Quantification of Heparan Sulfate Disaccharides Using Ion-Pairing Reversed-Phase Microflow High-Performance Liquid Chromatography with Electrospray Ionization Trap Mass Spectrometry

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The glycosaminoglycan (GAG) family of biomacromolecules is composed acidic and linear chains of repeating disaccharide units. Quantitative disaccharide composition analysis is essential for the study and characterization of GAGs. Heparan sulfate and heparin consist of multiple disaccharide units and can be well-separated by ion-pairing reversed-phase microflow high-performance liquid chromatography (IPRP-Mf-HPLC). Each disaccharide can be detected and its mass confirmed by electrospray ionization mass spectrometry (ESI-MS). Isotopically enriched disaccharides were prepared chemoenzymatically from a uniformly 13C,15N-labeled N-acetylheparosan (−GlcA(1→4)GlcNAc−) obtained from the fermentation of E. coli K5. These isotopically enriched disaccharides have identical HPLC retention times and mass spectra as their unlabeled counterparts and were used in liquid chromatography—mass spectrometry (LC—MS) as internal standards. The ratio of intensities between each pair of enriched and nonenriched disaccharides showed a linear relationship as a function of concentration. With the use of these calibration curves, the amount of each disaccharide (≥2 ng/disaccharide) could be quantified in four heparan sulfate samples analyzed by this method.

Heparan sulfate (HS)/heparin participate in many important biological processes, including blood anticoagulation, viral and bacterial infection and entry, angiogenesis, inflammation, cancer, and development.1–3 HS/heparin carry out their biological functions primarily by their interaction with proteins in which the sulfonic acid groups electrostatically interact or hydrogen bond with basic amino acids of the target protein.4–6 In a number of cases, HS/heparin have been demonstrated to bind specifically and with high affinity to proteins regulating their biological functions.4

HS/heparin are acidic linear polysaccharides having closely related structures. Both are isolated from animal tissues and are members of the glycosaminoglycan (GAG) family. These GAGs have an average molecular weight of 10–15 kDa and contain chains in size ranging from 5 to 50 kDa, corresponding to a polydispersity of 1.05–1.6.7 HS/heparin are both composed of a repeating disaccharide structure of 1,4-linked hexuronic acid and glucosamine residues. Heparin has a simpler structure with its most common disaccharide unit being 2-O-sulfo-L-iduronic acid (IdoA2S) 1→4-linked to 6-O-sulfo, N-sulfo-α-L-glucosamine (GlcNS6S), −IdoA2S(1→4)GlcNS6S−. HS has a similar but more highly variable and less sulfated structure with its most common disaccharide unit being β-D-glucuronic acid (GlcA) and N-acetyl-α-L-glucosamine (GlcNAc), −GlcA(1→4)GlcNAc−.8 HS/heparin from different tissues and various animals can differ substantially in their disaccharide composition and, hence, often have very different activities.9

The characterization of HS/heparin with various sequences and structures is critical in elucidating the functions corresponding to these GAGs. Characterization is still a challenge for analysts, because of the structural complexity and heterogeneity of these GAGs. Nuclear magnetic resonance (NMR) spectroscopy has been used for the disaccharide analysis of HS/heparin, but it is limited by the required sample amount, sample molecular weight, polydispersity, and sequence heterogeneity. High-performance liquid chromatography (HPLC) and capillary electrophoresis (CE)-based disaccharide analysis has been widely used over the last couple

