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Analysis of Heparan Sulfate Oligosaccharides with Ion Pair-Reverse Phase Capillary High Performance Liquid Chromatography-Microelectrospray Ionization Time-of-Flight Mass Spectrometry†

Balagurunathan Kuberan, Miroslaw Lech, Lijuan Zhang, Zhengliang L. Wu, David L. Beeler, and Robert D. Rosenberg*

Contribution from the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, and Department of Molecular Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215

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Abstract: Heparan sulfate, a cell surface bound glycosaminoglycan polysaccharide, has been implicated in numerous biological functions. Heparan sulfate molecules are highly complex and diverse, yet deceivingly look simple and similar, rendering structure−function correlation tedious. Current chromatographic and mass spectrometric techniques have limitations for analyzing glycosaminoglycan samples that are in low abundance and that are large in size, due to their highly acidic nature arising from a large number of sulfate and of carboxylate groups. A new methodology was developed using capillary ion-paired reverse-phase C18 HPLC directly coupled to ESI-TOF-MS to address the above issues. On the basis of HS disaccharide analysis, dibutylamine was found to be the best suited for HS analysis among many ion-pairing agents investigated. Next, analysis of oligosaccharides derived from heparosan, the precursor for heparan sulfate, was undertaken to demonstrate its greater applicability in a more complex structural analysis. The established chromatographic conditions enabled the characterization of heparosan oligosaccharides of sizes up to tetracontasaccharide with high resolution in a single run and were amenable to negative ion electrospray MS in which sodium adduction and fragmentation were avoided. To date, these are the largest nonsulfated HS precursor oligosaccharides to be characterized by LC/MS. Finally, the current methodology was applied to the characterization of the biologically important ATIII binding pentasaccharide and its precursors, which differ from each other by sulfation pattern and/or degree of sulfation. All of these pentasaccharides were well-resolved and characterized by the LC/MS system with $^{34}SO_4$ as a mass spectral probe. This newly developed methodology facilitates the purification and rapid characterization of biologically significant HS oligosaccharides, and will thus expedite their synthesis. These findings should undoubtedly pave the way in deciphering multiple functional arrangements, ascribed to many biological activities, which are predictably embedded in a single large chaotic, yet well-organized HS polysaccharide chain. Development of newer techniques for HS oligosaccharide analysis is greatly needed in the postgenome era as attention shifts to the functional implications of proteins and carbohydrates in general and HS in particular.

Introduction

Proteoglycans are dominant glycoconjugates located on the cell surface and in extracellular spaces and consist of a core protein with one or more glycosaminoglycan side chains covalently linked. Heparan sulfate (HS) belongs to the family of glycosaminoglycans. HS has been assigned a variety of physiological and pathological functions, such as cell−cell adhesion, cell−matrix adhesion, cell proliferation, motility and differentiation, lipoprotein metabolism, blood coagulation, inflammation, tissue regeneration, tumor progression and invasion, pathogenic infection by bacteria, protozoa, and viruses, through their specific interaction with a wide array of proteins, ligands, receptors, and pathogens.[1−3]

HS is a highly acidic polysaccharide with repeating disaccharide units consisting of a glucosamine and hexuronic acid (ido- and/or gluco-). HS is biosynthesized in the golgi by addition of nucleotide sugars to the reducing end of the growing

* Address correspondence to this author at Massachusetts Institute of Technology. E-mail: rdrrosen@mit.edu.

† Abbreviations: ESI TOF MS, electrospray ionization time-of-flight mass spectrometry; cHPLC, capillary high performance liquid chromatography; GlcN, glucosamine; GlcA, glucuronic acid; dp, degree of polymerization; FAB, fast atom bombardment; GAG, glycosaminoglycan; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; HS, heparan sulfate; ATIII, antithrombin III; PAPS, 3-phosphoadenosine-5-phosphosulfate; 3-OST1, heparan sulfate 3-O sulfotransferase 1; 6-OST1, Heparan Sulfate 6-O sulfotransferase1; DS, dermatan sulfate; CS, chondroitin sulfate.

