Published in final edited form as:

J Am Soc Mass Spectrom. 2020 October 07; 31(10): 2061–2072. doi:10.1021/jasms.0c00178.

Peracylation coupled with tandem mass spectrometry for structural sequencing of sulfated glycosaminoglycan mixtures without depolymerization

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Abstract

The structures of glycosaminoglycans (GAGs), especially the patterns of modification, are crucial to modulate interactions with various protein targets. It is very challenging to determine the fine structures using liquid chromatography-mass spectrometry (LC-MS) due in large part to the gas-phase sulfate losses upon collisional activation. Previously, our group reported a method for fine structure analysis that required permethylation of the GAG oligosaccharide. However, uncontrolled depolymerization during the permethylation process due to esterification of uronic acid lowers the reliability of the method to resolve structures of GAGs, especially for larger oligosaccharides. Here we describe a simplified derivatization method using propionylation and desulfation. The oligosaccharides have all hydroxyl and amine groups protected with propionyl groups, then have sulfate groups removed to generate unprotected hydroxyl and amine groups at all sites that were previously sulfated. This derivatized oligosaccharide generates informative fragments during CID that resolve the original sulfation patterns. This method is demonstrated to enable accurate determination of sulfation patterns of even the highly sulfated pentasaccharide fondaparinux by MS² and MS³. Using a mixture of dp6 from porcine heparin, we demonstrate that this method allows for structural characterization of complex mixtures, including clear chromatographic separation and sequencing of structural isomers, all at high yield without evidence of depolymerization. This represents a marked improvement in reliability to structurally characterize GAG oligosaccharides over permethylation-based derivatization schemes.

Graphical Abstract

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Author contributions

H.L. performed all propionylation method development and optimization, gathered SEC-UV and LC-MSⁿ data and performed initial interpretation. Q.L. performed the permethylation experiments and gathered SEC-UV data. All authors participated in the writing of this manuscript. J.S.S. designed the method, supervised all experiments, and guided experimental design and data analysis.

Supporting Information

Tables and figures detailing experimental results including sample derivatization, compositional screening, and annotated MS/MS spectra of derivatized dp6 structures. Details are also included describing the process of spectral interpretation of isomeric dp6 structures.

Keywords

glycosaminoglycans; heparin; carbohydrates; derivatization; LC-MS/MS

Introduction

Glycosaminoglycans (GAGs) are linear polysaccharides which are usually covalently linked to the protein forming proteoglycans (PGs). GAGs are present on the cell surface and in the extracellular matrices (ECMs) of almost all mammalian cells [1]. Based on the differences in the repeating disaccharide building blocks, GAGs are typically separated into five classes: hyaluronan (HA), keratan sulfate (KS), chondroitin sulfate (CS), dermatan sulfate (DS), and heparin/heparan sulfate (Hp/HS) [2]. The disaccharide motif of Hp/HS is comprised of either β -D-glucuronic or α -L-iduronic acid (GlcA and IdoA, respectively) and α -D-N-acetyl-D-glucosamine (GlcNAc) connected by 1 $\rightarrow$ 4 linkages. The uronic acid (UA) can be 2-O-sulfated and epimerized, while the GlcN can be 3-O-sulfated, 6-O-sulfated, N-deacetylated and/or N-sulfated [3]. While Hp and HS share the same backbone repeat, they differ by both protein core (HS is found on many proteins and virtually all mammalian cells, while Hp is solely found on serglycin in mast cells and some hematopoietic cells) and the extent of modification (Hp is typically more heavily sulfated with more iduronic acid, while HS is more heterogeneous) [4]. These modifications are incomplete and untemplated during biosynthesis, making Hp/HS the most structurally complex member of GAG family [5]. The high degree of heterogeneity makes separation and structural sequencing a very challenging task.

Interactions between GAGs and proteins are involved in a wide range of physiological and pathophysiological processes, including cell signaling, tumor progression and viral

attachment and entry [6–10]. It is widely believed that GAG polysaccharides with specific sulfation patterns play important roles in many of these bio-processes. For example, not only the total amount but also the amount of specific sulfation of Hp/HS are significantly decreased in renal tumor tissue, which promotes tumor growth, invasion and stimulates angiogenesis [8]. Therefore, the structural sequencing of functional active oligosaccharides rather than just disaccharide composition analyses is essential to understand the functions of GAGs and design novel therapeutics based on these interactions.