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of decades. These methods are generally more sensitive and efficient than NMR analysis. In these analyses, HS/heparin are first completely or nearly completely depolymerized using either nitrous acid or a mixture of heparin lyases and reduced to obtain a mixture of disaccharides. After disaccharide separation by HPLC or CE,14–16 the resulting disaccharides are often detected by absorbance, fluorescence (requiring either precolumn or postcolumn derivatization), or radioisotope (requiring precolumn labeling) methods to quantify the disaccharides comprising an individual HS/heparin sample. These disaccharide analyses require the use of standards, some of which are commercially available but some of which need to be prepared.17,18 However, because these approaches provide no additional structural information, these methods are difficult to use for the analyses of mixtures containing larger oligosaccharides (i.e., resistant tetrasaccharides) or peptide and other impurities. The sensitivity of such methods can also be affected by gradient solvent systems often required for elution and the requirement for precolumn or postcolumn derivatization steps. Mass spectrometry (MS) and liquid chromatography–mass spectrometry (LC–MS) have been successfully applied to analyze disaccharides and oligosaccharides derived from HS/heparin and can provide sensitive disaccharide analysis along with additional structural information.19–22 However, quantification is often a bottleneck of MS analysis, and quantification is critical for understanding the disaccharide content of biological samples. An unnatural internal standard disaccharide, UA2S–GlcNCOEt6S (IP) has been used in several studies for the identification and quantification of mixtures of HS/heparin disaccharides in MS and LC–MS.22,23 Unfortunately, different disaccharide structures, in particular sulfation level and pattern, result in very different ion intensities in MS.23–25 Different ionization methods, solvent systems, or matrixes, and even instrumentation result in major differences in disaccharide signal levels. Thus, a better method for disaccharide quantitative analysis by LC–MS would use an internal standard for each disaccharide present in an HS/heparin sample. Our laboratory has recently reported the chemoenzymatic synthesis of HS/heparin.26–30 Moreover, in this synthesis it was possible to introduce stable isotopes into the structures of HS/heparin and its precursors having a variety of disaccharide compositions.

In this paper, the most common heparin/HS disaccharides were analyzed by ion-pairing reversed-phase microflow high-performance liquid chromatography (IPRP-Mf-HPLC) using electrospray ionization MS (ESI-MS) detection. Seven uniformly 13C,15N-labeled disaccharides were prepared by chemoenzymatic synthesis from a uniformly labeled [13C,15N]-N-acetylheparosan (–GlcA(1–4)GlcNAc–) prepared from E. coli K5. These internal standards with same retention time and ionization patterns were applied in LC–MS method to quantify the HS/heparin disaccharide composition. In addition, the sensitivity in this method improved to 2 ng/disaccharide.

**EXPERIMENTAL SECTION**

**Materials.** Unsaturated disaccharide standards of heparin/HS, ∆UA–GlcNAc (0S), ∆UA–GlcNS (NS), ∆UA–GlcNac6S (6S), ∆UA2S–GlcNAc (2S), ∆UA2S–GlcNS (NS2S), ∆UA–GlcNS6S (NS6S), ∆UA2S–GlcNac6S (2S6S), and ∆UA2S–GlcNS6S (Tris) were obtained from Iduron Co. Manchester, U.K. (where ∆UA is 4-deoxy-α-l-threo-4-enopyranosyluronic acid). HS-L1 and HS-L2 GAG chains, having low and high sulfation levels, were released from HS proteoglycans that had been extracted and purified from porcine liver. HS-B1 and HS-B2 GAG chains, having low and high sulfation levels, were released from HS proteoglycans that had been extracted from bovine brain in our laboratory.31,32 The protocol for the preparation of these HS samples is described in the Supporting Information.

**Preparation of Isotopically Labeled Disaccharide Standards.** Uniformly labeled [13C,15N]N-acetylheparosan polysaccharide was prepared by the fermentation of E. coli K5 on 13C glucose and 15N ammonium chloride as previously described.29

