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Ion-pair high-performance liquid chromatography for determining disaccharide composition in heparin and heparan sulphate

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Abstract

In this report we describe a convenient and sensitive HPLC method for separating and determining the non- and variously sulphated Δ-disaccharides derived from heparan sulphate, heparin and Fragmin, using heparin- and heparan sulphate lyases. This method is superior to others since it can separate and determine twelve different non-, mono-, di- and trisulphated Δ-disaccharides containing either N-sulphated, N-acetylated or unsubstituted glucosamine in a single HPLC run. The various types of Δ-disaccharides are separated by an ion-pair reversed-phase chromatographic procedure on a Supelcosil LC-18 column, using a binary acetonitrile gradient system with tetrabutylammonium as the ion-pairing reagent. The eluted peaks were recorded by dual wavelength at 232 and 226 nm and a linear detector response was obtained over the entire interval tested, i.e., to 50 μg of Δ-disaccharides. As little as 0.8-5 ng of Δ-disaccharides can be reliably detected and accurately determined. Following separate digestion with the heparin- and heparan sulphate lyases (heparin lyases I, II and III), the characteristic heparin Δ-disaccharides in the heparan sulphate chain, as well as the heparan sulphate Δ-disaccharides in the heparin polymer, can be identified. Using combined digestions with these three lyases, the glycosaminoglycan chains are degraded almost completely (>90%) to Δ-disaccharides, which are then determined by direct injections into the HPLC system and thus an almost complete spectrum of disaccharide composition can be obtained. By this method, it is possible to analyse and confirm that the heparan sulphate chain is defined as a glycosaminoglycan dominated by GlcNAc($\pm$6S)-GlcA disaccharides and by some copolymeric disaccharides, such as GlcNS-IdoA2S and GlcNS6S-IdoA2S, otherwise most common in heparin. Fragmin, which is a controlled depolymerized heparin fragment of M_r 5000, is made up mainly of trisulphated disaccharides of the GlcNS6S-IdoA2S type (88.8%). Using separate digestions with the specific heparin lyases, one can also distinguish between heparin and heparan sulphate.

Keywords: Heparin; Heparan sulphate; Saccharides; Glycosaminoglycans; Heparin lyases

1. Introduction
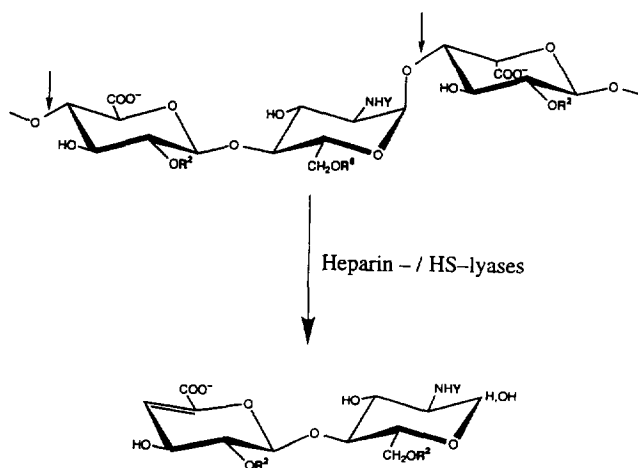
Heparin and heparan sulphate (HS) are highly charged, linear, sulphated glycosaminoglycans (GAGs) composed of a repeating disaccharide unit in which the uronic acid may be either D-glucuronic acid (GlcA) or L-iduronic acid (IdoA) and the D-glucosamine (GlcN) may be either N-acetylated

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(GlcNAc), N-sulphated (GlcNS) or unsubstituted (GlcNH). The two polysaccharides, heparin and HS, are the most highly charged polysaccharides among the naturally occurring GAGs in vertebrates, since they contain sulphate residues in both the uronic acid and glucosamine forms in a molar ratio of sulphate to disaccharide unit that usually exceeds one. Although heparin and HS have common structural features, the former contains a higher number of sulphates and IdoA residues than the latter. In addition, heparin contains sulphated glucosamine at

C-3, which is rarely found in HS. This C-3 sulphation has been shown to be an essential characteristic for the expression of heparin anticoagulation activity [1]. Chemical structures of all twelve known heparin and HS disaccharides are shown in Fig. 1.

Heparin and HS are both synthesised covalently bound to protein cores forming proteoglycans (PGs), which, for the heparin PGs, are present in intracellular granules and for the HSPGs are present both in the plasma membrane (Syndecan family) and in the extracellular matrix (Perlican) [2]. These PGs partici-

Heparin – / HS–lyases

No	Formulas	Suggested terminology	R^2	R^6	Y
1	ΔUA-[1→4]-GlcN	Δdi-nonS	H	H	H
2	ΔUA-[1→4]-GlcNAc	aΔdi-nonS	H	H	Ac
3	ΔUA-2S-[1→4]-GlcN	Δdi-mono2S	SO_3^-	H	H
4	ΔUA-2S-[1→4]-GlcNAc	aΔdi-mono2S	SO_3^-	H	Ac
5	ΔUA-[1→4]-GlcN-6S	Δdi-mono6S	H	SO_3^-	H
6	ΔUA-[1→4]-GlcNAc-6S	aΔdi-mono6S	H	SO_3^-	Ac
7	ΔUA-[1→4]-GlcNS	Δdi-monoNS	H	H	SO_3^-
8	ΔUA-2S-[1→4]-GlcN-6S	Δdi-di(2,6)S	SO_3^-	SO_3^-	H
9	ΔUA-2S-[1→4]-GlcNAc-6S	aΔdi-di(2,6)S	SO_3^-	SO_3^-	Ac
10	ΔUA-2S-[1→4]-GlcNS	Δdi-di(2,N)S	SO_3^-	H	SO_3^-
11	ΔUA-[1→4]-GlcNS-6S	Δdi-di(6,N)S	H	SO_3^-	SO_3^-
12	ΔUA-2S-[1→4]-GlcNS-6S	Δdi-tri(2,6,N)S	SO_3^-	SO_3^-	SO_3^-

