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Heparan Sulfate Separation, Sequencing, and Isomeric Differentiation: Ion Mobility Spectrometry Reveals Specific Iduronic and Glucuronic Acid-Containing Hexasaccharides

Matthew R. Schenauer[1], **John K. Meissen**[1], **Youjin Seo**[2], **James B. Ames**[2], and **Julie A. Leary**[1,2,*]

[1]The Departments of Molecular and Cellular Biology, University of California, Davis, One Shields Avenue, Davis, CA 95616

[2]The Department of Chemistry, University of California, Davis, One Shields Avenue, Davis, CA 95616

Abstract

We describe the resolution of heparan sulfate (HS) isomers by chromatographic methods and their subsequent differentiation by mass spectrometry (MS), ion mobility, and ^{1}H-NMR analysis. The two purified hexasaccharide isomers produced nearly identical MS spectra, quantitative disaccharide profiles, and partial enzymatic digestions. However, both tandem spectrometry (MS^2) and ion mobility spectrometry (IMS) indicated structurally differences existed. All data suggested the distinction between the two hexasaccharides resided in their uronic acid stereochemistries. Glucuronic (GlcA) and iduronic acids (IdoA) were subsequently defined by ^{1}H-NMR analysis completing the structural analysis and verifying the unique structures initially indicated by MS^2 and IMS. Our results suggest that IMS may be a powerful tool in the rapid differentiation of GlcA and IdoA containing isomers in the absence of prior structural knowledge.

Keywords

heparan sulfate; glycosaminoglycan; uronic acid; sequencing; chromatography; mass spectrometry; ion mobility; nuclear magnetic resonance

INTRODUCTION

Glycosaminoglycans (GAGs) are unbranched, anionic polysaccharides predominantly present at cell surfaces and in the extracellular matrix. These polysaccharides are composed of repeating disaccharide units and are so named for the fact that one residue within each disaccharide is an amino sugar; either N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc). With the exception of keratan sulfate, the residue to the non-reducing end of the amino sugar is an uronic acid; i.e. either of the C5 epimers iduronic acid (IdoA) or glucuronic acid (GlcA). GAGs play diverse roles in biology. Some of their functions include provision of mechanical support in the extracellular matrix, modulation of enzymatic activities, and immobilization of morphogen gradients during development. Many of the biological functions of the GAG family are carried out in some way or another through protein interactions, and physiologically the most relevant GAG involved in protein interactions is heparan sulfate (HS) [1–5].

*Corresponding Author: jaleary@ucdavis.edu.

Heparan sulfate synthesis takes place within the Golgi apparatus where the polymer is synthesized as a repeating array of [-GlcNAcα1–4GlcAβ1–4-]$_n$. During chain elongation, and perhaps most important for protein binding, nascent HS is acted upon by a series enzymes which chemically modify the polymer. The modification reactions on GlcNAc residues include *N*-deacetylation/*N*-sulfation, 6-*O*, and 3-*O* sulfation. GlcA residues may be modified by epimerization at C5 (converting the sugar to IdoA), and 2-*O* sulfation. These modification reactions do not approach completion resulting in the production of HS molecules with varying sequences of acetylation, sulfation, and IdoA content [6–10]. To a large extent, the average modification profile and structure of HS varies with the tissue where it was synthesized [11–13]. Despite the modification content being relatively constant within a tissue, specific sequences (for example those binding to antithrombin III [14–16], basic fibroblast growth factor [17–20], or phage display antibodies [21]) may only be found in select chains.

Overall HS has been implicated in a multitude physiologic and pathophysiologic processes, including blood coagulation [22–24], viral infectivity [25–27], cancer [28–33], inflammation [34–39], growth, and development [40–45]. For all of these roles, only a small number of HS protein-binding sequences have been solved [2, 4, 5, 7, 46–48]. The reasons for this are many and include difficulties in isolating large amounts of pure sequence, inherent difficulties of HS chemical synthesis, the fact that some proteins can bind multiple HS sequences, and that only a relatively small number of methods for defining any purified HS structure exist. Traditional methods for HS sequencing have relied upon either 2-D NMR, or incorporation of radio or chemical labels followed by extensive enzymatic processing [49–54]. While such methods have utility in specific contexts, no single method is well suited for all applications. For instance, while 2-D NMR can provide 3-dimensional structural information, the technique often requires milligram quantities of material which may not be available. Labeling methods rely on the use of radioisotopes in cell culture or chemical labeling reactions which may proceed with varying efficiencies depending on the substrate and matrix.

To further facilitate HS sequence analysis, we present an efficient method of sequencing that relies heavily, but not exclusively, on mass spectrometry (MS). Our method is significantly more sensitive than 2-D NMR analysis and similarly sensitive to end labeling methods [51, 52, 54]. We describe the sequencing of two HS hexasaccharide isomers whose structures had been previously resolved following different isolation and sequencing methods [49, 54, 55]. We also report, for the first time, ion mobility resolution of HS sequences based solely on uronic acid identity. In our method, chromatographically-purified isomers were analyzed by nanoelectrospray MS, IMS, and MS^2 time-of-flight (TOF) mass spectrometry. MS-based compositional analysis was used to identify and quantify the disaccharides comprising the hexasaccharides [56, 57], while their linear sequences were reconstructed by partial enzymatic digestions. Finally, 1-D NMR was used to verify the IMS data suggesting differentiation between the iduronic and glucuronic acid isomers, thus completing the sequence analysis. In light of the importance of HS in biology in medicine, and given that resources, materials, and technical capabilities will undoubtedly vary from one research lab to another, we present this efficient, new approach to HS sequencing. Results presented herein indicate that IMS may be a powerful, untapped resource for HS analysis.

EXPERIMENTAL SECTION

Materials

Porcine intestinal heparin sulfate was purchased from Celsus Laboratories (Cincinnati, OH). Bio-Gel® P-10 Gel was obtained from Bio-Rad (Hercules, CA). The IonPac® AS7 anion-exchange column was purchased from Dionex (Sunnyvale, CA). The heparinase III (heparitinase, EC 4.2.2.8) used for preparative digestion was a gracious gift from Professor Jian Liu, UNC School of Pharmacy, Chapel Hill, NC. Standards used in disaccharide

compositional analysis were purchased from Calbiochem (La Jolla, CA), Sigma-Aldrich Corp. (St. Louis, MO), and Dextra Laboratories (Reading, UK). Heparinases I (EC 4.2.27), II (no EC number), and III (EC 4.2.2.8) used in quantitative disaccharide compositional analysis were obtained from Seikagaku Biobusiness Corp. (Tokyo, Japan). All other chemicals were purchased from Fisher Scientific or Sigma-Aldrich chemical co.

Preparative and Analytical HS Depolymerization

Heparinase III digestion of intact HS was performed essentially as previously described [58]. Briefly, HS (50 mg/ml in 50 mM sodium phosphate, pH 7.5) was extensively depolymerized at 37° C [59, 60]. The reaction was monitored by UV-absorption until the product concentration reached 18 mM (ε = 5,500 $M^{-1}cm^{-1}$1 in 0.03 M HCl at 232 nm 61). The reaction was quenched by addition of 50% methanol, boiled for 10 minutes, and then frozen and lyophilized. Partial digests for analytical purposes were performed using approximately 0.5 mU of heparinase I, II, or III, and 1–2 nMol of each hexasaccharide in 50mM NH_4OAc. These reactions were quenched by the addition of the nanoelectrospray ionization (nESI) solvent containing 1:1 MeOH: H_2O and 0.1% acetic acid.

