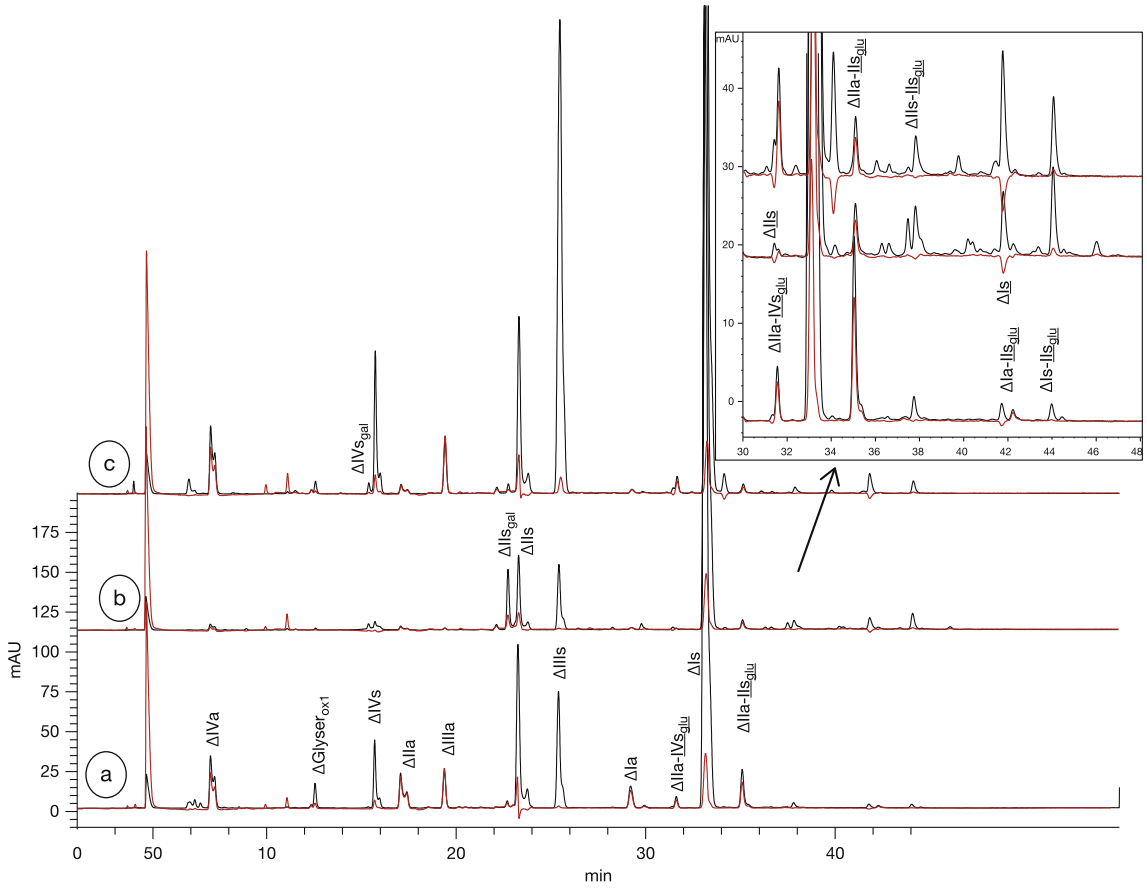


Fig. 5. Comparison of digests from 3 heparins: (a) porcine intestinal mucosa; (b) bovine lung; and (c) bovine intestinal mucosa. Black line: 232 nm; red line: 202–242 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Conclusions

The SAX chromatographic method described in the USP and Ph. Eur. pharmacopeias for the quantification of 1,6-anhydro derivatives in enoxaparin after digestion by heparinases [5,6] can also be used to quantify the building blocks of heparin after its digestion by a mixture of heparinases I, II, and III. In addition to the compositional assessment of the 8 naturally occurring building blocks that can be used to identify the animal source, this method can be used to detect markers of polysaccharide backbone denaturation resulting from the heparin manufacture process as well as control material consistency. Here we provide a comprehensive description of key manufacturing process fingerprints, such as glycoserine derivatives, 3-O-sulfated disaccharides and tetrasaccharides, desulfated building blocks, and sulfite-modified disaccharides in order to enable crucial analysis of heparin sources and suppliers. Although not currently requested by the pharmacopoeias, it is hoped that monitoring the building blocks and fingerprints of heparin will help safeguard its quality and ensure suitability for drug product use. The method described here therefore provides a heparinoid analysis technique complementary to ^{1}H NMR spectroscopy, which strengthens the barriers against adulteration and process deviations at any stage of heparin production and LMWH-derived products.

Conflict of interest

All authors are employees of Sanofi.

Acknowledgments

This study was funded by Sanofi. Editorial support in the preparation of this article was provided by R. Spice, Excerpta Medica, funded by Sanofi.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ancr.2014.12.001.

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