a = acetylated

Fig. 1. The chemical structures of heparin and HS and of Δ-disaccharides produced by the action of heparin- and HS lyases. Δ-disaccharides differ in the patterns of sulphation and the presence of GlcNAc, GlcNS or unsubstituted GlcNH. Arrows indicate the sites cleaved by heparin- and HS lyases.

pate in many biological processes, such as blood coagulation, cell differentiation, adhesion, cell matrix interaction, recognition and extracellular matrix organization, via interactions with the GAG chains [3,4]. It is well known that heparin has been widely used during the past five decades as an anticoagulant in clinical practice. To eliminate the pathological side effects of heparin, attention in the last decade has focused on the low-molecular-mass heparins, such as Fragmin, which have better antithrombotic activity and bioavailability [5].

The sulphation of GAG chains has been shown to be an important factor in the expression and regulation of biological and physiological functions of heparin and HSPGs. The main components in heparin are GlcNS6S and IdoA2S and the most frequent unit is →4)-α-D-GlcNS6S-(1→4)-α-L-IdoA2S-(1→, while in HS the dominant components are GlcNAc and GlcA and the most common repeating units are →4)-α-D-GlcNAc($\pm$6S)-(1→4)-β-D-GlcA-)1→ and →4)-α-D-GlcNS-(1→4)-β-D-GlcA-(1→ or α-L-IdoA-(1→. This sulphation variability of heparin and HS is biosynthetically regulated by the various sulphotransferases in the Golgi apparatus, where the chain sulphation is an event that is subsequent and closely related to the C-5 epimerization of GlcA to IdoA and the deacetylation of GlcNAc. The various isomeric structures formed give the GAGs particular biological and physicochemical properties, such as those of importance to the anticoagulant activity of the heparin pentasaccharide [6]. It has been proposed [1] that the structural variability in the sulphate position is a result of non-random and controlled biosynthesis. For a better understanding of this structural variability and its biological importance, it is therefore essential to proceed to an extensive characterisation of heparin and HS structures on the disaccharide level.

The available enzymes that specifically cleave glycosidic linkages within the heparin and HS chains, resulting in Δ-di- and oligosaccharides, are heparin lyase I, commonly referred to as heparinase (EC 4.2.2.7), heparin lyase II (no EC number) and heparin lyase III, commonly referred to as heparitinase (EC 4.2.2.8). Each one of the different lyases has specificity in respect of the uronic acid moiety and the sulphation [7–12]. Their availability makes it possible to degrade heparin and HS specifically to Δ-oligosaccharide fragments. By combining these three lyases, it is also possible to cleave most of the polysaccharides to a large extent (>90%) into Δ-disaccharides [13–16]. These three lyases can therefore be used to determine the composition of disaccharides in the chain, considering their sulphation and hexuronic acid content (IdoA or GlcA). Using these enzymes, in combination with techniques such as thin-layer- and gel permeation chromatography, the disaccharide composition of HSPG from endothelial cells [17] and that of HS from mollusc [18] have been determined. Variously sized and sulphated oligosaccharides and disaccharides have been analysed by a number of HPLC techniques [19–24] and by gel electrophoresis [25–27] as well as capillary electrophoresis techniques [13–15], which have been reviewed by Novotny and Sudor [28] and El Rassi and Mechref [29]. With these techniques, the sulphation patterns of porcine intestinal mucosal heparin and HS [30] and the HSPG from human skin fibroblasts [26,31] and liver [32] have been examined.

Recently, we have reported [33,34] high-resolution HPLC and HPCE methods for the determination of the disaccharide composition of the chondroitinase-degradable GAGs, chondroitin sulphate, dermatan sulphate and hyaluronan. Our group has also described an ion-pair HPLC method [35] for the identification of longer IdoA- and GlcA-containing oligosaccharide sequences in dermatan sulphate after fragmentation with specific enzymes, such as chondroitinase ABC, AC and B. Reversed-phase ion-pair HPLC has been used previously by Linhardt et al. [9] and Loganathan et al. [36] to separate four differently sulphated Δ-disaccharides and larger oligosaccharides for testing the substrate specificity of commercially available heparin- and HS lyases. Although a modification of this method has also been successfully employed by Moffat et al. [37], several elution systems were used to determine and characterise the variously sulphated Δ-disaccharides and therefore there is still a need for the development of HPLC methodology, with which all known and commercially available heparin- and HS-derived Δ-disaccharides can be easily and accurately determined in a single HPLC run. In this paper, we extend the use of ion-pair HPLC to establish a method by

which all twelve commercially available heparin- and HS-derived Δ-disaccharides can be reliably separated and determined by a simple procedure. These Δ-disaccharides include those where the GlcN is either N-sulphated, N-acetylated or unsubstituted.