Size Exclusion Chromatography

The depolymerized HS was redissolved in 1 M NH_4HCO_3. The solution was centrifuged to remove any particulates and then loaded onto a 170 × 1.5 cm Econo-Column® (Bio-Rad, Hercules, CA) packed with P-10 size exclusion gel. The separation was performed under gravity flow at room temperature in 40 mM NH_4HCO_3 and ~1 ml fractions were collected using a BioLogic LP system (Bio-Rad, Hercules, CA). Initial separation was carried out between 0.5 and 1.0 ml/hour which served to remove an otherwise high concentration of disaccharides (dp2, degree of polymerization-2, etc.) from the rest of the oligosaccharide products. The fractions containing the larger oligosaccharides were pooled, freeze-dried, re-dissolved in 1 M NH_4HCO_3, re-loaded onto the column, and run at a flow rate of 3.5 ml/hour. Separation was monitored by UV absorption online at 254 nm, and later verified offline at 232 nm. Collected fractions were lyophilized using a Vacufuge™ (Eppendorf, Westbury, NY). Each of the dried fractions was then re-dissolved in 100 μl of Millipore H_2O, quantified by UV-absorption, and analyzed by nESI TOF MS.

Strong Anion-Exchanged Chromatography

Fractions obtained from SEC corresponding to hexasaccharides (dp6) were pooled and re-quantified. SAX chromatography was run on a Waters Delta 600 System (Waters Corp., Milford, MA) using multistage, linear gradients as follows: solvent A, H_2O (pH 3.5); solvent B, 4 M NaCl (pH 3.5); 1–5 min, 0% B; 6–75 min, 8–15% B; 75–85 min, 15–17% B; 85–100 min, 17–19% B. Eluting peaks were detected by UV-absorption at 232 nm. Between 100 and 200 μg of material were injected for individual runs, with run to run variability generally less than one minute. Peak fractions were collected, lyophilized, and re-dissolved in the smallest possible volume of H_2O limited by the solubility of NaCl. Re-dissolved fractions were desalted by dialysis, at room temperature, first against degassed 500 mM NH_4OAc, and then against Millipore H_2O using 1kDa MWCO (molecular weight cut-off) Dispo-Biodialyzers™ (The Nest Group, inc., Southborough, MA). The desalted fractions were concentrated and quantified by spectrophotometry.

IMS and MS of Intact and Partially Digested Hexasaccharides

Borosilicate glass capillaries (# 1B120F-4) were purchased from World Precision Instruments, Inc. (Sarasota, FL). The capillaries were pulled on a Sutter P-97 Flaming/Brown micropipette puller, and gold coated using a Quorum Technologies SC7640 sputter coater (Ringmer, East Sussex, UK). MS and MS^2 analyses were performed on a Waters *Synapt*™ *HDMS*™ system

(Waters Corp., Milford, MA). The following parameters were used: nESI capillary voltage, 0.6 kV; sample cone, 10 V; extraction cone, 0 V; source temperature, 40°C; trap CE, 1 V; transfer CE, 3 V; trap gas flow, 1.0 ml/min; IMS gas flow, 24 ml/min; trap direct current bias, 7 V; pusher interval, 45 μs; IMS wave velocity, 400 m/s; IMS wave height, 8.0 V; transfer wave velocity, 350 m/s; transfer wave height, 5.0 V. For mass to charge-selected ions, LM and HM resolutions were each set to 14.0 or higher with the quadrupole set to isolate the *m/z* of interest. Collision induced dissociation (CID) of hexasaccharides was performed using a trap collision energy of 16 V, and for all experiments the time of flight analyzer was calibrated with a solution of NaI. For IMS analysis, the *Synapt G2*™ q-IMS-TOF mass spectrometer was used. *Synapt G2*™ parameters were generally similar to the parameters provided for the first generation *Synapt*™ mass spectrometer provided above with the following exceptions: extraction cone, 2 V; source temperature, 50°C; trap CE, 4 V; transfer CE, 0 V; trap gas flow, 1.5 ml/min; IMS gas flow, 180 ml/min; helium gas flow, 200 ml/min: trap direct current bias, 40 V; pusher interval, 50 μs; IMS wave velocity, 1600 m/s; IMS wave height, 4.7–5.1 V ramp over 10 % of IMS cycle; transfer wave velocity, 206 m/s; transfer wave height, 4.0 V.

Quantitative Disaccharide Compositional Analysis

Quantitative disaccharide compositional analysis was performed essentially as previously described [56, 57]. Briefly, 5 μg of hexasaccharide from each of the fractions were fully digested to disaccharide constituents in 20 μL of 7.5 mM NH_4OAc (pH 7.5) containing 1 mM $Ca(OAc)_2$ and 2 mU each of Heparin Lyases I, II, and III. The reactions were incubated at 37° C for 16 hours and then stopped by the addition of 180 μl of quench solution to create a final solution containing 1:1 MeOH/H_2O, 10 mM NH_4OH, and 5 μM I-P (ΔUA2S-GlcNCOEt6S) internal standard [56, 57]. Samples were analyzed by ESI-MS^n without further purification. Mass spectra were collected via ESI-linear ion trap MS (LTQ, Thermo Finnigan LTQ, San Jose, CA) using Xcaliber Version 2.0 data acquisition software. All spectra were obtained in negative ion mode with a 3.5 kV spray voltage and a 225° C capillary temperature. Samples were introduced by flow injection analysis at 10 μL/min with 1:1 MeOH/H_2O containing 10 mM NH_4OH. MS and MS^2 data acquisition and quantitative analysis of compositions were also performed as previously described [56, 57]. Heparin from bovine lung and HS from bovine kidney were used as controls for all aspects of the experimental method. Measurements were obtained in triplicate and results are reported as an average ± standard deviation.

NMR Spectroscopy

NMR experiments were performed on a Bruker Avance III 600 MHz spectrometer, equipped with a cryogenically cooled triple resonance (TCI) probe. Proton chemical shifts are reported with respect to external DSS (2,2-dimethyl-2-silapentane-5-sulfonic acid). Experiments were performed at 298 K using 512 scans, a 2 s recycle delay, and a 6600 Hz sweep-width (32,000 complex points). Water suppression was accomplished using pre-saturation of the water resonance during the recycle delay. Data were processed using Bruker TopSpin software. NMR samples contained approximately 0.1 mM oligosaccharide each and were dissolved in 99.9% D_2O to remove exchangeable protons.