Mass spectrometry (MS) and nuclear magnetic resonance (NMR) are two powerful analytical tools to resolve the detailed structural information [11, 12]. The application of NMR is usually limited by the low sensitivity, requiring significant amounts of pure oligosaccharide for fine structure assignment [13]. In contrast, the advantages of MS are often more desirable for sequencing of these highly heterogeneous mixtures, with a high sensitivity and direct compatibility with on-line liquid chromatography (LC) separations [14] and capillary electrophoresis [15]. There are several MS compatible LC separation methods that have been used to analyze GAG oligosaccharides, including size exclusion chromatography (SEC), hydrophilic interaction chromatography (HILIC), porous graphitized carbon chromatography (PGC) and ion-pair reversed phase chromatography (IPRP) [16–19]. The complexity of Hp/HS could be simplified prior to MS analysis using on-line separation [20]. However, accurate sequencing of the sulfation patterns of oligosaccharides remains a major challenge in the fine structural characterization of Hp/HS oligosaccharides using MS and MS².

Collisional induced dissociation (CID) is by far the most prominent method of tandem mass spectrometry [21]. However, CID usually results in extensive sulfate loss [22]. Several pioneering manuscripts have been published to address this issue. Amster and co-workers have stabilized the liable sulfate groups via Na⁺/H⁺ exchange to get informative glycosidic and cross-ring fragment ions [23]. However, the conditions, especially deprotonation and combination with on-line LC separation, need to be optimized for each sulfated GAG making it very difficult to use in a general experimental method. Other ion activation methods have been applied to the fine structural characterization of Hp/HS including negative electron transfer dissociation (NETD), electron detachment dissociation (EDD) and recently ultraviolet photodissociation (UVPD) [24–27]. Questions remain regarding the robustness of these techniques, and some of these dissociation techniques are not currently commercially available, but they remain promising methods of dissociation for underivatized GAG oligosaccharides.

In our previous reports, we have designed a chemical derivatization method coupled with tandem mass spectrometry for structural analysis of Hp/HS oligosaccharides using standard C18 chromatography coupled to CID MS/MS [28–31]. This method involves a permethylation protection step, gentle desulfation by solvolysis, and pertrideuteroacetylation to label sites of sulfation. The derivatized Hp/HS oligosaccharides are separated by C18 reversed phase LC (RPLC) and sequenced by CID MS/MS analysis, allowing fine sequence identification using only glycosidic bond cleavages. With this sequencing method, we could observe sufficient fragment ions to resolve the sequence and have first identified a Robo1-specific Hp/HS octasaccharide sequence [32]. We recently improved the method's ability to

sequence mixed NS/NAc domains by replacing the trideuteroacetylation with propionylation, allowing us to separate tetrasaccharide isomers that differed by the placement of NS/NAc groups [29]. While these methods could successfully sequence mixtures of small oligosaccharides, formation of the uronic acid methyl ester promotes cleavage of the glycosidic bond through β -elimination, especially under conditions required for high permethylation yield [33]. In order to more reliably sequence these essential GAGs, we present an approach here that prevents depolymerization side reactions while still offering high reaction yields. As shown in Figure 1, this method consists of an initial acylation protection step, installing propionyl groups on all non-sulfated/non-acetylated amine and alcohol positions. After solvolytic desulfation, the positional information of the original sulfation pattern will be represented by the newly formed hydroxyl or amine groups. The derivatized analyte can be well-retained on C18 RPLC and sequenced via MS² and MS³. This methodology provides a more robust option for the fine structure analysis of longer GAG oligosaccharides using commonly available commercial LC-MS systems.