2. Experimental

2.1. Materials

Fragmin (Na$^+$ salt), a fragment of controlled heparin depolymerization with an average M_r of 5000, and heparin (Na$^+$ salt) from porcine intestinal mucosa (25 000 IU/ml) were from Pharmacia (Uppsala, Sweden). Heparan sulphate (Na$^+$ salt) from bovine kidney and heparin lyase II (heparinase II, no EC number) derived from *Flavobacterium heparinium* were purchased from Sigma (St. Louis, MO, USA). Standard preparations of twelve different sulphated Δ-disaccharides containing either GlcNAc, GlcNS or GlcNH were also obtained from Sigma. Their trivial formulae and suggested terminology are shown in Fig. 1. Heparin-lyase I (heparinase, EC 4.2.2.7) and heparin lyase III (heparitinase, EC 4.2.2.8) from *Flavobacterium heparinium* were purchased from Seikagaku Kogyo (Tokyo, Japan). Tetrabutylammonium hydrogen sulphate (TBA, 0.5 M solution, pH 7.5) was obtained from Alltech (Deerfield, IL, USA).

2.2. Enzymic degradation of GAGs

Digestions of HS, heparin and Fragmin were performed in 20 mM acetate buffer, pH 7.0, containing 1 μmol of calcium acetate and 0.05 units each of heparin lyases I, II and III per 25 μg of uronic acid, as determined by the borate–carbazole method of Bitter and Muir [38], at 37°C overnight [15,39]. Digestions were terminated by boiling for 1 min, and following centrifugation in a Beckman (San Raman, CA, USA) microfuge (10 000 g) for 5 min, aliquots were taken for direct ion-pair HPLC analysis of variously sulphated Δ-disaccharides.

2.3. HPLC conditions for the separation of variously sulphated Δ-disaccharides

Separation of the variously sulphated Δ-disaccha-

rides, derived from the degraded polymers by the various heparin- and HS lyases, was carried out by ion-pair reversed-phase chromatography. This chromatography was performed on a Beckman HPLC system, using a 250×4.6 mm I.D. Supelcosil LC-18 (Supelco, Bellefonte, PA, USA) column connected to a 40×4.6 mm I.D. RP-18 precolumn (Brownlee Labs.). A gradient elution was performed, using a binary solvent system composed of 20% (v/v) aqueous acetonitrile (eluent A) and 75% (v/v) aqueous acetonitrile (eluent B), both containing 0.01 M TBA (the ion-pairing reagent)–phosphate at a final pH of 6.7, prepared as previously described [35]. The flow-rate was 1.2 ml/min and the following elution programme was used: Isocratic elution with 100% A for 8 min, gradient elution to 53.3% B over 15 min, followed by another gradient elution to 100% B over 20 min. The column was then washed and re-equilibrated by further elution with 100% B for 2 min, returning to 100% A over 1 min and continuing with 100% A for 10 min. The absorbance of the column eluate was monitored at 232 and 226 nm and the peak heights and/or peak areas were recorded by a Hitachi recording integrator.

2.4. Determination of Δ-disaccharides by HPLC

The types of the various Δ-disaccharides, produced by the action of heparin- and HS lyases, on heparin, Fragmin and HS, were determined by coinjecting standard disaccharides, and their composition comparing the peak heights and/or peak areas obtained with those from external standard disaccharides. The total amount of Δ-disaccharides produced by the combined actions of the three lyases on heparin, Fragmin and HS were also determined on the basis of their absorbance at 232 nm ($\varepsilon_{232} = 5500 \ M^{-1} \ cm^{-1}$) [15], due to the double bond of the unsaturated $\Delta^{4,5}$ site of the uronic acid at the non-reducing end, in conjunction with the carboxyl group at C-5.

2.5. Linearity and detection sensitivity

The linearity of the detector response was tested using precisely known amounts of all Δ-disaccharides dissolved in digestion buffer to give a stock solution containing 1.6 mg/l of each. Standard Δ-disaccharide solutions of 0.005, 0.01, 0.1, 0.25, 0.5,

1.0, 2.0, 4.0, 8.0, 16.0, 32.0 and 50.0 μg/ml were then prepared by appropriate dilutions of the stock solution. The calibration graphs were constructed by plotting the peak heights of each Δ-disaccharide signal against their concentration. The detection limit was estimated as the quantity of Δ-disaccharide giving a signal-to-noise ratio of 2:1.

2.6. Agarose gel electrophoresis

The different specificities of the various heparin- and HS lyases used were evaluated by gel electrophoresis, using an Oncor horizontal electrophoresis apparatus with a 1% agarose gel, according to the procedure of Bjornsson [40]. Electrophoresis was performed in 10 mM Tris–acetate, pH 7.3, at 90 V for 60 min and the polysaccharides were stained with 0.02% (w/v) toluidine blue in 3% acetic acid containing 0.5% (v/v) Triton X-100 and the gel was destained in 3% acetic acid.