RESULTS AND DISCUSSION

Purification of HS Hexasaccharide Isomers

Intact HS was digested to oligomers ranging from dp2 to d12 using heparinase III [58–60]. The products were subsequently separated by size exclusion chromatography (SEC), and oligosaccharides differing in length by at least one disaccharide were near baseline resolved (Figure 1). The resultant product fractions containing hexasaccharides (dp6) were pooled and subjected to strong anion exchange (SAX) chromatography using an IonPac AS7 column. This column type was chosen as it had previously been shown useful in differentiation of polyvalent

anions [62], but to our knowledge had not been used in GAG analysis. When running a shallow gradient, the column proved effective in separating both isomeric hexasaccharides as well as those differing in sulfate number (Figure 2). Of particular interest is the observation that isomeric hexasaccharides with three sulfate groups and a single acetyl group eluted at distinct retentions times between 22 and 36 minutes (we use sulfate to denote either sulfate or sulfamate groups on the saccharide). A strong peak with a prominent shoulder is observed around 22 minutes along with two well-resolved peaks centered around 27 and 28 minutes, respectively. Meanwhile, a smaller series of incompletely resolved peaks are present eluting between 30 and 34 minutes. The peak at 22 minutes and its shoulder represent a single chemical entity, interconverting conformers, or anomers as evidenced by identical MS^2 spectra and verified by reinjection of individually collected peaks onto the column. The peaks present at 27 and 28 minutes displayed the same relationship. Given the conformational flexibility in iduronate rings [63–65], and the fact that reducing-end residues are expected to be present as both α and β anomers [2, 5], either possibility could explain the split peaks. Taking this into consideration, the fraction at 22 minutes and its shoulder were pooled and analyzed as a single entity, as were the fractions collected at 27 and 28 minutes. For simplicity, the pooled fractions are referred to as fractions 22 and 27, respectively.

The full-scan MS spectra of fractions 22 and 27 were obtained with isotopic resolution providing a molecular mass of 1293.18 Da (Figure 3). The HOST macro was used to convert the molecular mass to the composition of a hexasaccharide with 3 sulfate groups and 1 acetyl group [66]. Both purified fractions were judged to be extremely pure based on the mass spectra. It is also of interest that under the gentle nanospray conditions employed, sulfate loss, which can lead to overestimation of the components in a mixture, was non-existent.

MS^2 and Ion Mobility Analysis of Intact Hexasaccharides

Following observation of distinct retention times, the second indicator that we had purified isomeric saccharides came from differences in the MS^2 spectra of isolated species. Interestingly, the same product ions were formed for both fractions 22 and 27, but differed in relative abundances (figure 4). A similar relationship had been observed for uronic acid residue isomers in chondroitin and dermatan sulfate, but had not previously been observed in HS [67]. The most intense product ions for each species are labeled in figure 4 using the Domon and Costello nomenclature [68], while all product ions present at greater than 1 % relative abundance are listed in supplemental table 1. Product ions were assigned based on cleavage patterns previously observed in HS disaccharides [56, 69] and extrapolated to the larger hexasaccharides in the present study. Given that sulfate loss is observed upon MS^2 (Table S1), it is precarious to assign sulfate group positions from MS^2 of an intact oligosaccharides alone. Acetyl modifications are, however, relatively stable in the gas phase and the MS^2 fragments observed (X and Y ions) localize this modification to the reducing end of both hexasaccharides (Table S1). It is of particular note, that we observe differences in ion mobility arrival time distributions (ATDs) for the two purified isomers. The hexasaccharide in fraction 22 arrived at 7.02 ms while the hexasaccharide in fraction 27 arrived earlier at 6.77 ms (Figure 5). Under identical instrumental conditions, IMS arrival times are influenced by structure (as collision cross section), mass, and charge [70–72]. Since both analytes have identical mass and charge, and were analyzed under identical conditions, these ATD differences are a clear indicator of their difference in molecular structure.

Disaccharide Compositional Analysis

The hexasaccharides in fractions 22 and 27 were completely digested to disaccharides using a mixture of heparinases I, II, and III. Each disaccharide was then quantified via flow injection analysis using ΔUA2S-GlcNCOEt6S as an internal standard, and isomeric disaccharides were distinguished and quantified by their diagnostic ions in MS^2 spectra [56, 57]. The results obtained

for fractions 22 and 27 are presented in table 1. Of note is that for both fractions, the disaccharides IV-A (ΔUA-GlcNAc), IV-S (ΔUA-GlcNS), and III-S (ΔUA2S-GlcNS) each make up approximately 30% of the mixture. While these data indicate the possibility of unique hexasaccharide structures in the range of 90% purity, the data provide neither the linear sequence of the disaccharides nor the stereochemistry at C5 of the uronic acids. The latter stereochemical information is lost through the use of heparinases I, II, and III which cleave via beta-elimination resulting in a C4–C5 double bond with loss of chirality at these stereogenic centers [46, 60].

Partial Enzymatic Digestion of Hexasaccharides

Partial enzymatic digestion is an effective way to generate sequence information between known disaccharide blocks present in longer oligosaccharides. Differential cleavage sites can be used to identify neighboring disaccharides and generate their linear sequence in the oligosaccharide. Two sets of partially cleaved hexasaccharides were generated for fractions 22 and 27 using heparinases II, and III. The results, presented in Figure 6, can be taken with the tandem MS data of the intact hexasaccharides (which localized the N-acetyl group to the reducing end) and the disaccharide compositions to generate linear sequences for each hexasaccharide, however devoid of knowledge of uronic acid identity. Interestingly the hexasaccharides in both fractions showed identical cleavage patterns (Figure 6). Heparinase II cleaved both hexasaccharides generating products at *m/z* 207.5 (2^- charge state), 247.5 (2^- charge state), and 291.1 (3^- charge state, Figure 6 A and B). These mass to charge ratios deconvolute to molecular masses of 417.1, 497.5, and 876.2 Da respectively. The first two masses correspond to the disaccharides IV-S and III-S identified in the compositional analysis, but provide no new information. However, the mass of 876.2 Da corresponds to a tetrasaccharide (with 2 sulfates and 1 acetyl group) linking the III-S and IV-A disaccharides (M_{theo} 876.13 Da). Since the MS^2 data of the intact hexasaccharides (Figure 4) positioned the acetyl group at the reducing end, these corresponding tetrasaccharides can be sequenced as ΔUA2S-GlcNS-HexA-GlcNAc (HexA is used to indicate a lack of knowledge of C5 stereochemistry indicating either GlcA or IdoA). Meanwhile, heparinase III cleavage also generated identical cleavages between the two fractions producing ions at *m/z* 227.5 (4^- charge state) and 303.7 (3^- charge state), which both deconvolute to a molecular weight of 914.1 Da (Figure 6 C and D). This ion is indicative of a tetrasaccharide (with 3 sulfate groups and no acetyl groups) linking IV-S and III-S disaccharides (M_{theo} 914.07 Da). Since the Heparinase II and MS^2 data localized the HexA2S-GlcNS and HexA-GlcNAc as the central and reducing end disaccharides, respectively, the heparinase III data confirmed the sequence ΔUA-GlcNS-HexA2S-GlcNS and localized the IV−S disaccharide to the non-reducing end. Thus hexasaccharides in fraction 22 and 27 *both* possess linear sequences of ΔUA-GlcNS-HexA2S-GlcNS-HexA-GlcNAc. One-dimensional (1-D) ^{1}H-NMR was subsequently used to define the uronic acid stereochemistries.

