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Use of graphitised carbon negative ion LC–MS to analyse enzymatically digested glycosaminoglycans

Niclas G. Karlsson[a,*], Benjamin L. Schulz[a], Nicolle H. Packer[a], John M. Whitelock[b]

[a] *Proteome Systems Ltd., Unit 1, 35-41 Waterloo Road, North Ryde, 2113 Sydney, NSW, Australia*
[b] *Graduate School of Biomedical Engineering, University of New South Wales, Kensington, 2052 Sydney, NSW, Australia*

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Abstract

Capillary liquid chromatography–mass spectrometry using graphitised carbon stationary phase and ion trap mass spectrometry was shown to be a powerful technique for analysing glycosaminoglycans digested with endoglycosidases. Commonly found disaccharides from heparin/heparan sulphate digests at sub nanomole levels were found to be separated by mass and/or retention time and detected by negative ion electrospray mass spectrometry predominantly as $[M-H]^-$ ions using a standard electrospray interface and flow rate between 6–10 μL/min. Graphitised carbon liquid chromatography–fragmentation mass spectrometry provided sequence data of disaccharides and oligosaccharides. Sequence information was obtained from either collision of the $[M-H]^-$ ions (low sulphated disaccharides) or of the $[M+Na-2H]^-$ ions (highly sulphated disaccharides). This separation and identification method of endoglycosidase digestion and sample preparation using a combination of cation exchange and graphitised carbon, was used to successfully analyse digests of keratan sulphate (keratanase) and heparin (heparinase) standards, and hyaluronic acid (hyaluronidase) from synovial fluid samples.
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1. Introduction

Glycosaminoglycans are a complex group of polysaccharides that decorate the protein component of proteoglycans, usually by linkage to an oxygen on serine residues that are present in predefined and carefully controlled sequences on the protein core. Proteoglycan GAGs include chondroitin sulphate (CS), dermatan sulphate (DS), hyaluronan (HA), heparan sulphate (HS) and heparin (HP) which all consist of long and varying polymeric chains made of repeating disaccharide units of alternating hexosamine and hexuronic acids. Hyaluronan (HA) is different to other glycosaminoglycans in that it is produced as a free glycosaminoglycan and is secreted by many types of cells directly into the extracellular environment. Keratan sulphate (KS) is also a glycosaminoglycan, however, it is synthesised as repeating units of *N*-acetylglucosamine and galactose and has been shown to be present on N-linked as well as O-linked structures [1]. A further level of complexity is added to these biopolymers via the activity of a family of deacetylases and sulphotransferases, which alter the addition of sulphate and acetyl groups on various positions on the lengthening polymeric chain and an epimerase, which converts glucuronic acid residues into iduronic acid residues. The action of this epimerase is critical in the production of DS, HS and HP as these three families have various amounts of iduronic acid in their chains, which have been shown to be critical for many of their suggested biological interactions. HP is a specific HS produced by mast cells that decorates the proteoglycan serglycin. It has the same basic structure as HS except that it is produced mainly in a highly sulphated form that consists of large stretches of tri-sulphated disaccharides [2]. It has been shown to interact with many proteins on an ionic basis and is poorly characterised with respect to the length of the individual polymer

* Corresponding author. Tel.: +61 2 9889 1830; fax: +61 2 9889 1805.
E-mail address: Niclas.Karlsson@proteomesystems.com (N.G. Karlsson).

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chains. This has led investigators to suggest that many of these interactions are “non-specific”. HS, on the other hand, is present in the pericellular and extracellular space decorating proteoglycans both on the cell surface, like the glypicans and syndecans [3] and in the matrix such as perlecan [4].

The variable length of the glycosaminoglycan chains isolated from cells and tissues together with the variability in sulphation (and hence charge), and the low levels usually present has forced many investigators to work with heterogeneous GAG samples that were isolated from a pool of the different types of proteoglycans, which has made detailed analysis very challenging. Compositional analysis has taken advantage of a group of bacterial endoglycosidases that depolymerise the glycosaminoglycan chains into their constituent disaccharides. These disaccharide fractions can then be analysed by conventional gel electrophoresis [5], or HPLC methods followed by comparison with standards [6]. Mass spectrometric (MS) methods have also successfully been applied to GAG analysis. Due to the presence of sulphate and hexuronic acid in these molecules, negative ion mode MS has usually been the method of choice. Historically, fast atom bombardment and liquid secondary desorption/ionisation techniques for analysing glycosaminoglycans or depolymerised glycosaminoglycans [7–10], have been substituted by negative ion electrospray (ES) mass spectrometry [11–18]. Matrix assisted time-of-flight (MALDI-TOF) mass spectrometry has also been successfully used for this analysis [19], including coupling of the negatively charged glycosaminoglycans to positively charged polypeptides enabling glycosaminoglycans to be detected in positive ion mode [20,21]. The sulphate groups attached to GAG oligosaccharides have shown to provide a challenge for MS. Sulphate is easily lost from the oligosaccharide due to in-source decay and “ghost” oligosaccharide from pseudomolecular ions of undersulphated compunds are detected when highly sulphated oligosaccharides are analysed. In addition, the liability of sulphate hinders sequencing of oligosaccharides by MS/MS since the loss of sulphate becomes the major fragmentation, and glycosidic sequence ions are scarce or absent in the MS/MS spectra. In order to minimize the in-source desulphation, mild declustering in the ion source needs to be applied [22], and increasing the amount of negative charge on the pseudomolecular ions has been shown to improve MS/MS quality [17,18,22].