3. Results and discussion

3.1. Resolution of variously sulphated Δ-disaccharides

The method presented in this paper is a modification of those methods previously described [9,26,37]. It differs in the use of a gradient elution system and a separating column, such that all twelve known Δ-disaccharides could be separated and determined in a single run. The chromatogram obtained from the ion-pair HPLC for the standard non-, mono-, di- and trisulphated Δ-disaccharides containing either GlcNAc, GlcNS or GlcNH are shown in Fig. 2. All tested Δ-disaccharides, with various numbers of sulphate groups as well as with differences in their polarities because of the presence or absence of acetyl groups at the $-NH_2$ group of glucosamine, were completely separated under these conditions, giving sharp peaks without peak broadening. As shown in Fig. 2, the increased number of sulphate groups resulted in higher retention times. This is explained by the higher number of TBA cations that interact with the anionic charges of the saccharides – the carboxyl and the sulphate groups – and the hydrophobic sites of the C_{18} column.
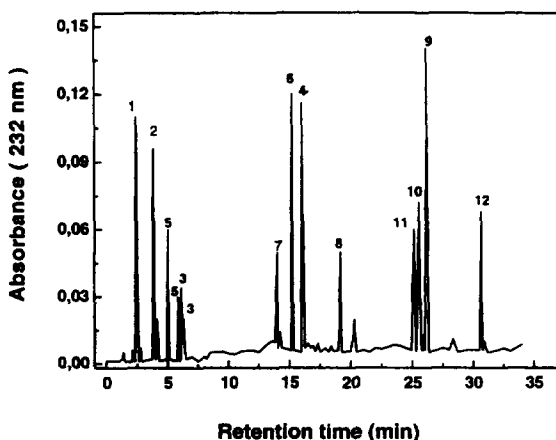

Fig. 2. Typical HPLC profile of non- and variously sulphated heparin and HS Δ-disaccharides obtained by ion-pairing reversed phase HPLC. The peaks were recorded at 232 nm. The column (Supelcosil LC-18) was eluted with a binary acetonitrile gradient system, as described in Section 2.

3.2. Method repeatability, linearity and detectability

The retention times of the various Δ-disaccharides found by performing analyses over a three-month period using the same column, but with different analysts, are reported in Table 1. These results indicated that the method described had excellent repeatability, which is a problem commonly associated with analyses in gradient HPLC runs.

Table 1
Retention times obtained for heparin and HS Δ-disaccharides by ion-pair reversed-phase HPLC[a]

Disaccharide type	Retention time(s)[b]±S.D.
Δdi-nonS	2.4±0.07
aΔdi-nonS	3.8±0.1
Δdi-mono6S	5.0/5.75±0.12/0.12
Δdi-mono2S	6.1/6.3±0.15/0.15
Δdi-monoNS	14.0±0.2
aΔdi-mono6S	15.2±0.25
aΔdi-mono2S	16.0±0.3
Δdi-di(2,6)S	19.15±0.4
Δdi-di(6,N)S	25.1±0.5
Δdi-di(2,N)S	25.55±0.55
aΔdi-di(2,6)S	26.1±0.6
Δdi-tri(2,6,N)S	31.15±0.65

[a] Values are the average of twelve HPLC runs. Variations in retention times were less than 5% for all Δ-disaccharides.
[b] Double peaks with two retention times most probably represent the separated α- and β-anomeric forms of Δ-disaccharides.

The sensitivity and linearity tests performed for all available Δ-disaccharides showed that the peak heights and the peak areas obtained were linear up to 50 μg of each Δ-disaccharide injected into the column, i.e., over the entire interval tested (Fig. 3). The precision of the method was tested by nine repeated determinations of all Δ-disaccharides. Therefore, when 2 nmol of each Δ-disaccharide was measured, the relative standard deviations for the various Δ-disaccharides ranged from 2.7 to 3.6% of the registered value, showing that the method is precise. The detection limits of Δ-disaccharides ($\varepsilon_{232} = 5500$ M cm^{-1}), expressed as twice the baseline noise, corresponded to 0.8 to 5 ng, depending on the disaccharide type. Thus, those disaccharides bearing GlcNAc were more sensitive than those lacking the acetyl group. The detectability of the latter, however, can be increased to the same level as that of the acetylated group by measuring their absorbance at 226 nm (not shown). Therefore, dual wavelength recording is also more useful for detecting and reliably determining the non-acetylated and/or N-sulphated Δ-disaccharides. To obtain an accurate determination of the disaccharide composition in triplicate, within a 95% confidence interval, as little as 0.1 μg of the GAG is required.