1-D ^{1}H-NMR Analysis and Assignment of Uronic Acid Residues

Aliquots of fractions 22 and 27 in deionized H_2O were lyophilized via freeze-drying overnight. The following morning the samples were dissolved in 99.9% D_2O to remove exchangeable protons and the samples were subjected to 600 MHz ^{1}H-NMR analysis. As for most oligosaccharides, the majority of the ring proton resonances cluster in a congested, narrow spectral range between 3.2 and 4.0 ppm (data not shown) [73–78]. The data outside this range was interpreted in accordance with published ^{1}H chemical shifts of protons in similar chemical environments to those of our saccharides [51, 52, 79–83]. All signals resonating above 4 ppm were identified (Figure 7), the majority of which correspond to the anomeric protons of the individual sugar residues which are shifted downfield due to unshielding by the ring oxygen atom [78]. Of note is that in both spectra of fractions 22 (Figure 7A) and 27 (Figure 7B) resonances corresponding to a 2-*O*-sulfated IdoA are observed. Peaks *c* (5.23 ppm), *g* (4.36 ppm), *i* (4.27

ppm), and *j* (4.08 ppm) correspond to IdoA2S H1, H2, H3, and H4 respectively [51, 52, 79, 82, 84]. Given that both isomers contained one 2-*O*-sulfated uronic acid (the central HexA2S), these data identify the central uronic acid as IdoA2S in both hexasaccharides. Also of note is that only the spectrum of hexasaccharide-22 showed resonances for an unsulfated iduronic acid. Peaks labeled *u* (5.39 ppm), *v* (5.02 ppm), *w* (4.78 ppm), and *x* (4.15 ppm) correspond to GlcNS H1 (when linked to IdoA), IdoA H1, IdoA H5, and IdoA H3 respectively [11, 79, 80, 85]. Meanwhile the spectrum of hexasaccharide-27 displays resonances unique to a saccharide containing GlcA. Peaks labeled y (5.60 ppm) and *z* (4.60 ppm) corresponds to GlcNS H1 (when linked to GlcA), and GlcA H1 respectively [79, 80, 82, 83]. Summarizing, the common internal HexA2S in both hexasaccharide-22 and -27 is identified as IdoA2S. Conversely the uronic acid in the reducing end disaccharide is IdoA in hexasaccharide-22 and GlcA in hexasaccharide-27. All other common peaks in the reporter group region were consistent with our MS-derived structures including ΔUA H4 (peak *a* 5.80 ppm), GlcNS H1 (when linked to IdoA2S; peak *b*, 5.36, ppm), GlcNAcα H1 (*d*, 5.23 ppm), ΔUA H1 (*e*, 5.12 ppm), GlcNAcβ H1 (*f*, 4.73 ppm), and ΔUA H3 (*h*, 4.32 ppm) [79, 80, 82, 84]. Thus by combining the MS data of the intact hexasaccharides, the disaccharide compositional analysis, partial enzymatic digestions, and finally 1-D ^{1}H-NMR we were able to define the purified structures in fractions 22 and 27 as ΔUA-GlcNS-IdoA2S-GlcNS-IdoA-GlcNAc, and ΔUA-GlcNS-IdoA2S-GlcNS-GlcA-GlcNAc, respectively (inset Figure 7). In defining the full sequence for both hexasaccharides, it is interesting to note that the enzymatic cleavage product, ΔUA-GlcNS-IdoA2S-GlcNS, obtained from the hexasaccharide in fraction 22 (Figure 6C) may not have been predicted based on some published accounts of heparinase III specificity [86]. Nevertheless, the use of all three heparinases (regardless of specificity), in addition to the all the supporting experiments, indicates that the two derived sequences for fractions 22 and 27 are the only reasonable possibilities. Thus, our results are consistent with studies suggesting that the GlcNS-IdoA linkage is, in fact, a viable target for the heparinase III enzyme [59, 87].

In identifying the complete structure of two highly specific hexasaccharide structural isomers, we have shown that MS and ion mobility can be powerful tools in the initial screening process of complex libraries of biologically derived GAG. IMS was capable of differentiating HS isomers with changes in the axial vs. equatorial positioning of a single carboxylate functional group. Thus, this methodology may be useful in differentiating GAG structures for which there is no knowledge uronic acid chemistry, *a priori*. Research from other groups has shown that MS can be used to differentiate uronic acid residue isomers when conditions are optimized from known starting structures [67, 88], although the MS^2 spectra in these cases are quite complicated. IMS has been applied previously for GAG analysis, however saccharides differing in multiple chemical modifications were investigated making specific structural interpretation difficult [89]. Given the data presented herein, one might envision a role for IMS-MS in the rapid, complete analysis HS oligosaccharides based on a combination of experiment and theory without prior knowledge. In such a scenario, MS-based methods, like those described in this manuscript, would supply the majority of the structural information, while molecular modeling methods [89] would correlate variations in uronic acid stereochemistry with the collision cross sections obtained by IMS. Applications such as this would represent a significant advance in HS-GAG analysis.

Conclusions

Given the vast array of HS-interacting proteins and the number of physiologic and pathophysiologic processes in which those interactions are significant, new methods to define structural motifs within HS should continue to be developed. In recent years the first therapeutic based on specific knowledge of a HS-protein interaction emerged [14, 90, 91], and more are expected [92]. Knowledge of HS structure will undoubtedly contribute to these developments. In the present study we applied a combination of chromatography, MS, and 1-D ^{1}H-NMR

techniques to sequence two isomeric HS hexasaccharides. The method developed herein will be amenable to the analysis of longer and more complex saccharides as they are purified, and will thus facilitate additional HS sequence determinations. We also showed that IMS was capable of resolving HS isomers which differed at only a single stereogenic center (C5 of uronic acids). Given their abilities to rapidly distinguish closely related species, MS and IMS are expected play increasingly important roles in GAG analysis and structural elucidation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ABBREVIATIONS

HS	heparan sulfate
MS	mass spectrometry
NMR	nuclear magnetic resonance
MS^2	tandem spectrometry
IMS	ion mobility spectrometry
GlcA	glucuronic acid
IdoA	iduronic acid
HexA	either glucuronic or iduronic acid
GAGs	glycosaminoglycans
GlcNAc	N-acetylglucosamine
GalNAc	N-acetylgalactosamine
TOF	time-of-flight
NH_4OAc	ammonium acetate
ESI	electrospray ionization
nESI	nanoelectrospray ionization
dp	degree of polymerization (dp2 = disaccharide, etc.)
SEC	size exclusion chromatography
SAX	strong anion exchange chromatography
MWCO	molecular weight cut-off
$Ca(OAc)_2$	calcium acetate
DSS	2,2-dimethyl-2-silapentane-5-sulfonic acid
ATDs	arrival time distributions