Uniformly 13C,15N-labeled polysaccharides, N-sulfolheparosan (–GlcA–GlcNS–), N-acetyl-6-O-sulfopentosan (–GlcA–GlcNac6S–), N-sulfo-6-O-sulfopentosan (–GlcA–GlcNS6S–), undersulfated heparin (–IdoA2S–GlcNS–), and heparin (–IdoA2S–GlcNS6S–) were prepared from N-acetylheparosan (–GlcA–GlcNAc) using chemoenzymatic synthesis previously reported methods (Scheme 1).26–30 [13C,15N]-UA–GlcNAc, (0S), [13C,15N]-UA–GlcNS (NS), [13C,15N]-UA–GlcNac6S (6S), [13C,15N]-UA2S–GlcNS (NS2S), [13C,15N]-UA2S–GlcNS6S (NS6S), and [13C,15N]-UA2S–GlcNS6S (Tris) were prepared by digestion of corresponding polysaccharides using recombinant, E. coli–expressed heparin lyases 1, 2, and 3 (Hep 1, 2, and 3).29 The digestion products were purified using anion-exchange high-pressure liquid chromatography (SAX-HPLC) on a SAX S5 Spherisorb column (Waters, Milford, MA) with a 0–1 M NaCl (pH 3.5) linear gradient elution.33 The purified fractions were collected, using desalted P2 column (BioRad, 4350 Analytical Chemistry, Vol. 81, No. 11, June 1, 2009

Scheme 1. Scheme Used to Chemoenzymatically Prepare Isotopically Labeled Disaccharides Standards†

\[
\begin{align*}
\text{13C-glucose and } & ^{15}\text{NH}_4\text{Cl} \\
\text{GlcA-GlcNAc} & \text{Hep 1,2,3} \\
\text{Hep 1,2,3} & \text{ΔUA1(4)GlcNAc (0S)} \\
\text{GlcA-GlcNAc6S} & \text{ΔUA1(4)GlcNS (NS)} \\
\text{Hep 1,2,3} & \text{ΔUA2S(1,4)GlcNS (NS2S)} \\
\text{ΔUA2S(1,4)GlcNAc6S (2S6S)} & \text{N-desulfonation & N-acetylation} \\
\text{ΔUA2S(1,4)GlcNAc6S (2S6S)} & \text{ΔUA2S(1,4)GlcNAc (0S)} 
\end{align*}
\]

† \( n = 20 \) to ∼50.

Rabenstein, D. L., and freeze-dried. \([^{13}\text{C},^{15}\text{N}]\)-ΔUA2S-GlcNAc6S (2S6S) was prepared by N-desulfonation and re-N-acetylation from TriS\(^{1}\).\(^{34}\) TriS\(^{1}\) (40 µg) was passed through a cation-exchange column (AG50W-X8, H form, Bio-Rad, U.S.A., 0.5 mL), neutralized with pyridine, and freeze-dried. The dried sample was dissolved in 20 µL of 5% DMSO in H\(_2\)O and incubated in 50 °C for 1.5 h. After N-desulfonation, the sample was freeze-dried and dissolved in 10 µL of H\(_2\)O containing 10% MeOH, 5% uniformly labeled \([^{13}\text{C}]\)acetamide (Sigma Co., St. Louis, MO). The re-N-acetylation was performed at 4 °C for 2 h, and the final product was purified by SAX-HPLC.

Quantification of GAGs by Carbazole Assay. GAGs were subjected to carbozole assay\(^{35}\) to quantify the amount of GAG in each sample using HS as standard.

Enzymatic Digestion. The Hep 1, 2, and 3 (5 mUnits each) were added to HS samples (5 µg) and incubated at 37 °C for 10 h. The products were recovered by centrifugal filtration (10 000 g using a YM-3, 3000 MWCO membrane, Millipore, Bedford, MA), and the HS/heparin disaccharides were recovered in the flow-through, freeze-dried, and redissolved in 10 µL of H\(_2\)O for LC–MS analysis.