3.3. Degradation of HS, heparin and Fragmin with heparin and HS lyases and determination of disaccharide composition

This study on the structures of heparin and HS was based on the different specificities of the three lyases, the actions of which have been reviewed by Dietrich et al. [7] and more recently in Ref. [41], which uses the excellent reports by Desai et al. [10,11], Jandick et al. [12], Rice and Linhardt [8] and Linhardt et al. [42]. The disaccharides produced by the action of the three lyases on heparin and HS have been reported by Griffin et al. [30]. These
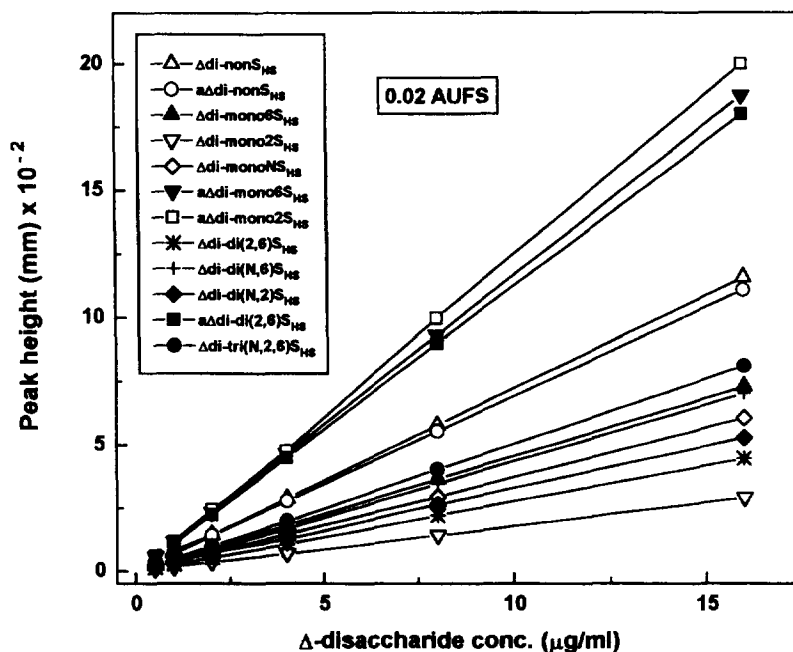

Fig. 3. Calibration curves for the analysis of disaccharides 1–12, showing detector response, expressed as peak heights versus Δ-disaccharide concentrations (μg/ml). The detector response (AU $2 \cdot 10^{-2}$) corresponds to the following equations for the respective disaccharides: 1, $y=(4.4\pm2.0)+(71.9\pm0.3)x$; 2, $y=(1.9\pm0.9)+(69.2\pm0.1)x$; 3, $y=(-1.3\pm0.8)+(18.1\pm0.1)x$; 4, $y=(-7.7\pm4.5)+(125.4\pm0.6)x$; 5, $y=(-0.6\pm2.0)+(71.9\pm0.3)x$; 6, $y=(-0.6\pm0.35)+(45.7\pm0.05)x$; 7, $y=(-1.9\pm1.8)+(37.8\pm0.2)x$; 8, $y=(-0.65\pm1.1)+(27.7\pm0.15)x$; 9, $y=(-0.5\pm0.9)+(112.5\pm0.1)x$; 10, $y=(23.2\pm10.35)+(31.2\pm1.4)x$; 11, $y=(-2.6\pm1.5)+(43.9\pm0.2)x$; 12, $y=(1.0\pm0.7)+(50.6\pm0.1)x$.
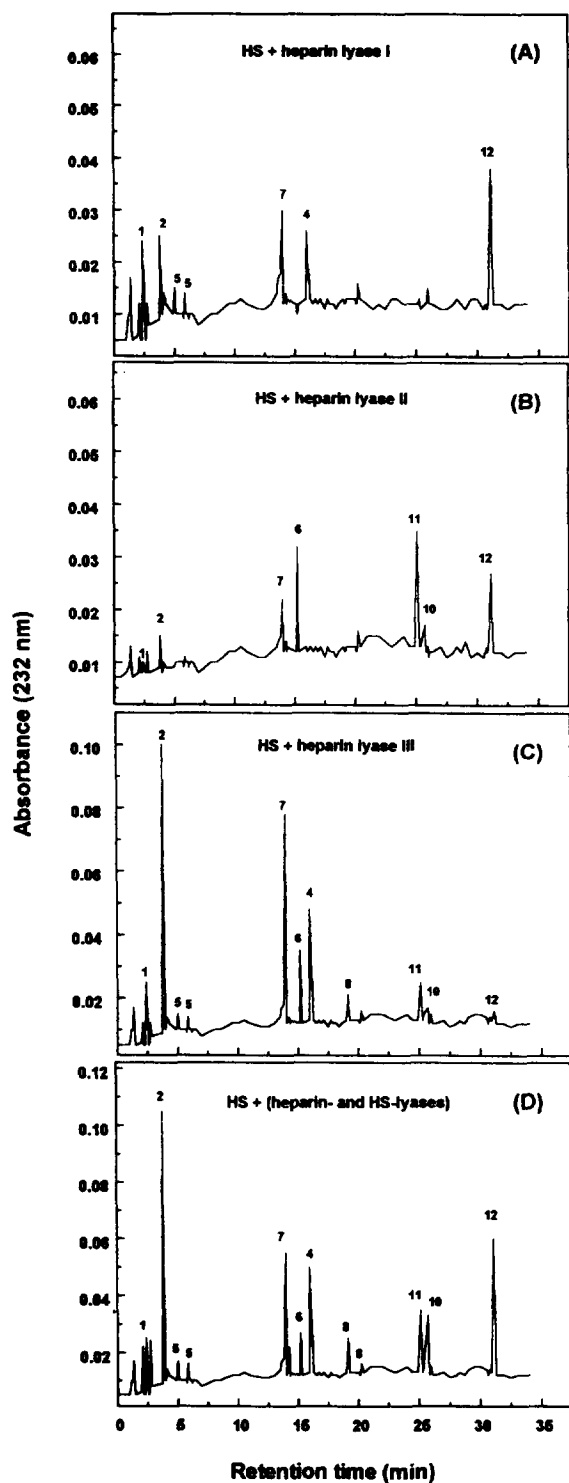
articles have clearly established that heparin lyase III (heparitinase), otherwise referred to as HS lyase, catalyses the eliminative degradation of only HS at the glycosidic linkage between GlcNAc($\pm$6S) or GlcNS($\pm$6S) and GlcA. Heparin lyase I, or heparinase, catalyses the eliminative cleavage of heparin at the glycosidic linkage: GlcNS($\pm$6S) ($\pm$3S) α1-$\downarrow$-4IdoA2S as well as the cleavage of HS, including this heparin-like structure. This enzyme produces the more highly sulphated disaccharides Δdi-tri(2,N,6)S and Δdi-di(2,N)S, which are dominant in heparin and of low frequency in HS. Heparin lyase II is an enzyme of broader specificity. It catalyses the eliminative cleavage of heparin and HS at the glycosidic linkage: GlcNAc($\pm$NS) ($\pm$6S) ($\pm$3S)α1-$\downarrow$-4IdoA($\pm$2S) or GlcA($\pm$2S), resulting primarily in disaccharides that have a higher level of sulphation (one to three sulphates per disaccharide unit) than those obtained by heparin lyase I.