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polysaccharide chain followed by subsequent modification by different enzymes in a concerted fashion. The nascent chain may be epimerized at the C-5 position and/or sulfated at the C-2 position of uronic acid residues, and may be *N*- or *O*-sulfated and/or *N*-acetylated in glucosamine residues. While core proteins have fairly homogeneous compositions, the lengths and compositions of HS chains are highly variable. The structural diversity of HS poses significant challenges in the field of structural and functional glycobiology. In most cases, HS oligosaccharides, obtained by enzymatic or chemical depolymerization, were resolved to homogeneity by liquid chromatography to identify specific sequence/functional groups at the origin of its biological activity and then subsequently were characterized by NMR or MS. The current existing high-field NMR instruments require that oligosaccharides be available in large quantities, from 100 nanomoles to micromoles, whereas MS analysis requires as little as a few femtomoles, a billionth of the sample required for NMR. Hence mass spectrometry is the method of choice for analyzing samples of biological origin, which are often procurable only in minute quantities.

Purified HS or HS-like oligosaccharides have been analyzed with a number of mass spectrometric techniques. HS-like oligosaccharides have been analyzed with fast atom bombardment (FAB) mass spectrometry, and up to octasaccharides have been detected at the 10-nmol levels. Derivatization of sulfated GAG samples has also been investigated for fast atom bombardment mass spectrometry (FAB MS). Disaccharides and tetrasaccharides derived from HS can be analyzed in the amount of less than 10 μg by FAB-MS, which can provide information not only on molecular ions but also on certain sulfate position and linkage position.[4,5]

During the past decade, advances made in mass spectrometry (MS) have led to the development of soft ionization techniques, namely electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), which are becoming indispensable tools in HS analysis and are replacing FAB MS analysis.

Although matrix-assisted laser desorption ionization (MALDI)-MS is far more sensitive than FAB ionization, sulfated HS oligosaccharides ionize poorly when mixed directly with any of the matrixes commonly used for MALDI-TOF-MS. MALDI analysis can be facilitated, however, by pairing the oligosaccharide with a basic polypeptide, to form a neutral complex, which has been observed to ionize readily.[6] The method, originally developed by Biemann, has been applied in conjunction with capillary electrophoresis to investigate the mechanisms of heparinases.[7] Sulfated HS oligosaccharides ionize readily by negative ion electrospray ionization (ESI) mass spectrometry and are detected as alkali or ammonium salts. The molecular mass distribution can be extracted from these spectra, and results have been reported for HS up to decasaccharide size,[8] DS up to dodecasaccharide,[9] and CS up to tetradecasaccharide size.[10]

ESI spectra of sulfated GAGs are very complex, due to several factors: (A) samples from biological sources often contain a mixture of different chain lengths; (B) each molecule is detected with multiple charge states; (C) the sulfate/carboxylate groups have a tendency to form stable adducts with such alkali earth metal cations as Na^+, K^+, and Ca^{2+} to varying degrees; and (D) the larger oligosaccharides are relatively fragile and may fragment or lose sulfate groups during ionization. Although all HS oligosaccharides produce abundant ions by negative ESI MS, it is often difficult to extract molecular mass information from the spectra due to the presence of overlapping peaks. Thus, removal of metal cations during sample preparation is mandatory to ensure the quality of mass spectra. In most of these studies, oligosaccharides were first purified and collected after LC separation and then characterized. These off-line approaches, however, suffer from requiring time-consuming fraction isolation, collection, and purification. Despite the fact that on-line approaches hold several advantages, as of today, only a few reports have appeared in which HS/CS/DS disaccharides/hexasaccharides and CS tetradecasaccharide have been reported.[10−12]

Liquid-phase separation techniques, such as liquid chromatography and electrophoresis, have always played a key role in the separation and detection of oligosaccharides. Moreover, miniaturized separation techniques, especially capillary high-performance liquid chromatography (capillary HPLC) and capillary electrophoresis, have had a profound impact on the modern practice of analyzing minute amounts of biopolymers contained in highly heterogeneous mixtures of biological origin. The characteristic flow rates are in the range of microliter and nanoliter per minute for capillary HPLC, making this separation technique ideally suited for direct conjugation to ESI-MS. Although anion-exchange HPLC has been the most frequently used chromatographic mode for the separation of oligosaccharides,[13,14] the salt gradients applied to elute the oligosaccharide from anion-exchange columns render them unamenable for direct coupling to ESI-MS and mandate on-line microdialysis or a similar ion-removing interface. Separation by ion-pair reversed phase high performance liquid chromatography (IP-RP-HPLC) is very useful for sample preparation of oligosaccharides as well because it not only enables removal of alkali or alkaline earth metal cations from oligosaccharide samples but also fractionates oligosaccharides in more complex samples that can otherwise not be directly analyzed by ESI-MS.