IPRP-Mf-HPLC–MS. LC–MS analyses were performed on an Agilent 1100 LC/MSD instrument (Agilent Technologies, Inc. Wilmington, DE) equipped with an ion trap, binary pump followed by microflow, and a UV detector. The column was a 2.1 mm × 150 mm, and a UV detector. The column used was a 5 mm × 250 mm (Agilent Technologies). Eluent A was water/acetonitrile (85:15) v/v, and eluent B was water/acetonitrile (35:65) v/v. Both eluents contained 12 mM tributylamine (TrBA) and 38 mM NH\(_4\)OAc with pH adjusted to 6.5 with HOAc. A gradient of 0% B for 20 min and 0–50% B over 25 min was used at 10 µL/min for disaccharide analysis. The column effluent entered the source of the ESI-MS for continuous detection by MS. In addition, another 5 µL/min acetonitrile was added just after column and before MS to make the solvent and TrBA easy to spray and easy to evaporate in the ion source. The electrospray interface was set in negative ionization mode with a skimmer potential of −40.0 V, a capillary exit of −40.0 V, and a source of temperature of 325 °C, to obtain the maximum abundance of the ions in a full scan spectrum (150~1500 Da, 10 full scans/s). Nitrogen was used as a drying (5 L/min) and nebulizing gas (20 psi).

Disaccharide Analysis. Unlabeled mixtures of disaccharide standards 1, 2, 5, 10, 20, and 50 ng per disaccharide were analyzed by LC–MS to test the sensitivity of this method, the linearity based on amount of disaccharide and peak intensity in mass spectrometry. Isotopically labeled disaccharide standards (15 ng/disaccharide) were mixed with unlabeled disaccharide standard mixtures (1, 2, 5, 10, 20, and 50 ng/disaccharide) and analyzed by LC–MS. Isotopically labeled mixtures of disaccharide standards (15 ng/disaccharide) were also mixed with 2 µL of disaccharides prepared from various HS samples and analyzed by LC–MS. All analyses were performed in triplicate.

RESULTS AND DISCUSSION

HS/heparin GAGs are heterogeneous with respect to molecular weight and disaccharide composition. HS/heparin GAGs consist of 10~50 repeating disaccharide units, and their chain length can vary based on the level endoglucuronidase processing\(^{36}\). The quantitative disaccharide compositions of HS/heparin have direct relationships with their important biological functions, including blood anticoagulation, viral and bacterial infection and entry, angiogenesis, inflammation, cancer, and development.\(^{1,36}\) The availability of small amounts of samples is often a bottleneck for quantitative compositional analysis. Sensitive and reliable methods for quantitative analysis are required to characterize the HS/heparin and to elucidate the structure–activity relationships of these GAGs.\(^{1,7,36}\)

IPRP-Mf-HPLC–MS Method. The most common disaccharides comprising HS/heparin are shown in Figure 1. These disaccharides can be separated by IPRP-Mf-HPLC and detected by extracted ion chromatography (EIC, Figure 2A). The separation observed in EIC resolved the α- and β-anomeric forms present in

6S, 2S, and 2S6S. NMR analysis confirms similar amounts of α- and β-anomers are present in N-acetylated HS/heparin disaccharides, whereas the α-anomeric form is predominant in the N-sulfo disaccharides (data not shown). IPRP-Mf-HPLC is widely used in pharmaceutical research as a result of its high resolution and high sensitivity.37–40 In addition to excellent separation, ESI-MS affords the mass of each disaccharide (Figure 2B–I). A peak was observed for each disaccharide at \( m/z \) 378.1, 416.1, 458.1, 496.0, 538.0, and 575.9 (Figure 2B–I, respectively). A single peak was observed at \( m/z \) 458.1 and 496.0 for pairs of the 6S/2S and NS6S/NS2S anomers, respectively. Because sulfo groups are relatively unstable, minor peaks corresponding to desulfonation were also observed in the MS spectra of di- and trisulfated disaccharide. In addition to the major peaks for NS6S/NS2S, 2S6S, and TrIS, minor peaks corresponding to monodesulfonated NS6S/NS2S, 2S6S, and TrIS were also observed at \( m/z \) 416.0, 458.0, and 538.0, respectively. Thus, the disaccharides comprising HS/heparin could be unambiguously identified by both their retention times and their masses using IPRP-Mf-HPLC–MS. Interestingly, no multiply charged ions were observed even for highly charged disaccharides. This may be due to the relatively high concentration of TrBA ion-pairing reagent, which helped avoid multiple ionization. Additional acetonitrile was injected in front of the ion source to increase the sensitivity of MS. The higher concentration of acetonitrile makes the solvent spray more efficiently. This efficient spraying affords more uniform and complete evaporation of solvent and volatile TrBA and ammonium acetate additives reducing the major source of background noise in the spectrum. Unlabeled disaccharide mixtures of equal mass amounts of disaccharides were prepared. Mixtures containing 1, 2, 5, 10, 20, and 50 ng of each disaccharide were analyzed by LC–MS (Figure 3). In the presence of pre-ion-source addition of acetonitrile, the peaks of disaccharides were broader but the disaccharides remained separated (Figure 3A). The EIC of disaccharide mixtures containing 1, 2, 5, 10, 20, and 50 ng of each disaccharide are shown. (G) The curves and linear equations of intensity as a function of concentration for each disaccharide are shown.