Various studies have been performed on the specificity of the various lyases on HS and heparin by polyacrylamide gel electrophoresis (PAGE) [30] and on composition of the disaccharides by capillary electrophoresis [13,14,30,31,43]. In this study, we examined these specificities on HS, heparin and Fragmin by ion-pair HPLC and agarose gel electrophoresis. Ion-pair HPLC is useful for the determination of disaccharide composition and electrophoresis is useful for the identification of high molecular mass fragments that are not degradable by the various lyases. Thus, heparin, Fragmin and HS were treated with each lyase separately and also with a mixture containing an equal amount of the three heparin lyases I, II and III (Figs. 4–6).

Agarose gel electrophoresis showed that heparin lyases I and II acted only to a small extent on the substrate, HS, giving HS fragments with large molecular masses and with slightly lower mobility than that of intact HS (not shown). Ion-pair HPLC of these digests also showed that the susceptibility of HS to degradation was low, because of the low yield

Fig. 4. Chromatograms showing the analysis of lyase-treated HS. This GAG was treated with heparin lyase I (heparinase) (A), heparin lyase II (B), heparin lyase III (C) and a mixture of the three lyases (D). Peaks were confirmed by coinjecting the sample with standard Δ-disaccharides (see Fig. 1 for peak identity).
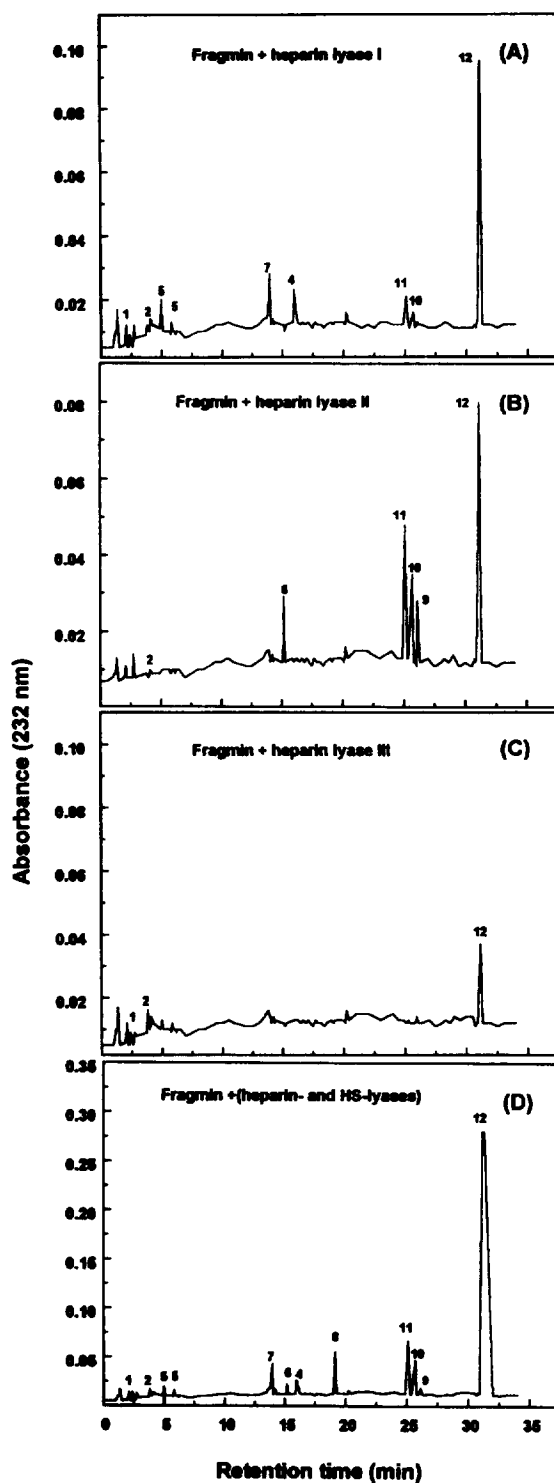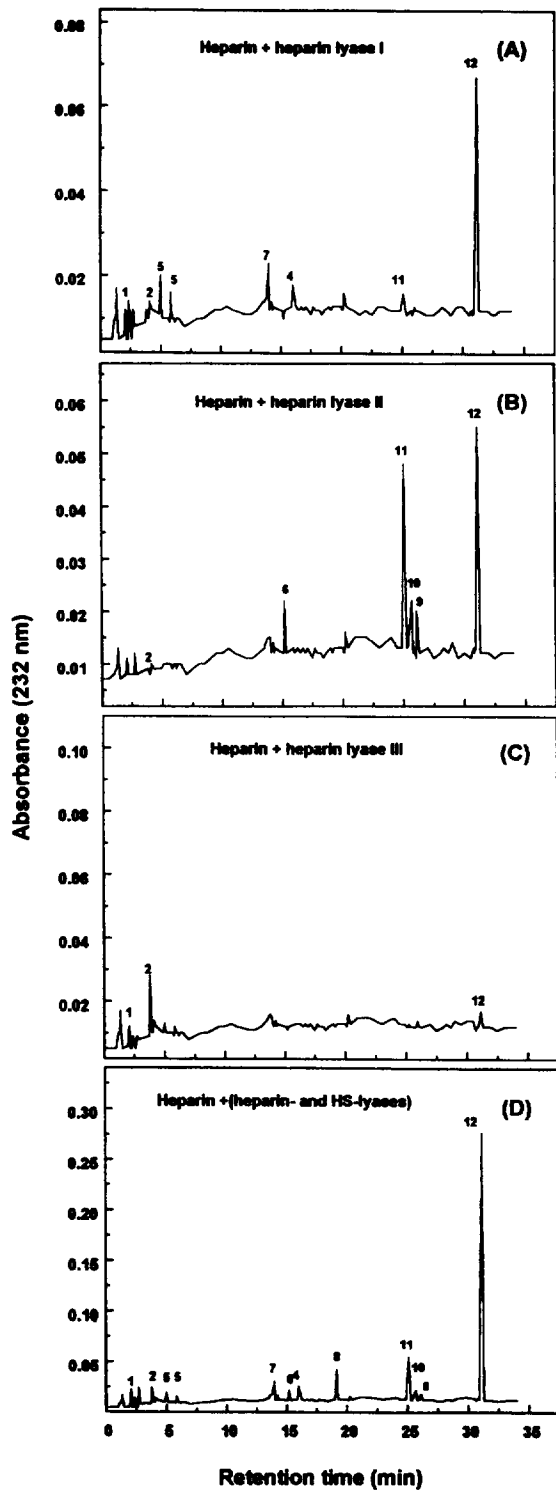
of Δ-disaccharides obtained in the chromatogram (32 and 38%, respectively). The disaccharides obtained by heparin lyase I consisted mainly of Δdi-tri(2,6,N)S (Fig. 4A, peak 12). Small portions of Δdi-monoNS (peak 7) and aΔdi-mono2S (peak 4) were also obtained (Fig. 4A). These results are in good agreement with those reported by Griffin et al. [30]. The action of heparin lyase II on HS resulted in Δ-disaccharides which, in addition to those obtained by heparin lyase I, involve Δdi-di(6,N)S (peak 11), Δdi-di(2,N)S (peak 10) and aΔdi-mono6S (peak 6; Fig. 4B).