Since isomeric oligosaccharides are generated in ezymatic GAG digests there is a need for up-front separation of the isomers for conclusive sequence assignment by MS/MS. We prefer to do this by LC–MS/MS. While normal phase amino columns have successfully been employed for this type of separation [23], recent progress with reversed phase chromatography together with ion-pairing of glycosaminoglycan derivatives with hydrophobic amines in the mobile phase shows promise [24,25]. Capillary electrophoresis-MS may also provide an alternative [12,26]. However, in all these approaches there are significant technical aspects that could prevent researchers within the field of GAG analysis to adopt it as a standard approach for analysis of GAG digests. We have previously shown that graphitised carbon LC–MS is a robust method for analysing commonly found O-linked and N-linked oligosaccharides released from glycoproteins [27,28], and as such could also be an attractive method for analysing GAG oligosaccharides. The disaccharides released from enzymatic depolymerisation present unique analytical challenges because of this high degree of acidity, sulphation and isomerization. This paper describes the analysis of depolymerised HS disaccharide standards using separation on graphitised carbon LC, with which isomers are shown to be baseline resolved prior to MS and MS/MS fragmentation

By incorporating specific digestion protocols for HP, HA and KS with their respective endoglycosidase, we extend the methods to the analysis of complex mixtures of GAG derived oligosaccharides. We are planning to expand these studies to include GAG samples isolated from a single biological proteoglycan with the aim of elucidating structures and establishing a simple and robust method for analysis of GAGs.

2. Experimental

2.1. Reduction of heparan sulphate disaccharide standards

Heparan sulphate standards (Table 1) (Sigma–Aldrich, St Louis, MO) (10 μg) were reduced in 20 μL 0.25 M sodium borohydride in 25 mM sodium hydroxide for 8 h at 50 °C. The reduction was quenched by adding 1 μL of glacial acetic acid and the oligosaccharides were desalted on a cation exchange microcolumn (10 μL AG50W-X8 (Biorad, Hercules, CA) packed in a ZipTip (Millipore, Bedford, MA) and eluted with 100 μL of water. After rotational evaporation of water, the remaining borate complex was removed by repeated extraction/evaporation in 1% acetic acid in methanol [27]. The dried disaccharides were dissolved in water (100 ng/mL) and analysed by LC–MS.

Table 1
Recovery of mixture of heparan-sulfate derived disaccharides after digestion and reduction, by cation exchange zip-tipping and carbon zip-tipping

Component	Sequence	[M − H]	% Yield
IV-A	ΔUA → GlcNAcol	380.2	47 ± 7
III-A	ΔUA-2S → GlcNAcol	460.2	41 ± 25
II-A	ΔUA → GlcNAcol-6S	460.2	53 ± 25
I-A	ΔUA-2S → GlcNAcol-6S	540.0	48 ± 24
II-S	ΔUA → GlcNSol-6S	498.0	30 ± 12
III-S	ΔUA-2S → GlcNSol	498.0	53 ± 18
I-S	ΔUA-2S → GlcNSol-6S	577.9	32 ± 12
	All		44 ± 18

2.2. Digestion of keratan sulphate and heparin

Keratan sulphate (bovine cornea, Seikagaku, Tokyo, Japan) (1 μg) or porcine heparin (10 μg) were digested with 10 milliunits keratanase (Seikagaku) or heparinase III (Seikagaku), respectively, in 10 μL PBS at 30 °C over night. Depolymerised oligosaccharides were reduced by adding 20 μL of 0.5 M sodium borohydride/50 mM sodium hydroxide and incubated at 50 °C for 12 h. After this time, the reduction reaction was halted with the addition of 1 μL of glacial acetic acid. The samples were desalted using cation exchange resin in micro-columns (as described above) and lyophilised. Dried samples were re-suspended in 5 μL water and desalted again using graphitised carbon micro-columns [28]. Graphitised carbon (∼1 μL) was added to a 5 μm C18 ZipTip. The column was washed with 20 μL of 0.1% trifluoroacetic acid in 90% acetonitrile, followed by 10 μL water. The applied sample was washed with 5 μL water, eluted with 10 μL 0.1% trifluoroacetic acid in 40% acetonitrile, dried, and re-suspended in 10 μL water for analysis using LC–MS.

2.3. Digestion of hyaluronic acid

Synovial fluid (100 μL) samples from a patients with osteoarthritis were reduced by 20 mM DTT in 0.375 M Tris–HCl buffer pH 8.8, 1% SDS and 10% glycerol (900 μL), and heated at 95 °C for 20 min. The sample was cooled and alkylated with 50 mM iodoacetamide at room temperature for 1 h. The sample (10 μL) was dot-blotted onto Immobilon PSQ (Millipore). The volume was allowed to dry and the dried area was cut out and incubated in 50% polyvinyl pyrolidone (Sigma) in 50% methanol (20 min), and washed with 3 μL × 100 μL of water, 5 min each wash. Hyaluronidase (Bovine testis, Sigma) was added (50 μL/10 units) in PBS containing 0.01% BSA, and incubated overnight at 37 °C. Samples were desalted using carbon micro-desalting columns as described above followed by washing with 20 μL of 25% acetonitrile, 0.1% trifluoroacetic acid, which eluted the bound oligosaccharides. Samples were then dried, redissolved in 10 μL water and subjected to LC–MS analysis.