disaccharide analytes, with the exception of their isotopic compositions. Internal standards that are identical in all properties but having different physical and chemical properties is to utilize labeled internal standards. (A) ESI-MS spectrum of isotopically labeled disaccharide 0S.

**Figure 4.** ESI-MS and MS/MS spectra of isotopically labeled disaccharide 0S. (A) ESI-MS spectrum of isotopically labeled disaccharide 0S. (B) MS/MS spectrum and scheme fragmentation of isotopically labeled disaccharide 0S.

Mixtures Containing Known Amounts of Disaccharides

Table 1. Linear Equations of Disaccharides Based on a Fixed Amount of the Corresponding Isotopically Labeled Internal Standards

<table>
<thead>
<tr>
<th>disaccharides</th>
<th>linear equations</th>
<th>correlation coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td>0S</td>
<td>( Y = 0.125X + 0.242 )</td>
<td>0.999</td>
</tr>
<tr>
<td>NS</td>
<td>( Y = 0.128X + 0.244 )</td>
<td>0.999</td>
</tr>
<tr>
<td>6S</td>
<td>( Y = 0.131X - 0.123 )</td>
<td>0.997</td>
</tr>
<tr>
<td>2S</td>
<td>( Y = 0.127X - 0.216 )</td>
<td>0.992</td>
</tr>
<tr>
<td>NS6S</td>
<td>( Y = 0.138X - 0.172 )</td>
<td>0.997</td>
</tr>
<tr>
<td>NS2S</td>
<td>( Y = 0.128X - 0.212 )</td>
<td>0.987</td>
</tr>
<tr>
<td>2S6S</td>
<td>( Y = 0.131X - 0.271 )</td>
<td>0.995</td>
</tr>
<tr>
<td>TriS</td>
<td>( Y = 0.131X + 0.145 )</td>
<td>0.998</td>
</tr>
</tbody>
</table>

A mixture of all of the isotopically labeled disaccharides, each in a fixed amount (15 ng), was analyzed by LC–MS five times in the presence of a mixture of the eight unlabeled disaccharides in five different amounts (2, 5, 10, 20, and 50 ng). The ratio of the intensity of the ion corresponding to each unlabeled disaccharide to the ion corresponding to the identical isotopically labeled disaccharide (Y) was plotted as a function of the amount of unlabeled disaccharide (X).

Table 2. Quantification of HS/Heparin Disaccharide Mixtures Containing Known Amounts of Disaccharides