Heparin lyase III degraded HS to a large extent, resulting in a very weakly stained HS fragment of high electrophoretic mobility (not shown). The action of this lyase on HS converted the polysaccharide to Δ-disaccharides (approximately 87%) and resulted mainly in aΔdi-nonS (major disaccharide, peak 2) and Δdi-monoNS. Considerable amounts of aΔdi-mono6S were also obtained (Fig. 4C). These results, in combination with the known specificities of heparin lyases, suggest that HS is dominated by GlcNAc (±6S)-GlcA, whereas it contains low amounts of GlcNS-IdoA2S as well as oversulphated disaccharides with GlcNAc-GlcA or IdoA as the basic core unit.

Heparin and Fragmin were much more sensitive than HS to heparin lyase I, giving Δdi-tri(2,6,N)S (Fig. 5A and Fig. 6A, peak 12) as the major peak and smaller amounts of mono- and non-sulphated disaccharides, in accordance with the lower content of the latter disaccharides in heparin preparations. Heparin lyase II degraded heparin and Fragmin to a much higher extent (84 and 88%, respectively), producing more Δ-disaccharides than those obtained with heparin lyase I (Fig. 5B and Fig. 6B). Thus, the action of this lyase on heparin resulted in high

amounts of Δdi-tri(2,6,N)S (peak 12), which was more dominant in Fragmin, as were Δdi-di(6,N)S (peak 11) and also Δdi-di(2,N)S (peak 10), aΔdi-di(2,6)S (peak 9) and aΔdi-mono6S (peak 6) (Fig. 5B and Fig. 6B). Heparin lyase III cleaved heparin and Fragmin, producing low amounts of Δ-disaccharides (11 and 14%) dominated by Δdi-tri(2,6,N)S and aΔdi-nonS (Fig. 5C and Fig. 6C). Taking into account the specificity of heparin lyases I, II and III and the results obtained by HPLC using these lyases separately, one may conclude that heparin is dominated by GlcNS (±6S)-IdoA2S, whereas units of the type GlcNAc (±6S)-GlcA have low frequencies.