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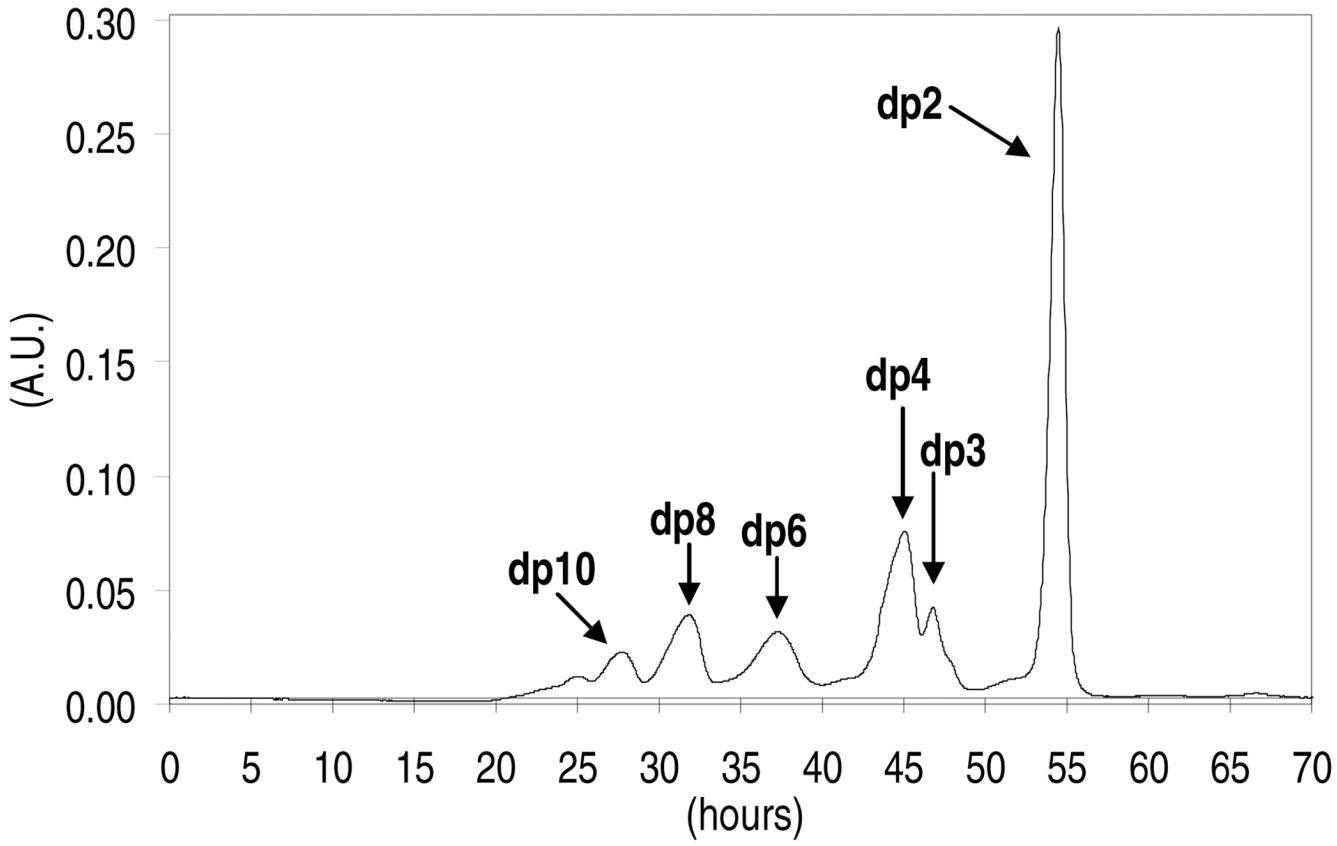

Figure 1.
SEC chromatogram of partially depolymerized HS. Peaks corresponding to di-through decasaccharides are labeled dp2 to dp10.

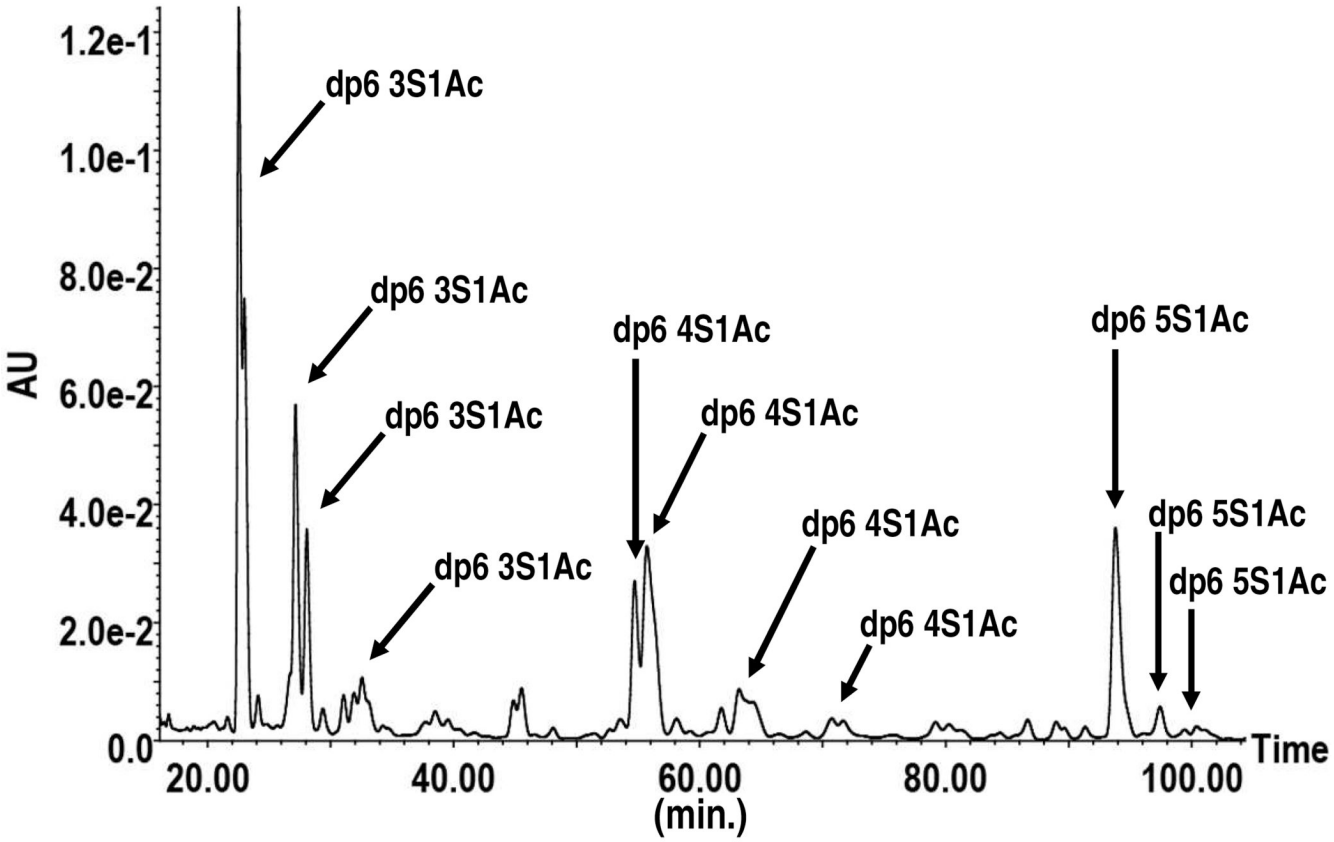

Figure 2.
IonPac AS7 SAX chromatogram of HS hexasaccharides (dp6). The number of sulfates and the number of acetyl groups present on the hexasaccharides in the collected peaks are represented by the numbers preceding ‘S’ and ‘Ac’, respectively.