Common ion-pairing agents used for IP-RP-HPLC are tetraalkylammonium salts, for example, tetrabutylammonium or tetrapropylammonium, and triethylammonium or trimethyloctadecylammonium salts, which are used mainly with UV or fluorescence detection. Direct coupling of ion-pair chromatography to mass spectrometry is made difficult by the contamination of the interface by the involatile tetraalkylammonium salts used as mobile phase modifiers. Additionally, sensitivity in HPLC-electrospray ionization-MS decreases rapidly with rising concentrations of ion-pairing agents in the HPLC eluent. The use of low concentrations of ion pairing agent did result in insufficient retention of the more polar analytes. Another disadvantage of tetraalkylammonium salts as mobile phase

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modifiers in HPLC-ESI-MS is their tendency to form cluster ions with anionic analytes.

For the coupling to mass spectrometry, volatile buffers are preferred to enhance retention and peak shape. Ammonium acetate is well suited for this purpose, since it does not interfere with mass spectrometric detection in concentrations normally applied for HPLC. However, acidic polysaccharides generally require more efficient ion pairing agents than ammonium acetate to obtain reasonable retention. Nevertheless, it is important to consider solution chemistry to achieve the best separation together with maximum analyte detectability, which usually implies that HPLC separation conditions need to be adjusted to be amenable for ESI-MS analysis. Usually, a compromise has to be found between optimum chromatographic and mass spectrometric conditions.

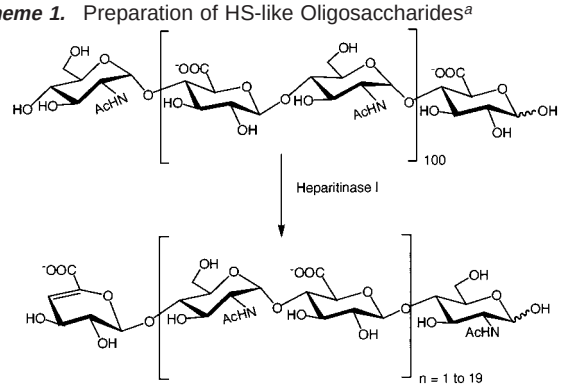
In this paper, we report on the feasibility of coupling this highly efficient IP-RP-capillary high performance liquid chromatographic separation system to electrospray ionization time-of-flight mass spectrometry. The negative ion detection is the obvious mode of choice for mass spectrometric analysis of HS oligosaccharides. We have applied octadecylated silica stationary phase capillary columns for high-resolution separation of HS di- and oligosaccharides. The influence of solution chemistry on chromatographic and mass spectrometric performance of disaccharides is studied with different volatile ion-pair reagents. Addition of the volatile amine suppresses multiply charged analyte ions in favor of less charged ones, thus resulting in the shift of peak centroids to higher masses providing molecular mass information. Thus, volatile aliphatic amines seemed to be suitable to provide the necessary retention for HPLC and to reduce ionic charge, thereby minimizing the occurrence of overlapping charge states in cHPLC-ESI-MS.

We therefore investigated the applicability of several aliphatic amines as mobile phase modifiers for the HPLC-ESI-MS analysis of HS disaccharides. The volatility of these ion-pairing agents overcame many of the obstacles usually experienced with ion pairing agents in HPLC-MS. The retention behavior as well as the influence of the concentration of the ion pairing on the mass spectrometric sensitivity of disaccharide compounds was studied. Finally, the optimized IP-RP-cHPLC-ESI-MS system is used to analyze HS-like oligosaccharides liberated from K5 Heparosan chains. The C18 ion-pair reverse phase chromatography (IP-RP-HPLC) LC/MS is shown to be effective for simplifying mass spectra produced from mixtures of Heparosan oligosaccharides, thus facilitating extraction of neutral masses and size profiles of oligosaccharides up to tetracontasaccharides. To date these are the largest oligosaccharides of any kind to be chromatographically resolved and characterized by mass spectrometry. Next, we demonstrate that the current built in LC/MS system is also useful in resolving oligosaccharides based on the degree of sulfation and based on sulfation pattern with antithrombin III binding pentasaccharide.