Using all three lyases in combination lead to HS, heparin and Fragmin being degraded completely, since neither macromolecular material nor oligomers were identified following gel electrophoresis (not shown). Treatment of all GAGs tested with a mixture containing equal amounts of all three lyases resulted in Δ-disaccharide products corresponding to all of the disaccharides, except Δdi-mono2S and aΔdi-di(2,6)S for HS (Fig. 4D) and Δdi-mono2S for heparin and Fragmin (Fig. 5D and Fig. 6D). The dominant disaccharide in HS was aΔdi-nonS, whereas in heparin and Fragmin, it was Δdi-tri(2,6,N)S. It should be noted that in heparin and Fragmin very small amounts of aΔdi-nonS are also present, but this does not correspond to more than one residue per heparin chain. Fragmin differed from heparin, as the former contained higher amounts of Δdi-tri(2,6,N)S (peak 12) and Δdi-di(2,N)S (peak 10) (Fig. 6D).

The disaccharide compositions of HS, heparin and Fragmin were determined by the chromatogram obtained using combined treatment with all three lyases (Fig. 4D, Fig. 5D and Fig. 6D). The disaccharide compositions of the GAGs tested are shown in Table 2. Values obtained for HS and heparin were closely related to the disaccharide composition obtained with other, previously reported, sulphation patterns studied by capillary electrophoretic methods [10,12]. It should be noted here that the commercially available bovine kidney HS preparation that we tested seems to be a mixture of HS and heparin, however, with much more HS than heparin, due to the much higher content of aΔdi-nonS and the lower content of Δdi-tri(2,N,6)S. Therefore, one should, perhaps, take into account the results concerning the disaccharide composition re-

Fig. 5. HPLC analysis of lyase-treated heparin. The polysaccharide was treated with heparinase (A), heparin lyase II (B), heparin lyase III (C) and a mixture of the three lyases (D) (see Fig. 1 for peak identification).

Fig. 6. Elution profiles showing the analysis of lyase-treated Fragmin. Fragmin was treated with heparinase (A), heparin lyase II (B), heparin lyase III (C) and a mixture of the three lyases (D) (see Fig. 1 for peak identification).

Table 2
Composition of Δ-disaccharides (in %) in heparin, Fragmin and HS[a]

Disaccharide	Heparin	Fragmin	HS
Δdi-nonS	0.15 (−)	tr[b] (−)	0.8 (−)
aΔdi-nonS	0.2 (0.15–11.1)[c]	0.1 (−)	34.5 (28.2–58.7)
Δdi-mono6S	3.5 (−)	1.7 (−)	0.7 (−)
Δdi-mono2S	ND[d] (−)	ND (−)	ND (−)
Δdi-monoNS	1.2 (0.8–2.9)	0.6 (0.5)	10.0 (5.5–20.9)
aΔdi-mono6S	2.5 (2.4–4.1)	1.2 (0.3)	6.0 (5.9–10.8)
aΔdi-mono2S	1.5 (≤1.8)	0.7 (0.4)	8.0 (≤8.9)
Δdi-di(2,6)S	2.35 (≤2.15)	1.2 (−)	11.0 (≤10.1)
Δdi-di(6,N)S	12.0 (10.3–14.4)	3.0 (3.2)	10.5 (2.5–11.2)
Δdi-di(2,N)S	4.7 (3.9–5.2)	4.7 (4.9)	9.8 (1.4–4.3)
aΔdi-di(2,6)S	3.9 (2.1–4.8)	2.6 (1.9)	ND (−)
Δdi-tri(2,6,N)S	68.0 (50.3–68.3)	84.2 (88.8)	8.7 (1.6–29.5)

[a] Results are the average of three separate experiments and are expressed as per cent of the total disaccharides recovered by HPLC after combined digestions with heparin lyases I, II and III. Less than 5% variations were noted in all cases.
[b] Traces.
[c] Values in parentheses for HS and heparin represent the range of literature values [12,13,15], whereas those for Fragmin are compared with those reported by Desai et al. [44].
[d] Not detected.

ported by Griffin et al. [30]. Thus, the HS we tested seems to resemble a mixture of HS-100 fractions 2, 3 and 4, prepared by Griffin et al. [30]. On the other hand, the disaccharide composition of the porcine intestinal mucosa heparin preparation is closely related to that of pure heparin (HS-100-5), prepared according to Griffin et al. [30], except that the former contains lower amounts of Δdi-tri(2,N,6)S than the latter, suggesting a commercially available preparation of pure heparin. Fragmin was found to be composed predominantly of Δdi-tri(2,6,N)S (88.8%, Table 2). This high content is in good agreement with the results obtained by Desai et al. [44], who, using capillary electrophoretic analysis of Fragmin, showed that this pharmaceutical preparation of heparin fragments (as well as others of low molecular mass) contained approximately 73–88% of Δdi-tri(2,N,6).

This report presents a method for determining the disaccharide composition of the Δ-disaccharides produced by separate action with heparin and HS lyases on heparin, Fragmin and HS. In all cases, the sulphation pattern of each liberated Δ-disaccharide could be determined directly by ion-pair chromatography, which is based on the number of TBA cations bound to charged sulphate and carboxyl groups. The HPLC method described is an accurate and precise technique that can be used easily to determine

heparin, low molecular mass heparins and the disaccharide composition of HS, using a mixture containing equal amounts of heparin lyases I, II and III in a HPLC run of 35 min and at a GAG level of a few ng.

Acknowledgments

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