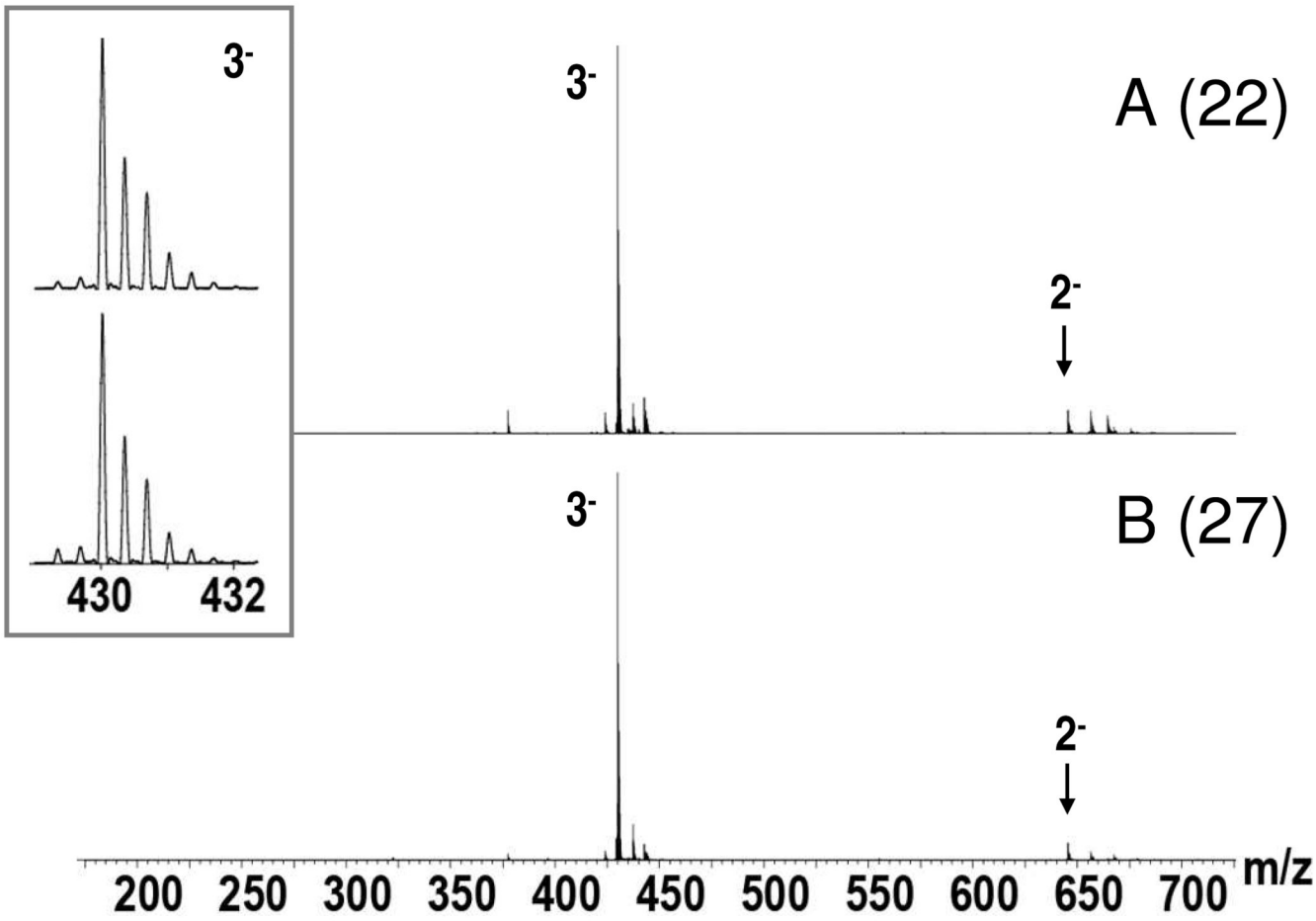

Figure 3.
Full scan mass spectra of fractions 22 (A) and 27 (B). The inset displays the isotopic resolution with which these spectra were acquired.

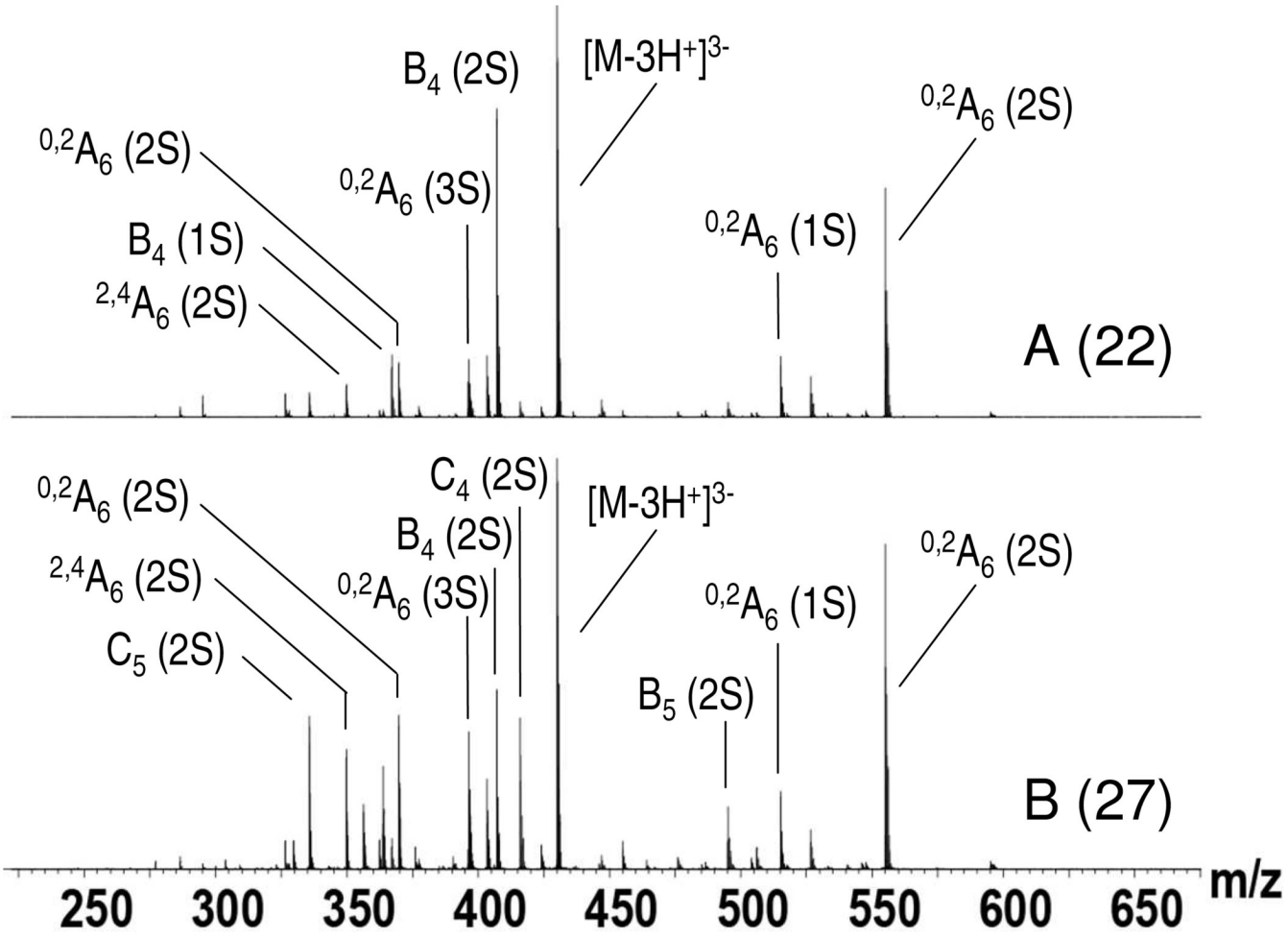

Figure 4.