<table>
<thead>
<tr>
<th>disaccharides</th>
<th>known amount</th>
<th>calcld amount</th>
<th>known amount</th>
<th>calcld amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>0S</td>
<td>40</td>
<td>41 ± 2</td>
<td>5</td>
<td>5 ± 0</td>
</tr>
<tr>
<td>NS</td>
<td>35</td>
<td>35 ± 0</td>
<td>10</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>6S</td>
<td>30</td>
<td>29 ± 1</td>
<td>15</td>
<td>16 ± 0</td>
</tr>
<tr>
<td>2S</td>
<td>25</td>
<td>24 ± 0</td>
<td>20</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>NS6S</td>
<td>20</td>
<td>21 ± 0</td>
<td>25</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>NS2S</td>
<td>15</td>
<td>15 ± 0</td>
<td>30</td>
<td>31 ± 0</td>
</tr>
<tr>
<td>2S6S</td>
<td>10</td>
<td>9 ± 1</td>
<td>35</td>
<td>34 ± 2</td>
</tr>
<tr>
<td>TriS</td>
<td>5</td>
<td>6 ± 1</td>
<td>40</td>
<td>41 ± 1</td>
</tr>
</tbody>
</table>

**Figure 5.** ESI-MS of disaccharides with corresponding isotopically labeled internal standards. (A–C) and (E–H) Mass spectra of equimolar mixtures of 0S with 0S\(^{1}\), NS with NS\(^{1}\), 6S with 6S\(^{1}\), NS6S with NS6S\(^{1}\), NS2S with NS2S\(^{1}\), 2S6S with 2S6S\(^{1}\), and TriS with TriS\(^{1}\), respectively. (D) Mass spectrum of 2S.

Quantitative Analysis. A single unnatural internal standard can be used in LC–MS disaccharide analysis to quantify disaccharides. An alternative method for quantifying disaccharides having different physical and chemical properties is to utilize internal standards that are identical in all properties to the disaccharide analytes, with the exception of their isotopic composition. Uniformly \(^{13}\)C,\(^{15}\)N-labeled HS/heparin polymers were synthesized chemoenzymatically in our previous work (Scheme 1).\(^{29}\) The isotopic purity of the fermentation product obtained from *E. coli* K5, N-acetylheparosan, the precursor to the HS/heparin polysaccharides, was 94% (\(^{13}\)C + \(^{15}\)N + \(^{16}\)O + H = 94%) based on MS spectrum of its disaccharide.\(^{29}\) The structures of N-acetylheparosan (GlcA–GlcNAc–) and HS/heparin polysaccharides, N-sulfoheparosan (GlcA–GlcNS–), undersulfated heparin (IdoA2S–GlcNS–), and heparin (IdoA2S–GlcNS6S–) were confirmed by NMR. The sequences of enzymatically derived disaccharides were also confirmed by MS and tandem mass spectrometry (MS/MS). The 0S\(^{1}\) disaccharide obtained on heparin lyase treatment of \(^{13}\)C,\(^{15}\)N-labeled N-acetylheparosan, for example, showed a molecular ion [M – H]\(^{-}\) at m/z 393.0 in its ESI-MS spectrum (Figure 4A), 15 amu greater than the mass of the unlabeled 0S disaccharide (Figure 2B). Two cross-ring cleavages are observed in the MS/MS spectrum at m/z 269.0 and 287.0 corresponding to \(^{15}\)NS and \(^{15}\)NSH\(_2\), respectively (Figure 4B).

The fragmentation observed in the MS/MS of 0S\(^{1}\) is identical to that observed for the unlabeled 0S disaccharide.\(^{41}\) The disaccharides NS\(^{1}\), NS2S\(^{1}\), and NS2S6S\(^{1}\) were also derived from corresponding polysaccharides by heparinase treatment and their structures (Scheme 1) confirmed by LC–MS. They have identical retention times as the corresponding unlabeled disaccharides (data not shown). Again, the MS of these disaccharides showed a peak of 13 amu greater than the corresponding MS. The disaccharides NSI, NS2SI, and NS2S6SI were also derived from corresponding polysaccharides by heparinase treatment and their structures (Scheme 1) confirmed by LC–MS. They have identical retention times as the corresponding unlabeled disaccharides (data not shown). Again, the MS of these disaccharides showed a peak of 13 amu greater than the corresponding MS.