Experimental

Materials

Fondaparinux sodium (Arixtra) was purchased from Mylan Inc. (Canonsburg, PA, USA). Sep-Pak C18 Plus Short cartridges (360 mg Sorbent per cartridge, 55–105 μ m particle size, 50/pk) were purchased from Waters (Milford, MA, USA). Enoxaparin sodium injection (Winthrop/Sanofi, Bridgewater, NJ, USA) was used as provided. Triethylamine (TEA) was purchased from Fisher Scientific (Hampton, NH, USA) while tetrahydrofuran (THF) was purchased from VWR International, LLC (Radnor, PA, USA). 4-(Dimethylamino) pyridine (DMAP), propionic anhydride and Dowex 50WX8–100 ion-exchange resin were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Water was purified by a MilliQ system (Millipore, Bedford, MA, USA). All other regular chemical reagents used in the chemical derivatization were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

Propionylation of HA dp10

3 nmols of dried hyaluronic acid (HA) dp10 were resuspended in 72 μ L toluene. 8 μ L of DMAP in toluene (1 μ g/ μ L) was added, followed by addition of 0.8 μ L TEA and 20 μ L propionic anhydride. The reaction was incubated at 25 °C for 2 days. The reaction was stopped by adding 1 mL methanol and dried under nitrogen gas flow. The propionylated HA dp10 was resuspended in water at a concentration of 1 μ g/ μ L. The sample was analyzed by HPLC SEC column (4.6 mm $\times$ 300 mm, 1.7 μ m, Waters) using a Dionex UltiMate 3000 LC system (Thermo Fisher Scientific) following the method of Zaia and coworkers [34]. UV detection was carried out at 232 nm.

Chemical Derivatization of Arixtra

To increase its solubility in THF, 11 μ g Arixtra was converted to triethylamine (TEA) salts by passing through a self-packed cation-exchange column with Dowex 50 W resin, followed by lyophilization. The dried TEA salts were resuspended in 500 μ L THF and added 160 μ L DMAP in THF (12 μ g/ μ L) followed by addition of 19 μ L TEA and 108 μ L propionic anhydride. The reaction was incubated at 45 °C for 4 days. The reaction was stopped by

adding 1 mL methanol. The propionylated products were dried and then purified with a 3 kDa Amicon Ultra centrifugal filter (Millipore, Temecula, CA) to remove the excess amount of DMAP and propionic anhydride. Although the molecular weight of Arixtra is below 3 kDa, it has reported that any size of the Hp/HS sample larger than tetrasaccharide does not pass through the 3 kDa membrane [35]. The purified products were made as pyridinium salts using the same cation-exchange resin and dried again as reported [30]. The pyridinium salts were dissolved in 10% methanol in dimethyl sulfoxide (DMSO) and incubated for 6 h at 100 °C to remove the sulfates [29]. The desulfated products were dried and resuspended in water at a concentration of 0.2 µg/ µL for analysis.

Yield estimation by HILIC LC-MS

As there is no chromophore on Arixtra for UV quantification, HILIC LC-MS was used to estimate the propionylation yield for Arixtra. The derivatized products were analyzed by LC-MS on a Thermo Orbitrap Fusion Tribrid (Thermo Fisher Scientific) coupled with a Dionex UltiMate 3000 liquid chromatography (Thermo Fisher Scientific). Buffer A was 10 mM ammonium formate (pH 4.4, adjusted with formic acid) and buffer B was 98% acetonitrile with 2% buffer A. Online HPLC was performed on a BEH Amide HILIC column (50 mm × 1 mm, 1.7 µm, ACQUITY UPLC® BEH Amide, Waters). The flow rate was set to 0.1 mL/min and a 3 µL injection at a sample concentration of 0.2 µg/ µL. The gradient started with 90% B for 5 min, with a linear gradient down to 30% B over 60 min, held at 30% B for 4 min and finally washed with 10% B for 10 min and re-equilibrated with 90% B for 10 min. The theoretical mass for each degree of substitution was generated via ChemDraw Professional software (version 16.0, PerkinElmer Informatics). The yield was estimated based on the peak area of the extracted ion chromatogram (EIC) of each product from HILIC-MS. This quantification method inherently assumes equal ionization efficiencies of all products, and should be viewed as semi-quantitative.