MS^2 spectra of fractions 22 (A) and 27 (B). Predominant fragment ions are labeled using the Domon and Costello nomenclature [68], whereas all fragment ions above 1% relative abundance are listed in supplementary table 1.

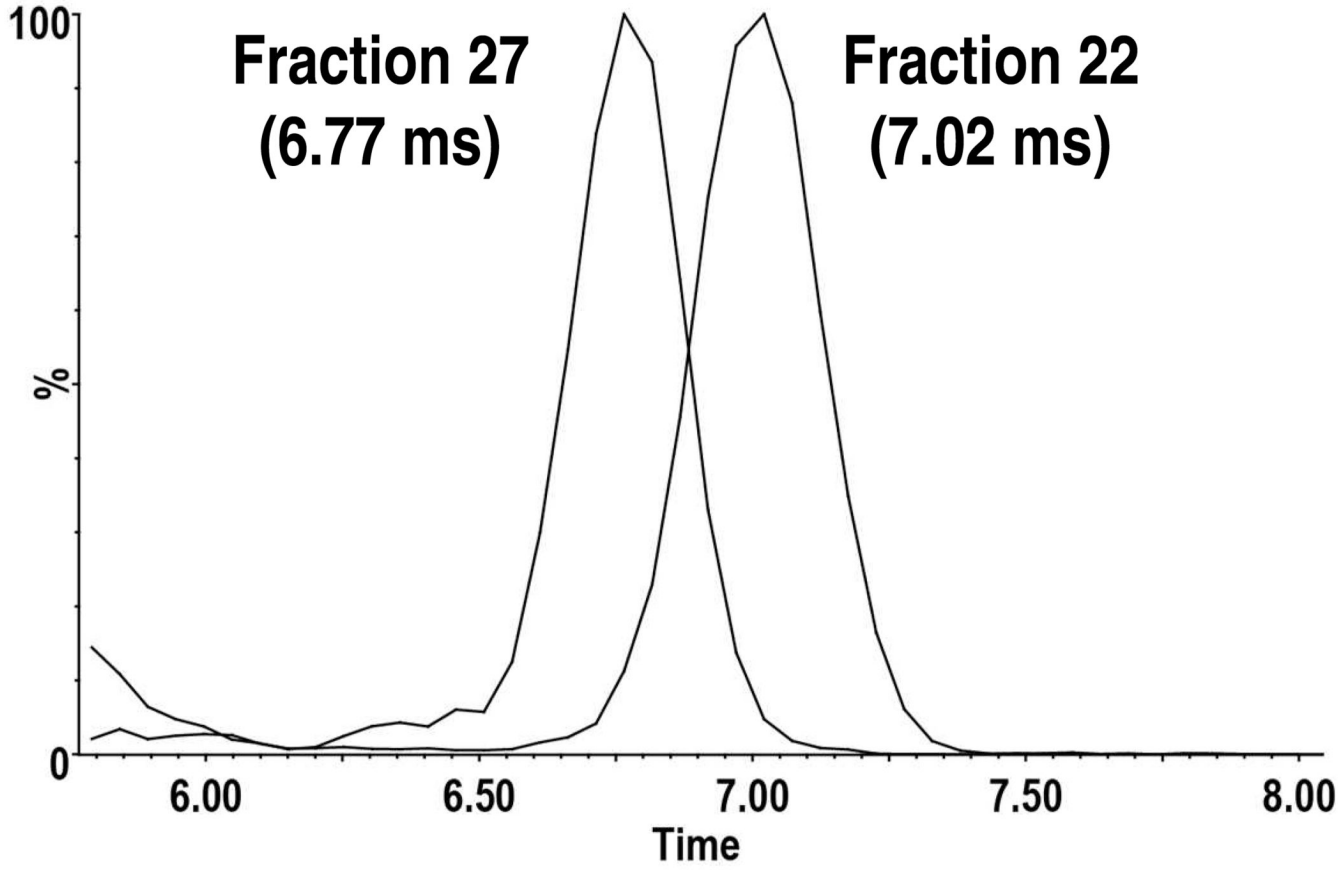

Figure 5.
Overlay of the ion mobility arrival time distributions for the 2^- charge states of the hexasaccharide isomers in fractions 22 and 27. Arrival times are reported in milliseconds

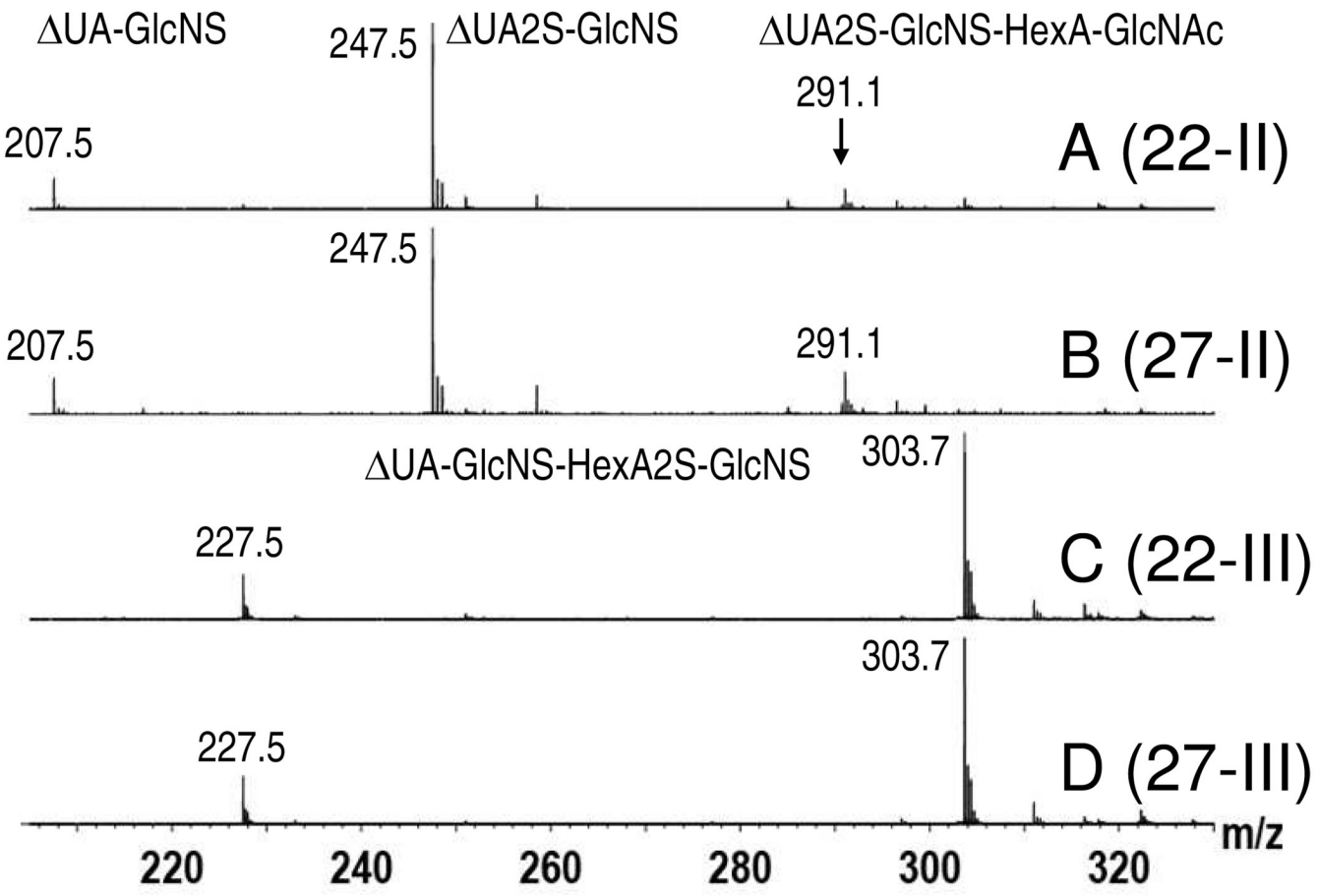

Figure 6.