Experimental Section

Materials. Standard mix containing six different disaccharides and Heparitinase I derived from *Flavobacterium heparinium* were purchased from Seikagaku USA (Cape Cod, MA). All ion-pairing agents, chemicals, and PAPS were from either Sigma (St. Louis, MO) or Aldrich (Milwaukee, WI). K5 Heparosan polysaccharide was prepared from *E.coli* K5.[15] Pentasaccharide 3 was a generous gift from Prof. Sinay. 3-OST1 and 6-OST1 sulfotransferases were cloned and expressed in baculovirus system.[16−18] Glucosamine 6-sulfatase was purchased from Glyko (Novato, CA). PAP^{34}S was prepared by using a slightly modified procedure.[19] DEAE-Sephacel material was purchased from Amersham Pharmacia (Piscataway, NJ).

Scheme 1. Preparation of HS-like Oligosaccharides[a]



[a] Partial digestion of Heparosan polysaccharide with Heparitinase I to prepare oligosaccharides for LC/MS analysis. HS-like oligosaccharides varied in size ranging from tetrasaccharide ($n = 1$) to tetracontasaccharide ($n = 19$).

Partial Digestion of Heparosan. Heparosan (10 μg) was digested with 0.33 mU of heparitinase I (EC 4.2.2.8) in 100 μL of 40 mM ammonium acetate buffer (pH 7.0) containing 3.3 mM $CaCl_2$ at 22 °C for 12 h and then the reaction mixture was taken out and boiled at 100 °C for 2 min to inactivate the enzyme and a fraction of reaction mixture (10 μL) was diluted to 100 μL with double distilled water and loaded to capillary HPLC for LC/MS analysis.

Selective 6-*O*-Desulfation. Removal of 6-*O*-sulfate from nonreducing terminal *N*-sulfoglucosamine residue (residue A, see Scheme 2) of synthetic AT III pentasaccharide was carried out by digesting pentasaccharides with glucosamine-6-sulfatase (EC 3.1.6.14) as follows: AT III 3-OH pentasaccharide (40μg) was taken into 125 μL of 50 mM sodium acetate buffer, pH 5.00, and treated with 10 μL of solution containing 1 mU of sulfatase enzyme and 0.1 mg/mL of BSA at 37 °C for 7 days. At the intervals of 48 h, 5 μL of enzyme solution (0.5 mU) was added three times, and prior to the addition of enzyme each time, a 0.5 μL aliquot was withdrawn from the reaction mixture for mass spectral analysis to monitor the completion of reaction; the reaction was terminated by heating the sample at 100 °C for 5 min. ATIII 3-*O* sulfated pentasaccharide (0.75 μg/10 μL) was taken into 65 μL of 50 mM sodium acetate buffer and digested with 5 μL of solution containing 0.5 mU of enzyme and 0.1 mg/mL of BSA at 37 °C, with subsequent addition of 0.2 mU of enzyme at 24 h intervals for 3 days. The desulfation was monitored and terminated as described above for 3-OH pentasaccharide.

Regioselective Sulfation with Sulfotransferases 3-OST1 and 6-OST1. The regioselective enzymatic sulfation reactions were carried out in the buffer having a final concentration of 25 mM MES (pH 7.0), 0.5% W/V Triton X-100, 2.5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 1.25 mM $CaCl_2$, 0.038 mg/mL protamine chloride, and 1 mg/mL BSA. A 25 μg sample of pentasaccharide in 100 μL of water was mixed with