Chemical derivatization of heparin dp6 mixture

A heparin hexasaccharide (dp6) mixture was collected by SEC separation. Briefly, 100 mg of commercial Enoxaparin Sodium Injection was injected onto the in-house packed P-10 SEC column (Bop-Rad®, 45–90µm), separated with 0.5 M ammonium bicarbonate, and the dp6 fraction was collected using a Frac-920 fraction collector (GE Healthcare Life Sciences, Chicago, IL, USA). Arixtra (5 µg) as an internal standard was added into the dried dp6 (50 µg) and the oligosaccharides were converted to TEA salts as described above for Arixtra. The dried TEA dp6 salts were resuspended in 650 µL THF and 5 mg of DMAP were added, followed by addition of 48 µL TEA and 1 mL propionic anhydride. The reaction was incubated at 45 °C for 4 days. The reaction was stopped by adding in methanol and purified with a 3kDa filter. The solvolytic desulfation process was completed as described above for Arixtra. The desulfated products were further dried and resuspended in water for LC-MS/MS analysis. Analysis of the Arixtra internal standard gave an estimated full reaction yield of 82% with no evidence of depolymerization (data not shown).

Structural sequencing with LC-MS/MS and LC-MS³ analysis

Online HILIC LC-MS was performed for hexasaccharide composition analysis of the enoxaparin dp6 mixture prior to derivatization. The detailed method for HILIC-MS was

identical to that described for Arixtra yield estimation. A theoretical mass list was generated manually to identify selected compositions using Xcalibur.

For LC-MS/MS and MS³, all samples were analyzed on a Thermo Orbitrap Fusion Tribrid (Thermo Fisher Scientific) coupled with a Dionex UltiMate 3000 liquid chromatography (Thermo Fisher Scientific). The reverse-phase separation was performed on a C18 PepMap 100 trap column (15 mm × 0.3 mm, 5 μm, Thermo Fisher Scientific) with an Acclaim PepMap 100 C18 column (15 cm × 75 μm, 2 μm, Thermo Fisher Scientific). Buffer A was 0.1% formic acid in water, and buffer B was 0.1% formic acid in acetonitrile. The flow rate was set to 0.25 μL/min. To sequence Arixtra, a 30 min gradient started at 20% B for 4 min, elevated to 70% at 4.5 min, with a linear gradient up to 98% B over 10.5 min, held at 98% B for 5 min and re-equilibrated with 20% B for 10 min. To sequence dp6, a 60 min gradient started at 20% B for 4 min, with a linear gradient to 98% B over 26 min, held at 98% B for 20 min and re-equilibrated with 20% B for 10 min. The derivatized products were analyzed in positive ion mode with the nanoelectrospray voltage set to 2.6 kV. Full MS scan range was set to 150–800 m/z at a resolution of 60000. RF lens was 6% and the automatic gain control (AGC) target was set to 1.0×10^5 . For the MS/MS scans, the resolution was set to 15000, the precursor isolation width was 3 m/z units and ions were fragmented by collision-induced dissociation (CID) at a normalized collision energy of 40%. For the MS³ scans for Arixtra, the resolution was set to 15000, the precursor isolation width was 2 m/z units and ions were fragmented by collision-induced dissociation (CID) at a normalized collision energy of 25%. Annotation of MS/MS spectra were carried out using the nomenclature of Domon and Costello [36]. -P represents neutral loss of C₃H₄O; -PA represents neutral loss of C₃H₆O₂; -OMe represents neutral loss of CH₃O.

Results and Discussion

The purpose of the chemical derivatization strategy is to protect the initial hydroxyl and free amine groups via an acylation reaction, and then remove all labile sulfate groups to leave free hydroxyl and/or amine groups only where the sulfates used to be as shown in Figure 1. Original sites of sulfation can then be identified by a lack of acylation in MS/MS. In order to differentiate naturally occurring GlcNAc from GlcN labeled during the acylation step, propionylation is used for the protection chemistry. After MS/MS, the original sites of sulfation can be determined based on the lack of propionyl groups on the monosaccharide.

Propionylation of HA dp10

The major drawback to our previously published derivatization scheme for sequencing GAG oligosaccharides was uncontrolled depolymerization, which increased with increasing oligosaccharide length. In order to test the ability to fully propionylate GAG oligosaccharides without the undesirable depolymerization previously identified with permethylation [28, 29], we propionylated dp10 of hyaluronic acid (HA) and used SEC LC-UV detection to identify any depolymerization products. As shown in Figure S1 in Supporting Information, HA dp10 undergoes permethylation-induced depolymerization due to esterification of the glucuronic acid. Because of the relatively homogeneous size, HA

dp10 allows us to screen our optimized acylation methodology to determine if it also results in depolymerization of the long oligosaccharide [29].