Partial enzymatic digestions of HS hexasaccharides. Fractions 22 (A) and 27 (B) were treated with heparinase II. Labeled peaks at *m/z* 207.5, 247.5, and 291.1 correspond to the sequences presented (see text for descriptions). Fractions 22 (C) and 27 (D) were also treated with heparinase III. Peaks at *m/z* 227.5 and 303.7 correspond to the 4^- and 3^- charge states of the sequence presented. HexA denotes an uronic acid, but lack of knowledge as to whether it is GlcA or IdoA.

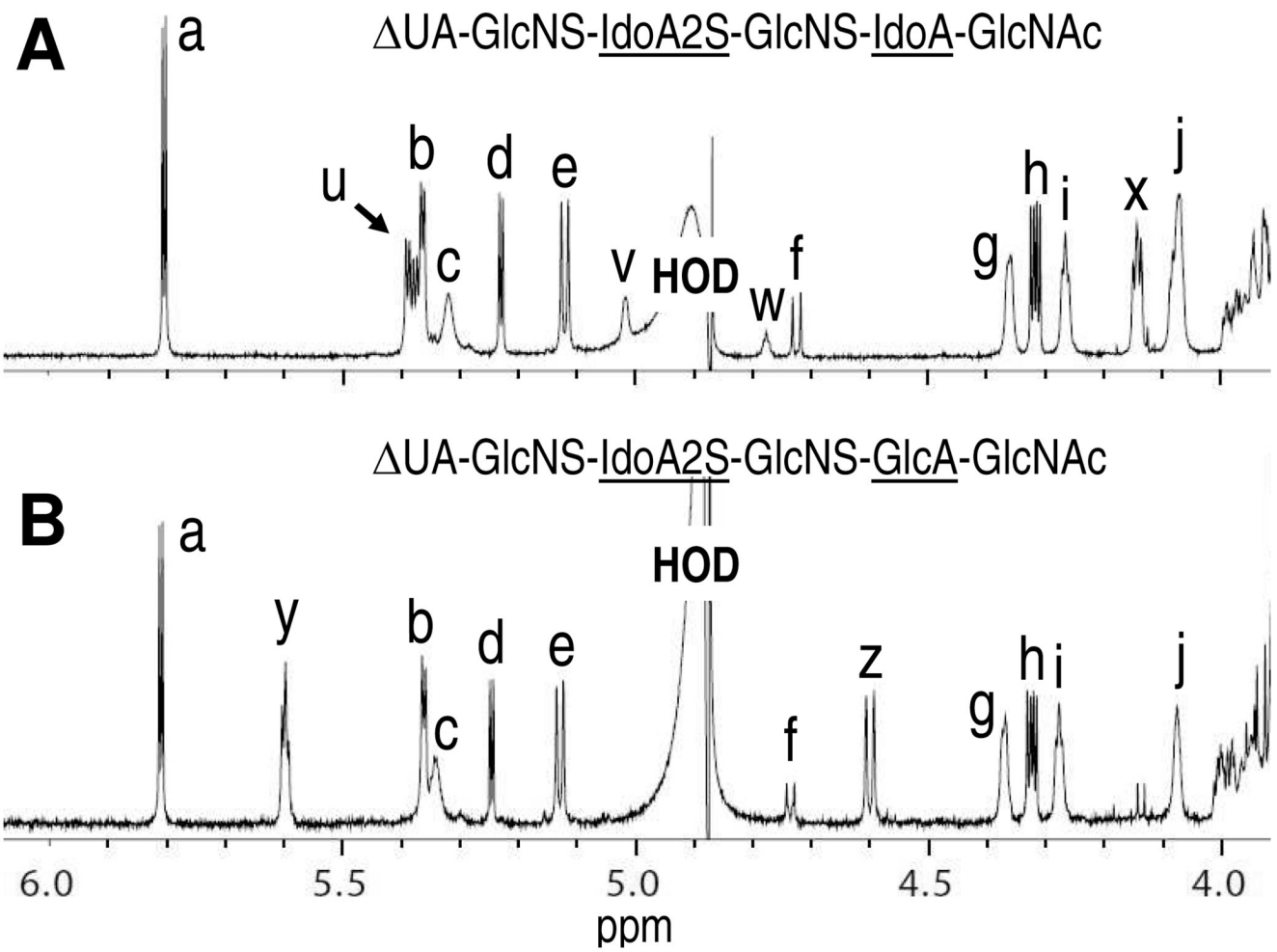

Figure 7.

1-D ^{1}H-NMR spectra for fractions 22 (A) and 27 (B). Common peaks are labeled as *a* ΔUAH4; *b*, GlcNS H1 (to Ido2S); *c*, IdoA2S H1; *d*, GlcNAcα H1; *e*, ΔUA H1; *f*, GlcNAcβ H1; *g*, IdoA2S H2; *h*, ΔUA H3; *i*, IdoA2S H3; and *j*, IdoA2S H4. Unique peaks are labeled as *u*, GlcNS H1 (to IdoA); *v*, IdoA H1; *w*, IdoA H5; *x*, IdoA H3; *y*, GlcNS H1 (to GlcA); and *z*, GlcA H1. The derived sequences for the hexasaccharide in each fraction, based combined data, are presented (inset).

Table 1

Disaccharide compositional analysis results for fractions 22 and 27.

Disaccharide		Fraction 22 Comp. (%)	Fraction 27 Comp. (%)
IV-A	**ΔUA-GlcNAc**	30.2 ± 0.2	31.2 ± 0.8
III-A	**ΔUA2S-GlcN**	0.3 ± 0.1	1.2 ± 0.1
II-A	**ΔUA-GlcNAc6S**	2.1 ± 0.5	2.1 ± 0.1
I-A	**ΔUA2S-GlcNAc6S**	0.1 ± 0.0	0.1 ± 0.0
IV-S	**ΔUA-GlcNS**	35.5 ± 0.9	35.8 ± 1.4
III-S	**ΔUA2S-GlcNS**	30.1 ± 0.3	27.7 ± 0.8
II-S	**ΔUA-GlcNS6S**	0.1 ± 0.0	0.1 ± 0.0
I-S	**ΔUA2S-GlcNS6S**	0.1 ± 0.0	0.1 ± 0.0
I-H	**ΔUA2S-GlcNH6S**	0.0 ± 0.0	0.0 ± 0.0
II-H	**ΔUA-GlcNH6S**	0.0 ± 0.0	0.0 ± 0.0
III-H	**ΔUA2S-GlcNH**	0.0 ± 0.0	0.0 ± 0.0
IV-H	**ΔUA-GlcNH**	1.5 ± 0.3	1.6 ± 0.3