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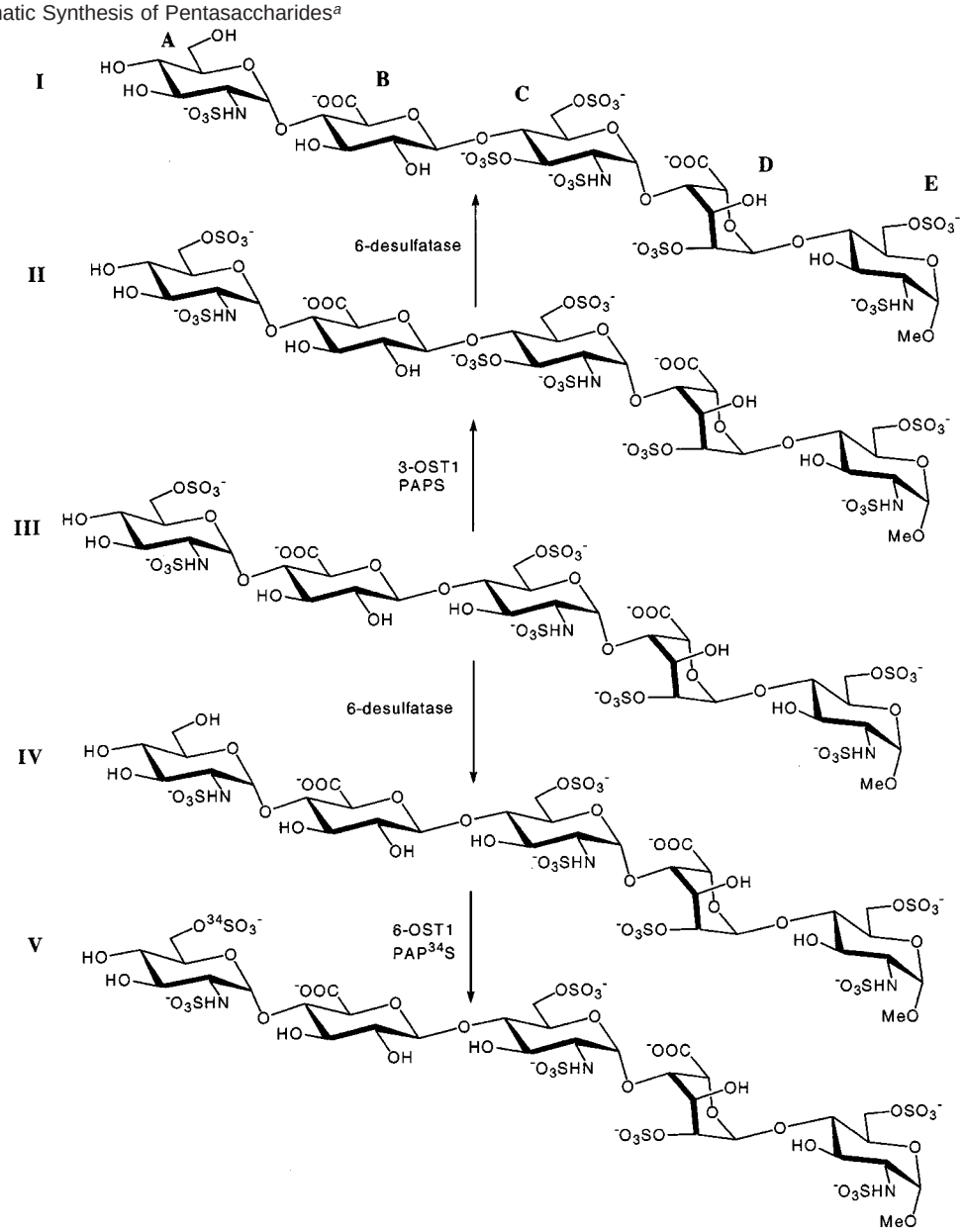
Scheme 2. Enzymatic Synthesis of Pentasaccharides[a]



[a] Enzymatic modification of Antithrombin binding pentasaccharide variants. 6-OST1 selectively sulfates the 6-OH group at the nonreducing end (residue A) whereas 3-OST1 regioselectively sulfates the 3-OH group of the middle sugar residue C. $PAP^{34}S$ was used in the preparation of Pentasaccharide V for LC/MS analysis.

112.4 μL of 2× buffer and 10.4 μL of 3.2 mM $PAP^{34}S$. A 2 μL sample of solution containing approximately 30 ng of the baculovirus expressed and purified recombinant 6-*O* sulfotransferase (6-OST1) was added. The reaction was incubated at 37 °C overnight and then stopped with heating at 100 °C for 4 min. The reaction was centrifuged at 10 000 g for 10 min and the supernatant was purified as before and loaded onto LC/MS. The 3-*O* sulfation of pentasaccharide was carried out with 3-*O* sulfotransferase under the same conditions applied for 6-OST1 catalyzed modifications.