2.4. Liquid chromatography–mass spectrometry analysis

Graphitised carbon (5 μm particles) liquid chromatography was performed on either a 100 mm × 0.32 mm column (ThermoHypersil, Runcorn, UK) or a 100 mm × 0.30 mm ProteCol column (SGE, Ringwood Australia). Chromatography (Surveyor HPLC and autosampler, ThermoFinnigan, San Jose, CA) was performed using a linear gradient of acetonitrile from 0 to 24% over 30 min in 20 mM ammonium bicarbonate, followed by a wash of 80% acetonitrile for 10 min. The flow rate was 4–7 μL/min through the column and samples of 10 μL were injected and analysed. Mass spectrometry was performed on an LCQ-XP+ (ThermoFinnigan) using the standard electrospray interface with a spray voltage of 3.5 kV. The mass spectrometer was scanned from *m*/*z* 200–800 followed by a data-dependent MS^2 scan of the most intense ion from the previously recorded full scan spectrum. Optimisation was performed using the trisulphated disaccharide I-S, where we found that an elevated temperature (380 °C) of the capillary was optimal for detection of the $[M - H]^-$ and gave less in-source desulphation. Fragmentation of all heparin disaccharide standards was found to be optimal between 35–40% normalised collision energy, where a lower energy resulted only in a higher amount of remaining precursor ion, with no significant difference in the relative fragment intensity, but a general lower intensity. Higher collision energy resulted in fragment ions of lower *m*/*z* values indicating an increase of multiple fragment cleavage events.

3. Results

3.1. Separation of heparin disaccharides

Graphitised carbon LC–MS has been used by us previously to analyse oligosaccharides isolated from both N- and O-linked glycoproteins. We have found that this chromatographic medium was capable of separating both isomeric sialylated as well as sulphated oligosaccharides, so we therefore thought that this approach could also be applicable to analyse the sulphated and acidic GAGs after specific endoglycosidase digestions. In the case of HS, endoglycosidase treatment with heparitinases (or heparinases) will generate disaccharides that have different N-sulphate or O-sulphate groups. A complication is that isomeric disaccharides may be O-sulphated in various positions. Hence, individual disaccharides cannot be identified by mass spectral analysis alone and fragmentation analysis (MS^2) was necessary to distinguish them. Graphitised carbon LC was capable of retaining and separating standard heparin disaccharides, with some disaccharides eluting as broad peaks or doublets (data not shown). This was overcome by chemical reduction of the oligosaccharides with borohydride to their alditols, suggesting that the graphitised carbon column could separate individual HS disaccharides differing only by the anomeric configuration of the C1 carbon on the reducing end GlcNAc. Hence, samples of reduced HS disaccharide were separated on graphitised carbon using a shallow acetonitrile gradient (0–24%) in a 20 mM ammonium bicarbonate buffer (Fig. 1). The two pairs of isomeric disaccharides II-A/III-A (Fig. 1C) and II-S/III-S (Fig. 1D) differing only in position of the sulphate were separated by the chromatographic conditions, and where the chromatographic resolution provided only partial separation, mass separation provided by the mass spectrometry provided additional resolution allowing MS^2 experiments to be performed. Analysis of each of these HS standards under these chromatographic conditions allowed us to distinguish between desulphation present in

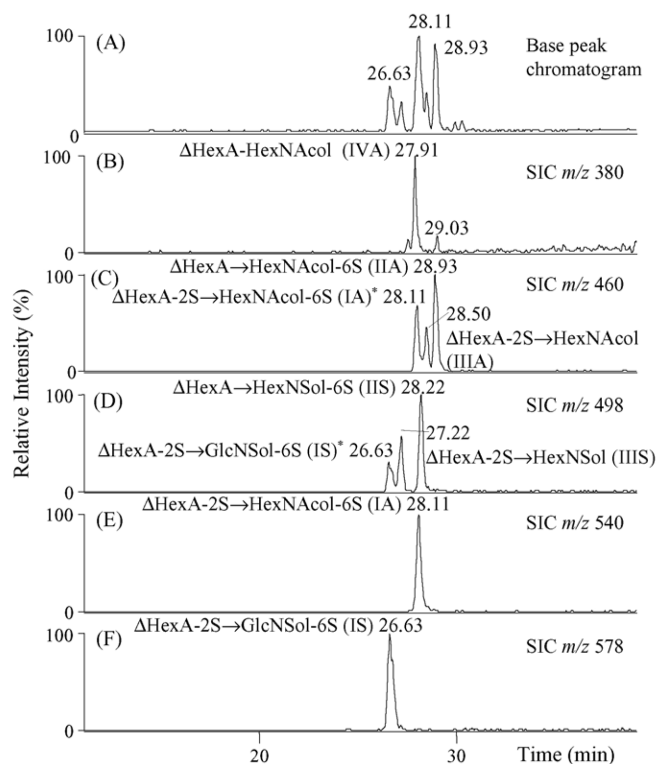


Fig. 1. Separation and detection of heparan disaccharides (100 ng each) using negative ion graphitised carbon LC–MS. Panes include base peak chromatogram and single ion chromatograms (SIC) of detected $[M-H]^-$ ions. Structures assigned with an asterisk (*) indicate that these structures were detected in these SIC due to in-source fragmentation with loss of sulphate (S) as $[M-H-S]^-$ ions.

our standards, and in-source desulphation induced by the electrospray conditions. This latter effect is identified by the compound having a single chromatographic retention time with two masses separated by 80 Da detected after ionisation in the MS. In the case of highly sulphated standards, it was not possible to totally prevent the loss of sulphate in the MS. For the disulphate standard I-A (RT 28.11 min, Fig. 1E) and the trisulphated standard I-S (RT 26.63 min, Fig. 1F), it was found that level of the $[M-H]-80$ (loss of SO_3) in-source generated fragments were 60 and 30%, respectively measured using the fully sulphated $[M-H]^-$ parent ions as a reference. For other disaccharides this level was below 10%. Without upfront chromatographic separation, in source desulphation would not have been detected, and the amount of less sulphated disaccharides would have been overestimated in the sample.