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unlabeled disaccharides (Figure 5, parts B, F, and H). The 6S1 and NS6S1 were synthesized by treating unlabeled disaccharides (Figure 5, parts B, F, and H). The 6S1 disaccharide was prepared from bovine brain disaccharides (A) HS-L1; (B) HS-L2 both prepared from porcine liver; (C) HS-B1; (D) HS-B2 both prepared from bovine brain.

Figure 6. EIC of disaccharide analysis of four HS samples. The ion chromatograms were extracted based on the mass of the unlabeled disaccharides: (A) HS-L1; (B) HS-L2 both prepared from porcine liver; (C) HS-B1; (D) HS-B2 both prepared from bovine brain.

unlabeled disaccharides were synthesized by treating N-acetylheparosan and N-sulfoheparosan using 6-O-sulfotransferase (OST) (Scheme 1). The resulting 6S1 and NS6S1 disaccharides again peaked at 15 and 13 amu higher than the unlabeled disaccharides, respectively (Figure 5, parts C and E). Disaccharide 2S6S1 was derived chemically from disaccharide 3S1 in a yield of ∼60%. The product was purified by SAX-HPLC, and LC-MS showed a peak of the same retention time with 15 amu greater mass than the corresponding unlabeled 2S6S disaccharide (Figure 5G). We were unable to prepare disaccharide 2S1, but its ionization is similar to that observed for 6S1 (Figure 3G). Rare disaccharides such as those containing 3-O-sulfo groups and resistant oligosaccharides having stable isotopic labeling would also be useful for disaccharide analysis and oligosaccharide mapping. Strategies involving the use of partial enzymatic digestion and 3OST are currently being evaluated for the preparation of these standards.

Fixed amounts of isotopically labeled disaccharides (15 ng/disaccharide) were combined with these two mixtures and analyzed by LC-MS. The quantity of each disaccharide in these two mixtures was calculated by the linear equations shown in Supporting Information Figure 1S and Table 1. The calculated amounts, given in Table 2, were consistent with the known amounts.

Quantitative Analysis of HS from Different Sources. Isotopically labeled standards (15 ng/disaccharide) were added to four HS samples isolated from animal tissues after their digestion with Hep 1, 2, and 3. The EIC of these samples were presented in Figure 6. The quantity of each disaccharide in these four samples was calculated by the linear equations shown in Supporting Information Figure 1S and Table 1. The disaccharide compositions of these four HS samples are given in Table 3. Absorbance (232 nm) detection, requiring 10-fold more sample, was also applied to determine disaccharide composition and was used to confirm the results from this method. In addition, carbozole assay was applied to quantify the total amount of HS-derived disaccharides. The compositions of these four HS samples obtained using UV detection are consistent with EIC results, which required 10-fold less sample. The quantification of these HS