Purification of Pentasaccharides. The reaction mixture, after termination of either desulfation or sulfation, containing the desired pentasaccharide was diluted to 1 mL with 0.25 M NaCl, 20 mM NaAc, 0.01% TX-100, pH 6.0 and then 1 mg of glycogen was added to minimize nonspecific interaction of oligosaccharides with the column matrix. The diluted reaction mixture was loaded on 1 mL of DEAE-Sephacel column, preequilibrated with 10 mL of washing buffer containing 0.25 M NaCl, 20 mM NaAc, 0.01% TX-100, pH 6.0. The column was washed with 10 column volumes of washing buffer and the pentasaccharide was eluted from the column with 5 mL of 1 M NaCl in 20 mM NaAc, pH 6.0. A 20 mL sample of absolute ethanol and 1 mg of glycogen were added to 5 mL of eluent in a 50 mL disposable polystyrene tube and incubated at 4 °C overnight to facilitate the precipitation of pentasaccharide. The precipitate was obtained by centrifuging in a RC3B centrifuge for 10 min at 3000 rpm. The obtained pellet was washed with 1 mL of 70% ethanol twice and finally dissolved in 200 μL of double distilled water for subsequent characterization.

Flow Injection Capillary Liquid Chromatography. An Ultimate capillary HPLC workstation (Dionex, Sunnyvale, USA) was used for microseparation. Ultimate consists of a high precision reciprocating pump with microflow processing to achieve precise delivery of micro- and nanoliter flow rates, a helium sparging device with four separately controlled lines for solvent degassing, a quaternary pressure gradient pump that allows the choice of up to 4 different solvent systems for mobile phase separation, a column thermostat for controlling column temperature (from ambient to 80 °C), and a scanning UV−vis spectrophotometer capable of concurrent monitoring of up to four

selectable wavelengths and requiring as little as 3 nL flow cell volume. UltiChrom software was used in data acquisition, analysis, and management. Flow injections were performed with the above HPLC system. To examine the effect of ion-pair concentration on ionization efficiency, the amines (triethylamine, dibutylamine, tributylamine, tripentylamine, tetrapropylamine, and tetrabutylamine) were dissolved in water−methanol (1:1) in the desired concentration containing 8 mM acetic acid. A gradient elution was performed, using a binary solvent system composed of water (eluent A) and 70% aqueous methanol (eluent B), both containing 8 mM acetic acid and 5 mM ion-pairing agent.

HPLC separations were performed on a 300 μm × 250 mm C18 polymeric silica column (Vydac, Hesperia, USA). The column temperature was maintained at 25 °C and the flow rate was set to 5 μL min^{-1}. Sample volumes of 5 to 100 μL were injected. For disaccharides and AT III pentasaccharide analysis, a 5 μL sample injection loop was used. For Heparosan oligosaccharide analysis, a 100 μL sample injection loop was used. The chromatographic conditions were optimized for disaccharides, AT III pentasaccharides, and Heparosan oligosaccharides. In brief, nonsulfated disaccharide was eluted with 100% A, single sulfated disaccharides were eluted with 10% B, isocratic elution with 20% B for double sulfated disaccharides, followed by isocratic elution with 35% B for triple sulfated disaccharide. The pentasaccharides were eluted with 60% B. When Heparosan oligosaccharide mix was loaded onto a column using 100 μL loop, 100% A was used for 20 min to ensure that oligosaccharides were concentrated before their elution. The column was washed and reequilibrated by further elution with 100% B for 10 min, returning to 100% A for 10 min at the end of the run. The absorbance of the column eluate was monitored at 232 nm. The commercially available standard disaccharides and heparosan derived oligosaccharides, obtained by heparitinase digestion, were determined on the basis of their absorbance at 232 nm (ϵ_{232} = 5500 M^{-1} cm^{-1}), due to the unsaturated double bond of the uronic acid at the nonreducing end, in conjunction with the carboxyl group at C-5.

Mass Spectrometry. Mass spectra were acquired on a Mariner BioSpectrometry Workstation ESI time-of-flight mass spectrometer (PerSeptive Biosystems, Framingham, MA). The ion path is 1.3 m in the reflectron mode. In the negative-ion mode, the instrument was calibrated with bis-trifluoromethyl benzoic acid, heptadecafluorononanoic acid, perfluorotetradecanoic acid, and oligothymidylic acids sodium salt [d(pT)$_6$ and d(pT)$_{10}$]. Nitrogen was used as a desolvation gas as well as a nebulizer. Resolution was measured as full width at half maximum (fwhm), which was at least 5000 for all spectra allowing isotopically resolved signals. The mass accuracy in the range from m/z 100 to 2000 u was better than 200 ppm. Conditions for ESI-MS were as follows: nebulizer flow 1 L/min, nozzle temperature 140 °C, drying gas (N_2) flow 0.6 L/min, spray tip potential 2.8 kV, nozzle potential 70 V, and skimmer potential 9 V. Negative ion spectra were generated by scanning the range of m/z 40−4000. During analyses, the indicated vacuum was 2.1 × 10^{-6} Torr. Total ion chromatograms and mass spectra were recorded on a personal computer with the Data Explorer software version 3.0 (Mariner). The parameters studied included the effect of concentration and structures of ion-pairing agents.