3.2. Sample preparation

In order to develop MS methods that could be used to analyse glycosaminoglycans in biological samples, we required that the protocols provided us with specific and sensitive detection of oligosaccharides together with reliability between sample preparations. Non-volatile salts cause problems for reliable analysis by MS. These salts are present in the isotonic salt-containing buffers used for the endoglycosidase digestions, as well as in the biological samples themselves and remain after the samples have been reduced to remove the complication of anomeric oligosaccharide peaks separated in the LC–MS profile. Along with non-volatile salts, detergents are also detrimental to LC–MS analysis and need to be removed. We developed a procedure that combined endoglycosidase digestion followed by sodium borohydride reduction, repeated acetic acid–methanol extractions of boric acid methyl esters and subsequent desalting over miniaturised cation exchange columns. The flow-through from the cation exchange columns was then passed through miniaturised graphitised carbon columns to recover the desalted oligosaccharides, by elution with acidified 40% acetonitrile. These samples were lyophilised, and re-dissolved in water prior to LC–MS analysis. We demonstrated the usefulness of this procedure by subjecting each of the heparin disaccharide standards to the above treatment and demonstrated good recoveries for each (Table 1). The relatively low yields demonstrate that excessive (>20 μL) washing of disaccharides bound to the carbon micro-column after desalting decreased the recovery significantly. The LC–MS chromatographic conditions showed that some of the disaccharides eluted between 5–7% acetonitrile, indicating that some of the disaccharides were eluted from graphitised carbon in the water wash, and controlled elution conditions need to be used.

3.3. MS fragmentation of heparin disaccharides

A major challenge for tandem mass spectrometry is to assign the position of the sulphate groups on the HS oligosaccharides, at the very least to a particular monosaccharide. MS^2 fragmentation spectra of the HS standard alditols are shown in Figs. 2 and 3. The spectra support the data obtained and discussed earlier that illustrate in-source decay proportional to the level of sulphation. This is illustrated by comparing the MS^2 spectra of the di- and tri-sulphated standards (Fig. 3) with the mono-sulphated standards (Fig. 2B and C). In the standard ΔUA-2S → GlcNAcol (Fig. 2B), the sulphate position could be verified being attached to the Δhexuronic acid moiety due to the presence of an intense B_1 fragment ion from a sulphated Δhexuronic acid of m/z 237. In the case of the other mono-sulphated standard ΔUA → GlcNAcol-6S (Fig. 2C), the sulphate was assigned to the HexNAcol due to the presence of an intense Y_1 fragment of a sulphated HexNAcol residue of m/z 302. However, we were unable to assign the locations of the sulphates in the di- and tri sulphated species, since the loss of sulphate was essentially the only fragmentation of the $[M-H]^-$ parent ion detected (Fig. 3, left panel), and neither lower nor higher collision energy significantly changed this feature.

It has been suggested that MS/MS sequence information of highly sulphated oligosaccharides requires a charge state

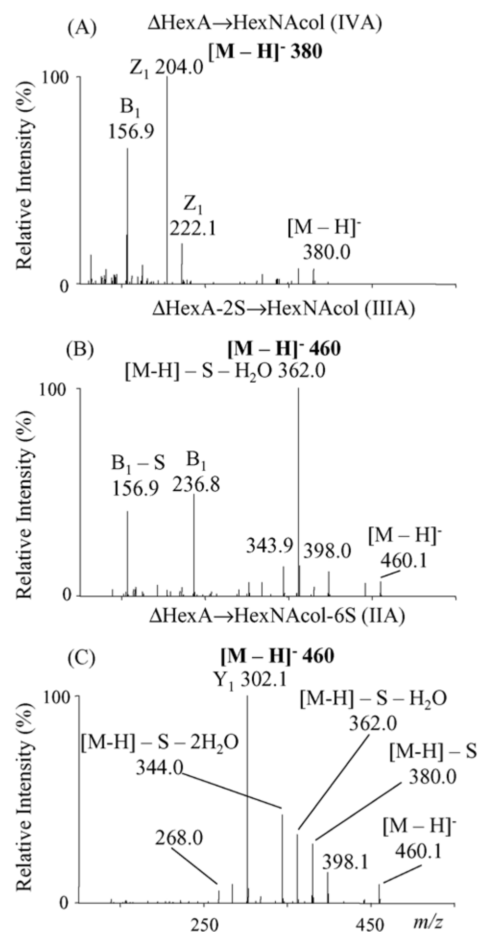


Fig. 2. Negative ion LC–MS^2 fragmentation analysis of low un- and mono sulphated heparan disaccharides (100 ng). Fragmentation assignment, according to Domon and Costello [32]. Sulphate is abbreviated as S.

of the parent ion equalling the number of sulphate groups on the oligosaccharide [22]. In order to obtain sequence information of highly sulphated disaccharides in the absence of these highly charged parent ions, we reasoned that the sodiated adducts of the oligosaccharide would provide similar stability to the sulphate-oligosaccharide bond. In the full scan spectrum, a portion of the di- and tri-sulphated disaccharides was detected as an $[M+Na-2H]^-$ pseudo-molecular ion. Collision-induced dissociation of these pseudo-molecular ions showed that the presence of sodium adducts stabilised the sulphate-saccharide linkage (Fig. 3, right panel), enabling glycosidic fragmentation to be detected in the MS^2 spectra. The tri-sulphated disaccharide I-S (Fig. 3D, right panel) was an exception, with the major fragment of the parent ion being due to the loss of sulphate, as predicted, since there was still a "free" proton present in the parent ion. Since the removal of "free" protons in the case of all the di-sulphated standards allowed glycosidic fragmentation (Fig. 3A–C, right panel), it could be predicted that fragmentation of tri-sulphated standard pseudomolecular ions without "free" protons such as $[M+2Na-3H]^-$ or $[M+Na-3H]^{2-}$ would produce more useful fragment ions. However, these ions were not detected in the full scan spectra.