As shown in Figure 2, the SEC HPLC chromatogram showed that after propionylation, the derivatized HA dp10 had a similar retention time as the underivatized sample. No evidence of depolymerization is evident in the acylated HA dp10, while depolymerized HA dominated the permethylated dp10 SEC chromatogram under both conditions tested.

Chemical derivatization of Arixtra

Unlike unsulfated HA, the high amount of sulfate and carboxyl groups made sulfated GAGs highly negatively charged. They also often form sodium salts, which prevents the complete dissolution in a less polar solvents [28]. Arixtra is often used to represent a challenging GAG oligosaccharide for methods development, as it has a very high sulfate density (eight sulfates in a pentasaccharide), has a non-repetitive structure, and includes an uncommon 3-*O*-sulfation which has been shown to be more labile to high pH in some analyses [37]. The polarity of the solvent had a large effect on the yield of acylation, with the rate of reaction accelerated in the less polar solvent. In order to solubilize highly-sulfated Arixtra in a non-polar solvent, Arixtra was converted to a TEA salt prior to propionylation [28, 38]. Even so, the highly sulfated TEA-Arixtra salts could not dissolve well in the toluene solvent used for HA propionylation. A prior report used THF as the solvent for acetylation [39], and the Arixtra-TEA salts dissolved well in this solution. The widely accepted mechanism for acylation is through the nucleophilic attack of DMAP at the anhydride carbonyl group and subsequent formation of the corresponding acyl-pyridinium cation. This cation reacted with the hydroxyl groups and yielded the final product, fully propionylated Arixtra [40]. The auxiliary base, TEA, was necessary to regenerate the protonated catalyst after the reaction. Thus, the amount of DMAP and TEA were important parameters for full propionylation. For the optimized protocol used here, the yield of full propionylation of Arixtra (+6 propionyl groups) was estimated to be 86% with most of the losses in the form of singly-underpropionylated (<10%) and singly-desulfated, singly-overpropionylated (<4%) products (Table S1, Supporting Information). The singly-overpropionylated side product is the result of a single desulfation, followed by full peracylation. Unfortunately, the abundance of this side product was too low to determine if a specific sulfate was lost, or if the desulfation could occur at multiple sites across Arixtra (data not shown). Permethylation was one of the most well-established chemical derivatization methods used in MS sequencing. However, the reported yields of full permethylation was low due primarily to depolymerization and work-up losses: approximately 30% for HS dp4 mixtures [31] and less than 50% for Arixtra [33]. Without using sodium hydroxide in the reaction, propionylation avoids depolymerization [38]. Together with a high yield and no depolymerization, propionylation shows advantage over permethylation.

LC-MS² and MS³ analysis of derivatized Arixtra

Compared to *N*-sulfation and 6-*O*-sulfation on GlcNAc, it is very rare to have 3-*O*-sulfated glucosamine naturally, occurring about 1/20 disaccharides in heparin and less than 1/100 disaccharides in HS [41]. Interestingly, 3-*O*-sulfation is essential for many Hp/HS-protein interactions, including antithrombin III [42], Herpes simplex virus-1 [43], cyclophilin B [44]

and fibroblast growth factor 7 [45]. Even though it is a rare modification, 3-*O*-sulfation is vital to diverse biological functions. A good structural sequencing technique needs to resolve the sulfation pattern, including the site of sulfation on each glucosamine (*N*-, or 6-*O*, and/or 3-*O*-sulfation). Unfortunately, there are few commercially available 3-*O*-sulfated Hp/HS standards. Arixtra is a synthetic, ultralow-molecular-weight heparin-based pentasaccharide that potentiates the innate neutralization of Factor Xa by antithrombin III (ATIII) and interrupts the blood coagulation cascade [46]. Arixtra has *N*-, and 6-*O*, and 3-*O*-sulfation sites in high sulfate density with variability among the GlcN residues, and is a challenging model to study the derivatization properties and validate the structural sequencing method.