Table 3. Quantity and Composition of HS from Porcine Liver and Bovine Brain

<table>
<thead>
<tr>
<th></th>
<th>0S</th>
<th>NS</th>
<th>6S</th>
<th>2S</th>
<th>NS6S</th>
<th>NS2S</th>
<th>2S6S</th>
<th>TriS</th>
<th>MS (ng)</th>
<th>carb (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS-L1*</td>
<td>UV</td>
<td>34.1±1.4%</td>
<td>38.3±2.1%</td>
<td>7.2%±0.5%</td>
<td>1.9%±0.4%</td>
<td>5.6%±0.3%</td>
<td>2.9%±1.1%</td>
<td>2.1%±0.5%</td>
<td>7.9%±0.8%</td>
<td>237±12</td>
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<tr>
<td></td>
<td>MS</td>
<td>33.0±1.7%</td>
<td>37.1±1.9%</td>
<td>7.9%±0.4%</td>
<td>2.7%±0.3%</td>
<td>4.3%±0.2%</td>
<td>4.0%±0.5%</td>
<td>2.3%±0.2%</td>
<td>8.6%±0.9%</td>
<td>131±11</td>
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<tr>
<td>HS-L2*</td>
<td>UV</td>
<td>30.0±0.9%</td>
<td>28.7±1.5%</td>
<td>10.1%±0.7%</td>
<td>4.1%±0.5%</td>
<td>4.6%±0.6%</td>
<td>5.2%±0.3%</td>
<td>7.9%±0.7%</td>
<td>9.4%±1.5%</td>
<td>237±12</td>
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<tr>
<td></td>
<td>MS</td>
<td>28.3±1.2%</td>
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<td>9.7%±0.6%</td>
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<td>6.1%±1.0%</td>
<td>5.6%±0.7%</td>
<td>8.8%±0.5%</td>
<td>8.1%±0.9%</td>
<td>131±11</td>
</tr>
<tr>
<td>HS-B1*</td>
<td>UV</td>
<td>25.7±0.9%</td>
<td>43.9±2.5%</td>
<td>9.2%±0.4%</td>
<td>4.4%±0.6%</td>
<td>4.6%±0.7%</td>
<td>2.9%±0.2%</td>
<td>6.6%±0.8%</td>
<td>2.7%±0.2%</td>
<td>188±15</td>
</tr>
<tr>
<td></td>
<td>MS</td>
<td>23.7±0.8%</td>
<td>42.8±1.7%</td>
<td>10.8%±0.9%</td>
<td>4.8%±0.8%</td>
<td>3.7%±0.8%</td>
<td>3.6%±0.6%</td>
<td>7.3%±0.4%</td>
<td>3.3%±0.4%</td>
<td>188±15</td>
</tr>
<tr>
<td>HS-B2*</td>
<td>UV</td>
<td>18.6±0.5%</td>
<td>32.1±0.7%</td>
<td>8.1%±0.7%</td>
<td>7.2%±0.9%</td>
<td>8.1%±0.5%</td>
<td>7.4%±0.7%</td>
<td>15.9%±0.4%</td>
<td>2.6%±0.2%</td>
<td>91±5</td>
</tr>
<tr>
<td></td>
<td>MS</td>
<td>17.7±0.7%</td>
<td>30.7±0.9%</td>
<td>7.7%±0.9%</td>
<td>8.4%±0.7%</td>
<td>7.7%±0.8%</td>
<td>6.9%±0.9%</td>
<td>16.8%±0.9%</td>
<td>4.1%±0.3%</td>
<td>91±5</td>
</tr>
</tbody>
</table>

a The total sulfate level of HS-L1 is 0.9/disaccharide. b The total sulfate level of HS-L2 is 1.1/disaccharide. c The total sulfate level of HS-B1 is 1.0/disaccharide. d The total sulfate level of HS-B2 is 1.3/disaccharide.
samples by carbazole assay was also similar to that obtained by LC–MS method. HS-L2 and HS-B2 have higher levels of 2-O-sulfo groups than HS-L1 and HS-B1. The two liver-derived HS samples showed a higher percentage of TrIS than the brain-derived samples but a lower total sulfate content.

CONCLUSIONS

The IPRP-Mf-HPLC–MS method demonstrated here is useful for the analysis of HS in tissue samples and is sensitive enough to be applied in the analysis of cell culture samples. With the growing interest in generating and evaluating therapeutic preparations of HS/heparin for possible clinical uses, there is need to develop fast and reliable methods for structural characterization and quantification of pharmaceutical heparin preparations and samples of native HS/heparin isolated from different biological sources. Quantitative disaccharide composition analysis is one of the most important ways to characterize the structures of HS/heparin. The complex structures of HS/heparin require such improved methodology. IPRP-Mf-HPLC–MS provides excellent separation of HS/heparin disaccharides and sensitivity of detection and quantification. The application of structurally defined, isotopically labeled, internal standards having identical physical and chemical properties as analyte disaccharides allows the accurate calibration each disaccharide in a mixture. This is a rapid, efficient, and reliable method to obtain the disaccharide composition quantitatively. This methodology should accelerate the study in GAG structures and glycobiology. Future work will examine the use of rare disaccharides and oligosaccharides with isotopic labeling.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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