The fragmentation spectra of the $[M-H]^-$ ion and $[M+Na-2H]^-$ ion of the di-sulphated disaccharide II-S (Fig. 3B) which is decorated with an O-sulphate on the C-6 and an N-sulphate on the C-2 of the reducing end HexNAcol indicate that it is not only the presence of a "free" proton in the pseudomolecular ion that destabilises the sulphate-oligosaccharide bond, but it is also its location. The spectra showed predominantly the loss of sulphate and only a small amount of glycosidic sequence ions in both cases. An explanation for this could be that the Δhexuronic acid moiety with its acidic group could pair up with the sodium adduct, giving a "free" proton available on the di-sulphated HexNAcol. The two sulphates in close proximity to each other, where one is pairing with a "free" proton, results in the destabilisation of either of the two sulphate-oligosaccharide linkages, and loss of sulphate in this case will be favoured before glycosidic fragmentation.

3.4. LC–MS analysis of heparin after heparinase digestion

Heparinase III should not significantly digest HP. However, using 10 μg of porcine HP we found that heparinase III liberated some low sulphated disaccharides (Fig. 4) supporting the poorly characterised nature of most commercial HP preparations. Along with the two disaccharides, ΔHexA → HexNAcol (IV-A) and ΔHexA → HexNSol (IV-S), we also detected several disaccharides with the sequence HexA → HexNAcol-S ($[M-H]^-$ of m/z 460) (Table in Fig. 4). Together with the more common disaccharide ΔUA → GlcNAcol-6S (II-A) (the major component at RT 24.18 min) deduced from the MS^2 spectra, two additional sequence isomers were detected at RT 25.75 and 27.18 both giving a strong Y_1 ion (50–100%) of m/z 302 indicating a sulphated HexNAcol unit (data not shown). This made us believe that these isomers differed in the location of the sulphate group and also possibly in the identity of the HexNAcol species (GlcNAcol or GalNAcol). Both HS and HP have been shown to contain sulphate linked to both carbon C-3 of GlcNAc as well as C-6 [2]. The detection of HP disaccharides potentially containing HexNAc moieties other than GlcNAc also raises questions regarding the purity of samples or the substrate specificities of the chosen heparinases.

3.5. LC–MS analysis of keratan sulphate oligosaccharides after keratanase digestion

Keratan sulphate can be found both as O-linked and N-linked oligosaccharides, consisting of a backbone of a polylactosamine $(-3Gal\beta1-4GlcNAc\beta1-)_n$ polymer. This backbone is then decorated with sulphate linked to C-6 of the GlcNAc and to lesser extent also to Gal. Digestion using keratanase II will generate predominantly the disaccharide S-6GlcNAc(β1–3)Gal, but the presence of sulphates attached