At the MS² level, oligosaccharides are mainly fragmented via glycosidic bond cleavage. Based on the glycosidic bond cleavage pattern of acylated and desulfated Arixtra, the number of sulfate groups on each saccharide was resolved. Fragmentation nomenclature is followed as described by Domon and Costello [36]. As illustrated in Figure 3, the mass differences between the product ion [Y₂+H]⁺ and [Y₁+H]⁺, and [B₃+H]⁺ and [B₄+H]⁺ both demonstrated that there was one propionyl group and one hydroxyl group, indicating one sulfate group on the GlcA close to the reducing end. Since only the 2-*O* position can be sulfated, we located the sulfate position on the reducing end uronic acid; the same strategy can be used to locate sulfation on any uronic acid. Similarly, the mass difference between the product ion [Y₃+H]⁺ and [Y₂+H]⁺ demonstrated that there was no acylation on the middle GlcN, indicating that this GlcN was initially (NS, 3S, 6S) trisulfated. With the MS² spectrum, we could resolve all uronic acid sulfations and the trisulfated GlcN by glycosidic bond cleavage. While Arixtra does not contain any GlcNAc residues, we would similarly be able to resolve any GlcNAc-containing residue using the same strategy (unsulfated or 6S).

The [B₁+H]⁺ product ion indicated that the GlcN on the non-reducing end was dipropionylated (and, therefore, originally disulfated). Similarly, the [Y₁+H]⁺ ion indicates that the GlcN on the non-reducing end was monopropionylated (and, therefore, originally disulfated). However, localization of sulfates in disulfated GlcN residues is more complex. The addition of 3-*O*-sulfation is one of the last modifications in biosynthesis, and has only been found from mammalian sources on GlcNS [47–49]. Due to biosynthetic constraints, disulfated GlcN has two possible structures: the common (NS, 6S) and the rare (NS, 3S). In order to resolve the sulfation positions on disulfated GlcN, we performed MS³ to generate cross-ring cleavage products. As illustrated in Figure 4, the MS³ of [B₃+H]⁺ gave three major product ions: [^{1,4}X₄+H]⁺, [B₃Y₄+H]⁺, and [^{1,4}X₄–H₂O+H]⁺. The unique ion, [^{1,4}X₄+H]⁺, indicates that there was a 6-*O* hydroxyl group on this GlcN. With these results, we located one sulfate group to the 6-*O* on the GlcN of the non-reducing end, placing the other sulfate group on the *N*-position on the GlcN of the non-reducing end.

Similarly, the annotation of the GlcN on the reducing end followed the same process. As illustrated in Figure 5, the MS³ of [Y₁-OMe+H]⁺ gave two major product ions [^{1,4}A₅+H]⁺, and [Z₁-OMe+H]⁺. The product ion, [^{1,4}A₅+H]⁺, shows that there is one propionylation, indicating an (NS, 6S) structure for the reducing end GlcN. All assignments are consistent with the known Arixtra structure, and no other possible assignments are supported by the data. While no monosulfated GlcN is present in Arixtra, it would be assigned in a similar fashion, using MS³ to differentiate between the common GlcNS and the rare GlcN6S.

LC-MS and LC-MS/MS analysis of heparin hexasaccharide mixture

To evaluate and apply our method for structural analysis of native heparin oligosaccharide mixtures, a heparin hexasaccharide mixture was collected from SEC separation as described above. Prior to chemical derivatization and LC-MS/MS analysis, the compositional information of dp6 was collected by HILIC LC-MS. Based on the compositional information, a theoretical mass list for the corresponding derivatization products of selected detected dp6 compositions were listed in Table S2. Prior to desulfation, the propionylated dp6 was analyzed by SEC-UV. As shown in Figure S2 in Supporting Information, dp6 was fully propionylated without evidence of depolymerization. After peracylation and desulfation, LC-MS/MS analysis was performed. As shown in Figure 6A, the resulting mixture of derivatized dp6 was incredibly complex. Figure 6B shows that even the number of structures was large, especially for certain composition. Complete analysis of this mixture by manual annotation is beyond the scope of this manuscript; we focused on six structures from five compositions to highlight the utility of our method. The mass spectrometry data have been deposited to the ProteomeXchange Consortium via the PRIDE [50] partner repository with the dataset identifier PXD021263. We annotated one pair of structural isomers (Figure 7 and Figure 8) and four other structures, with MS/MS spectra and assignments shown in Supporting Information: dp6 with 6 sulfo groups (Figure S3), dp6 with 7 sulfo groups (Figure S4), dp6 with 9 sulfo groups (Figure S5) and dp6 with 7 sulfo groups and 1 acetyl group (Figure S6). Development of automated analysis software as previously described for our previous permethylation-desulfation-acylation strategy [51, 52] will be required to allow for thorough characterization of these complex mixtures with this current strategy.

In order to test if our derivatization method allowed for chromatographic separation and assignment of structural isomers, we focused on two structures for dp6 with 8 sulfation sites. Figure 6c–d clearly shows that, after derivatization, we can achieve separation of structural isomers of this dp6. The structures illustrated in the pseudo-MRM traces in Figure 6c–d are assigned based on their MS/MS spectra, as shown in Figure 7 and Figure 8, respectively. A detailed description of the assignment process for this set of isomers is given in Supporting Information. Structure 1 assigned with 6-*O*- and *N*-sulfation on the reducing-end eluted around 22.0 min after derivatization, while Structure 2 assigned with only *N*-sulfation on the reducing-end eluted around 15.9 min after derivatization. The observed shift in retention time of almost 6 minutes from the movement of a single sulfation site from the reducing end to an internal residue makes it clear that chromatographic separation of even very complex mixtures is possible with this method, while the replacement of labile sulfo groups results in MS/MS spectra that are much more informative.

Conclusions

In this work, a new chemical derivatization strategy was designed to allow the structural sequencing of sulfated GAGs by MSⁿ fragmentation. The replacement of permethylation with acylation simplified the derivatization strategy from a three-step reaction to two-steps and generated informative product ions. While the method presented here has a longer derivatization time than previously published derivatization protocols [53–56] (~3 days

versus ~5 days), the current protocol is far less laborious, with most of the derivatization time being a single 4-day incubation. Even more notably, acylation eliminates the widespread depolymerization problem previously observed with permethylation. The resulting reaction yield was significantly increased. Therefore, without huge sample loss and the highest derivatization yield, the strategy would be more reliable to resolve the essential structures of unknown functional Hp/HS oligosaccharides.

After chemical derivatization, the sulfated GAGs retain well on a standard C18 column and elute with a typical reverse phase gradient, which was directly compatible to a common electrospray interface. The assignments based on product ions primarily generated by glycosidic bond cleavages reveal the amount of sulfo groups on each ring. The resulting cross-ring cleavage fragments could resolve the sulfation pattern on one partially sulfated (disulfated or monosulfated) glucosamine. Combining the low detection limit of nano C18 column (normally nmols to pmols), the resulting method is capable of sequencing even more complex sulfated oligosaccharides with trace amount and commonly LC-MS instruments.

While all methods have their own advantages at various aspects, there is still no universal methodology that can accurately sequence complex sulfated GAGs with various sulfation patterns. The method we describe here has its own limitations as well. While our experiments on heparin were able to generate a large amount of structural information on a large number of structures and likely structural assignments could be inferred based on the likelihood of certain modifications, complete and unambiguous assignment of all possible structures is not achieved with MS/MS. Unlike permethylation, MS³ is often necessary in this method to locate the sulfate groups on monosulfated or disulfated GlcN, such as GlcNS3S VS. GlcNS6S or GlcNS VS. GlcN6S. Some reports of chemoenzymatically synthesized Hp/HS oligosaccharides indicate that 3-*O*-sulfation can be forced onto GlcN residues under some conditions [57]; MS³ analysis may be required to structurally characterize monosulfated GlcN residues that derive from chemoenzymatic synthesis. The effect of epimerization of uronic acid on the fragmentation pattern of Hp/HS oligosaccharides derivatized as presented here is also currently unclear; further studies trying to understand these differences are currently underway. However, due to the high reaction yield, the lack of depolymerized side products, and the relative robustness of the chemistry, we anticipate that the method we present here will be more easily adopted by GAG researchers and more applicable to longer GAG oligosaccharides, opening up new areas of investigation in GAG structure-function analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institute of General Medical Sciences of the National Institutes of Health through the Research Resource for Integrated Glycotechnology (P41GM103390) and the Glycoscience Center of Research Excellence (P20GM130460-A1). LC-MS/MS was supported in part by a grant from the National Institute of General Medical Sciences (R01GM127267). H.L. acknowledges support for this work through a University of Mississippi Graduate Student Council Research Grant.