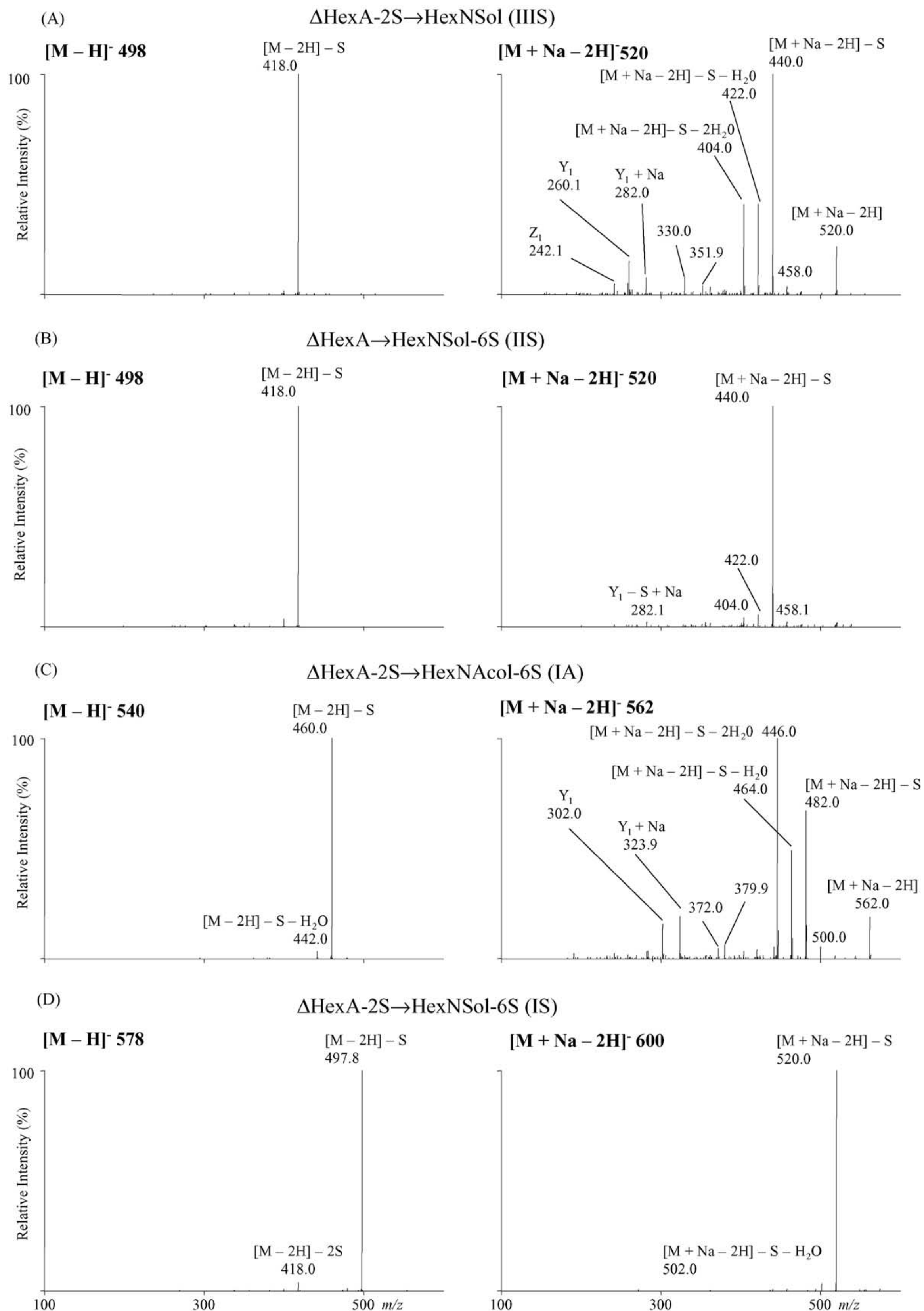


Fig. 3. Comparison of LC–MS^2 fragmentation of parent ions $[M-H]^-$ (right hand panel) and $[M-2H+Na]^-$ (left hand panel) of di- and tri-sulphated heparan disaccharides (100 ng). Fragmentation assignment, according to Domon and Costello [32]. Sulphate is abbreviated as S.

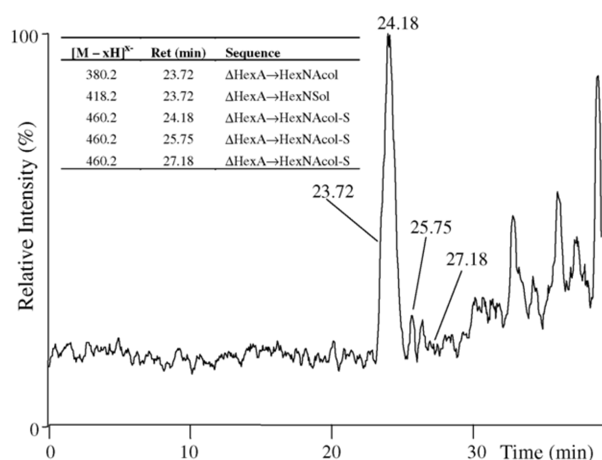


Fig. 4. LC–MS (base peak chromatogram) of heparinase III digested porcine heparin (5 μg) with detected structures. Sulphate is abbreviated as S.

to the galactose will prevent the enzyme hydrolysing some of the glycosidic linkages, resulting in the generation of larger fragments. At the non-reducing end sialic acids are frequently found [29]. LC–MS of keratanase digested porcine derived KS is shown in Fig. 5. Oligosaccharides were converted into alditols to prevent confusion in the interpretation of the anomers. The lower amount of sulphation (compared to highly sulphated heparin disaccharides) with only approximately one sulphate/disaccharide made them less prone to in-source desulphation. It was found that the dominant components present in the chromatogram were di-, tetra- and hexa-saccharides, with the larger two oligosaccharides containing sulphated hexoses that prevented further digestion. These oligosaccharides were probably originally part of the sulphated *N*-acetyl-polylactosamine backbone. Additional oligosaccharides were also detected containing either sialic acid or hexose on the non-reducing terminus. These epitopes have been demonstrated on other mammalian oligosaccharides, supporting the notion that the biosynthesis of KS shares common pathways with other glycoproteins. The fact that KS can also be linked to protein cores via N or O and that it uses a common mechanism of chain extension (*N*-acetyl polylactosamine backbone), indicates that KS may share evolutionary connections between proteoglycan oligosaccharides and the smaller, less sulphated and more common glycoprotein oligosaccharides.

Structural analysis using MS^2 fragmentation of highly sulphated oligosaccharides generated by keratanase will have similar limitations to that previously discussed for the HS standards. The presence of labile sulphates made the identification of the location of the sulphates difficult, but recent publication has shown that keratan sulphate oligosaccharides can be successfully sequenced when the charge state of the oligosaccharide is increased [18]. The fragmentation of a doubly charged trisulphated oligosaccharide is illustrated in Fig. 5B, where it is shown that glycosidic fragmentation is often accompanied by a loss of several sulphate groups. The presence of sialic acid in this particular oligosaccharide is deduced from the intense $Y_1 - S$ fragment, and the presence of a $B_1 + S$ fragment. This latter fragment is due to a re-arrangement within the molecule that results in the migration of the sulphate and attachment directly to the sialic acid. This phenomenon has been observed before in negative ion MS^2 fragmentation of oligosaccharides containing both sulphate and sialic acid residues [30], however the precise mechanism is poorly understood. Due to the pronounced desulphation in the fragmentation spectra, the interpretation of the oligosaccharide sequences from digested keratan sulphate was also based on the assumption that it was mostly sulphated on the HexNAc, and additional sulphates were found on Hex. Presence of terminal epitopes such as NeuAc and Hex was based on mass spectrometric evidence for those epitopes.

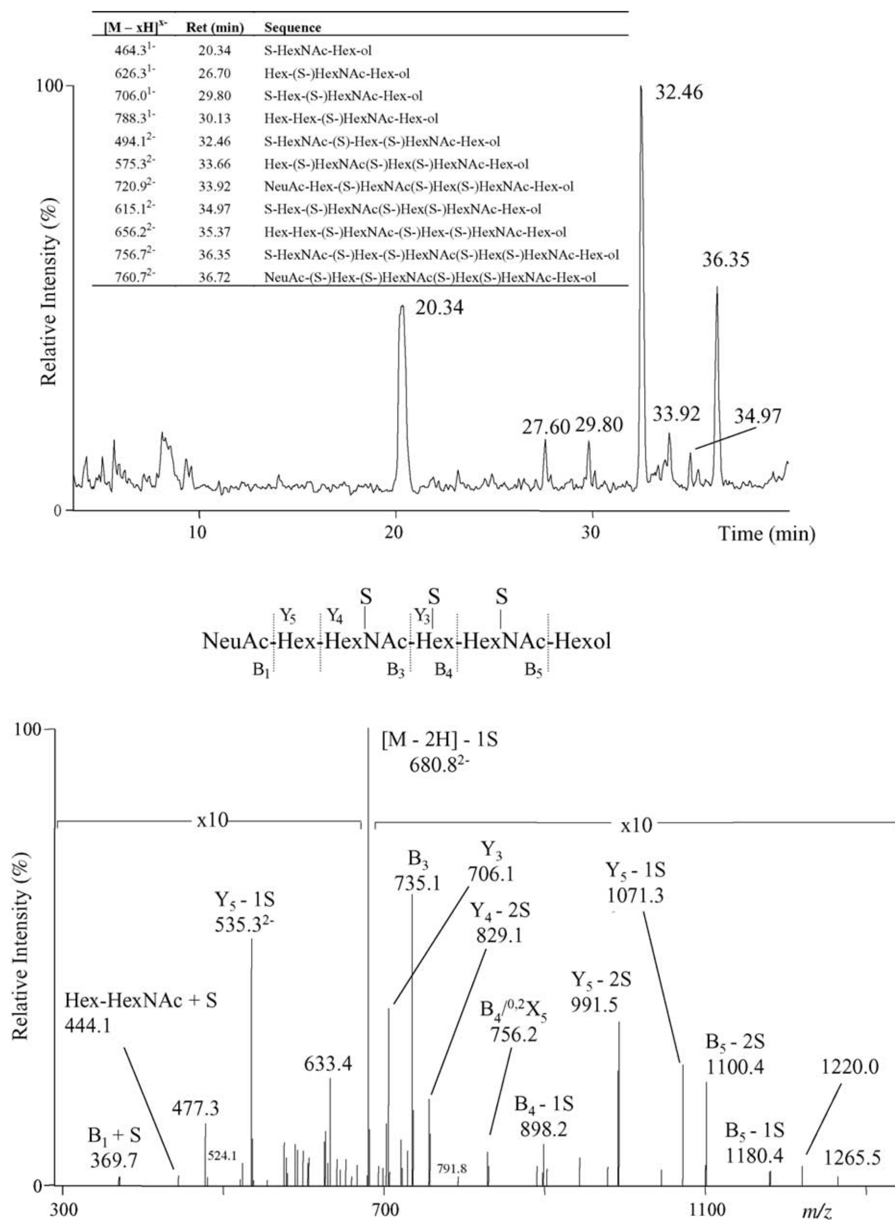
3.6. LC–MS analysis of hyaluronic acid derived oligosaccharides after hyaluronidase digestion

HA consists of large polymers of –4GlcAβ1–3GlcNAcβ1– and digestion of it with hyaluronidase isolated from bovine testis generates tetrasaccharides. HA is a major component of synovial fluid and its concentration and molecular weight has been shown to vary in diseases involving the joint [31]. Here we used synovial fluid as a source of HA and digested synovial fluid samples with hyaluronidase in order to test our detection methodologies with these types of glycosaminoglycans in complex biological samples. In Fig. 6, the digestion of 1 μL of synovial fluid from a patient with osteoarthritis is shown. The tetrasaccharide corresponding to GlcA(β1–3)GlcNAc(β1–4)GlcA(β1–3)GlcNAc ($[M - H]^-$ ion of *m/z* 775) could be detected together with a small amount of the hexasaccharides GlcA(β1–3)GlcNAc(β1–4) GlcA(β1–3)GlcNAc(β1–4) GlcA(β1–3)GlcNAc ($[M - H]^{2-}$ ion of *m/z* 577). Since the samples were not reduced prior to LC–MS analysis, the tetrasaccharide and hexasaccharide peaks eluted as broad peaks.

From the MS^2 fragmentation of the hyaluronic acid derived tetrasaccharide (Fig. 6B) it could be seen that an acidic group like that on the glucuronic acid is less labile than sulphate. The absence of sulphate in HA reduced the MS^2 fragmentation to simple glycosidic sequence fragment ions, and made interpretation of spectra relatively simple.