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Synopsis:

Extracted ion chromatograms showing complexity of heparin dp6 isomers, and ability of method to separate isomers chromatographically and identify structural differences by MS/MS

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Figure 1.

Chemical derivatization workflow. To enhance solubility in THF, Arixtra was first converted to its TEA salt by passing through a cation exchange resin, followed by propionylation with propionic anhydride. After propionylation, derivatized Arixtra was converted to its pyridinium salt. Sulfates were removed with solvolytic desulfation, leaving sites of sulfation marked by free hydroxyl or amine groups.

Figure 2. SEC chromatograms of **a.** reaction blank with no HA dp10, **b.** unmodified HA dp10, and **c.** fully propionylated HA dp10 with UV detection at 232 nm, respectively. After propionylation, there was only one GAG peak with a similar peak shape and retention time as the unmodified HA dp10. The SEC chromatogram demonstrated no depolymerization of HA dp10 during propionylation.

Figure 3.

Sulfation sequencing analysis of Arixtra by acylation/desulfation and MS/MS. **a.** The structure and fragmentation path for derivatized Arixtra; **b.** Tandem MS spectrum of $[M + 2H]^{2+} = 602.735$, corresponding to fully derivatized Arixtra. -P represents loss of a propionyl group (56.026 Da) and -OMe represents loss of methanol (32.026 Da) from the reducing end. The mass accuracy for each assigned product ion was below 10 ppm.

(b)

Figure 4.

Sulfation sequencing analysis of the non-reducing end of derivatized Arixtra by MS^3 . a. Fragmentation path of B_3 ion, $[B_3+H]^+$; b. MS^3 spectrum of $[B_3+H]^+$ of fully acylated, desulfated Arixtra. The mass accuracy for each assigned product ion was below 5 ppm.

Figure 5. Sulfation sequencing analysis of the reducing end of derivatized Arixtra by MS^3 . a. fragmentation path of Y_1 -OMe ion, $[Y_1\text{-OMe} + H]^+$; b. MS^3 of $[Y_1\text{-OMe} + H]^+$. The mass accuracy for each assigned product ion was below 5 ppm.

Figure 6.

LC separation of the derivatized heparin dp6 mixture. a. TIC of the derivatized dp6 mixture; b. EIC corresponding to a fully derivatized octasulfated dp6, doubly protonated ($m/z = 730.763$); c. MS/MS pseudo-MRM trace of $730.763 \rightarrow 274.128$, corresponding to the $[Z_1+H]^+$ product of a disulfated reducing end glucosamine as shown in Figure 7; d. MS/MS pseudo-MRM trace of $730.763 \rightarrow 330.1541$, corresponding to the $[Z_1+H]^+$ product of a monosulfated reducing end glucosamine as shown in Figure 8.

(a)

(b)

Figure 7.

Sulfation sequencing analysis of Structure 1 by acylation/desulfation and MS/MS. **a.** The structure and observed product ions for Structure 1; **b.** Averaged MS/MS spectrum of $[M + 2H]^{2+} = 730.763$ obtained between 21.90–22.26 min, corresponding to fully derivatized oligosaccharide illustrated in Structure 1. –PA represents the loss of propionic acid (74.037 Da). The mass accuracy for each assigned product ion was below 10 ppm.

Figure 8.

Sulfation sequencing analysis of Structure 2 by acylation/desulfation and MS/MS. **a.** The structure and observed product ions for Structure 2; **b.** Averaged MS/MS spectrum of $[M + 2H]^{2+} = 730.763$ obtained between 16.04–16.39 min, corresponding to fully derivatized oligosaccharide illustrated in Structure 2. The mass accuracy for each assigned product ion was below 10 ppm.