4. Discussion

Separation and detection of depolymerised glycosaminoglycans has been shown to be a challenge because of their complex sulphation, acidity and isomeric monosaccharides present. There is a need to be able to separate glycosaminoglycans before mass spectrometric analysis, where isomeric oligosaccharide structures make interpretation of sequence data from collision-induced fragmentation difficult. Graphi-



$[M-xH]^{x-}$	Ret (min)	Sequence
464.3^{1-}	20.34	S-HexNAc-Hex-ol
626.3^{1-}	26.70	Hex-(S-)HexNAc-Hex-ol
706.0^{1-}	29.80	S-Hex-(S-)HexNAc-Hex-ol
788.3^{1-}	30.13	Hex-Hex-(S-)HexNAc-Hex-ol
494.1^{2-}	32.46	S-HexNAc-(S)-Hex-(S-)HexNAc-Hex-ol
575.3^{2-}	33.66	Hex-(S-)HexNAc(S-)Hex(S-)HexNAc-Hex-ol
720.9^{2-}	33.92	NeuAc-Hex-(S-)HexNAc(S-)Hex(S-)HexNAc-Hex-ol
615.1^{2-}	34.97	S-Hex-(S-)HexNAc(S-)Hex(S-)HexNAc-Hex-ol
656.2^{2-}	35.37	Hex-Hex-(S-)HexNAc-(S-)Hex-(S-)HexNAc-Hex-ol
756.7^{2-}	36.35	S-HexNAc-(S-)Hex-(S-)HexNAc(S-)Hex(S-)HexNAc-Hex-ol
760.7^{2-}	36.72	NeuAc-(S-)Hex-(S-)HexNAc(S-)Hex(S-)HexNAc-Hex-ol

Fig. 5. LC–MS (base peak chromatogram) of keratanase digested keratan sulphate (1 μg) from bovine cornea with detected structures (upper panel), and fragmentation MS^2 of the non-reducing end terminal epitope of a sialylated trisulphated hexasaccharide $[M-2H]^{2-}$ ion of *m/z* 721 eluted at 33.92 min (lower panel). Fragmentation assignment, according to Domon and Costello [32]. Sulphate is abbreviated as S.

tised carbon has been shown to be a versatile separation media for various other oligosaccharides, and this report shows its applicability to glycosaminoglycan analysis. The data also indicates that partial digestion of glycosaminoglycans, where even larger oligosaccharide fragments (>2 monosaccharide units) are generated, can be analysed by LC–MS using graphitised carbon. This opens up the possibility of performing sequential exoglycosidase and sulphatase treatments and chemical degradation in combination with LC–MS in order to sequence glycosaminoglycans. In combination with LC–MS^2 this may be a powerful approach for sequencing glycosaminoglycans in order to relate biological activity to a particular oligosaccharide structure. Recent developments in negative ion LC–MS using graphitised carbon have shown that analysis can be performed in the low femtomole amount of N-linked oligosaccharides [28]. Implementing nanoflow LC–MS for analysis of glycosaminoglycans will allow analysis of samples where previously the sample amount has been the limiting factor. In addition, the enormous heterogeneity of sulphation, epimerisation and the large size of the oligosaccharide chains have encouraged us, and others, to develop specialised methods for sequencing of these macromolecules. The expansion of data from the increased amount of samples will demand automation of the data interpretation. In some respects the computerised interpretation of glycosaminoglycans fragmentation spectra is easier than

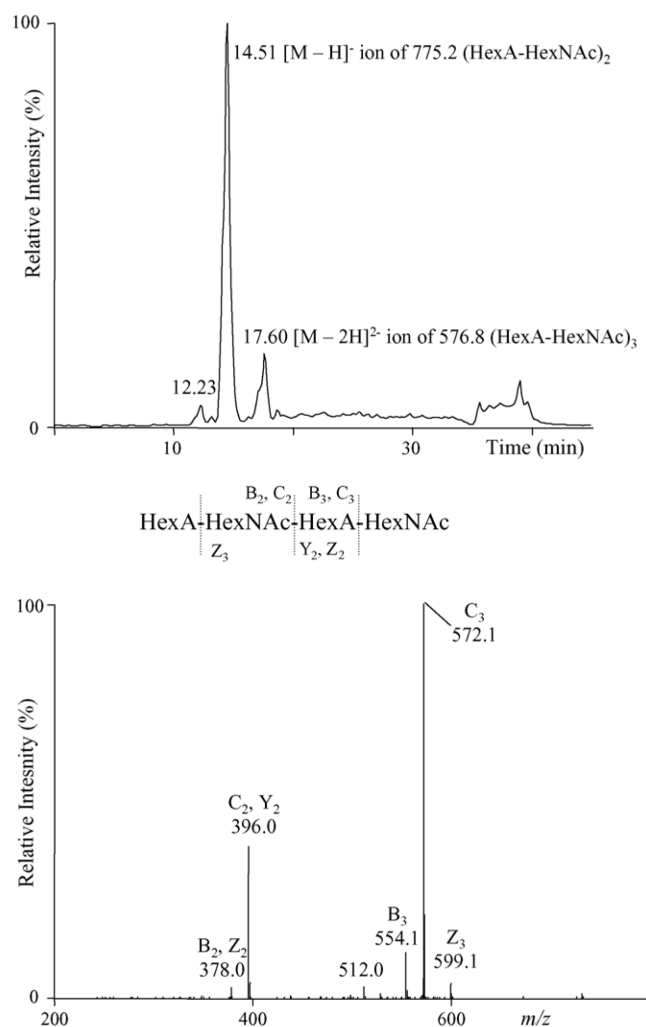


Fig. 6. LC–MS (base peak chromatogram) of hyaluronidase digested hyaluronic acid from human synovial fluid (μL) from a patient with osteoarthritis. Hyaluronic acid derived tetrasaccharides and hexasaccharides are shown in the upper panel), and fragmentation MS^2 of the hyaluronic acid derived tetrasaccharide ($[M-H]^-$ ion of m/z 775 eluted at 15.51 min) is shown in the lower panel. Fragmentation assignment, according to Domon and Costello [32].

methods for other oligosaccharides, due to the linear repeat nature of glycosaminoglycan chains. Rather than instrumentation driven discovery in the future, it can be predicted that bio-informatic interpretation of data will be the bottleneck for biological discovery. With regard to proteoglycan analysis, this development has only just begun.

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