Results and Discussion

Miniaturization of LC/MS. Conventional LC can be coupled with ESI interface by using either postcolumn splitting or optimized ESI interfaces to handle high flow rates. However, the former choice results in significant sample losses and the latter has not entailed noticeable sensitivity gains. Miniaturization of chromatographic column parameters brings a key advantage of improved detection sensitivities. A reduction in column diameter produces a higher sample peak concentration in the detector. The maximum peak concentration of the sample in the column eluate C_{max} is given by

$$C_{max} = m\left(\frac{N}{2\pi}\right)^{1/2}\left[\frac{1}{V_0(1+k)}\right]$$

C_{max} depends on the absolute amount of sample loaded on the column (m) and the column efficiency (N) and it is inversely proportional to the dead volume (V_0) of the column and the retention factor (k).[20] The spray performance can be improved by adjusting the flow-rate and tip diameters, which determine the formation and size of the droplets from which ions will be desorbed. An equation that describes droplet formation in ESI is given below

$$r_e = \left\{\frac{\rho}{4\pi^2\gamma\tan\left(\frac{\pi}{2}-\theta\right)\left[\left(\frac{U_T}{U_A}\right)^2-1\right]}\right\}^{1/3}\left(\frac{dV}{dt}\right)^{2/3}$$

where r_e is the radius of the emission region for droplets at the tip of the Taylor cone, γ is the surface tension of the liquid, θ is the liquid cone angle (for the classical Taylor cone model θ = 49.3°), ρ is the density of the liquid, U_T is the threshold voltage, U_A is the applied voltage, and dV/dt is the flow rate.[21] The combination of improved ion sampling efficiency and spray performance will result in a dramatic effect on the performance of the ESI source.

Influence of [Ion-Pair Agent] on Chromatographic Performance. In IP-RP-HPLC, retention of analytes is determined by several factors such as hydrophobicity of the stationary phase, charge, hydrophobicity, and concentration of the amphiphile, ionic strength and dielectric constant of the mobile phase, and concentration of the organic modifier.[22] According to the electrostatic model, retention is influenced by the electrostatic interactions between the positive surface potential generated by the amphiphilic ions adsorbed at the stationary phase, alkylammonium ions, for instance, and the negative surface potential generated by the carboxylate/sulfate groups of the HS di- and oligosaccharides. As a consequence of increasing the concentration of an organic modifier such as methanol, amphiphilic ions are desorbed from the stationary phase, resulting in elution of the retained analytes.

Tetrabutylammonium compounds are the most commonly used ion-pairing agents in IP-RP-HPLC[23,24] and tetrabutylammonium hydroxide reagent was used in LC/MS analysis of HS disaccharides.[11] At the outset, it was decided to study a panel of alkylammonium compounds (Figure 1) along with tetrabutylammonium hydroxide as ion-pairing agents to determine how their concentration and structure would affect LC/MS performance of HS fragments.

The influence of tetrabutylammonium concentration in the eluent on retention and resolution of disaccharides was undertaken. Heparitinase acts by eliminative cleavage to produce these disaccharides with an unsaturated C4−C5 bond on the uronic acid residue (Figure 2). To measure the retention times and resolution of disaccharides, a mixture containing 6 different HS disaccharide standards was eluted with a stepwise gradient of

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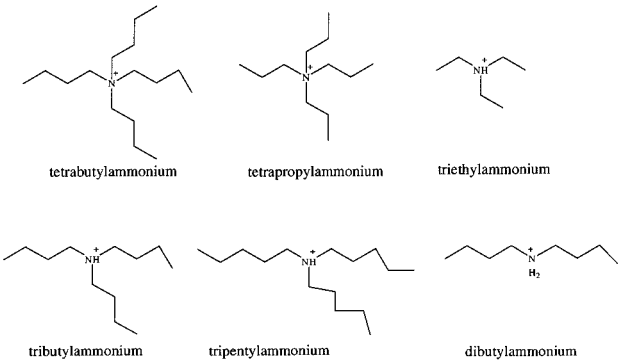

Figure 1. Ion-pairing agents studied in LC/MS analysis of HS disaccharides.

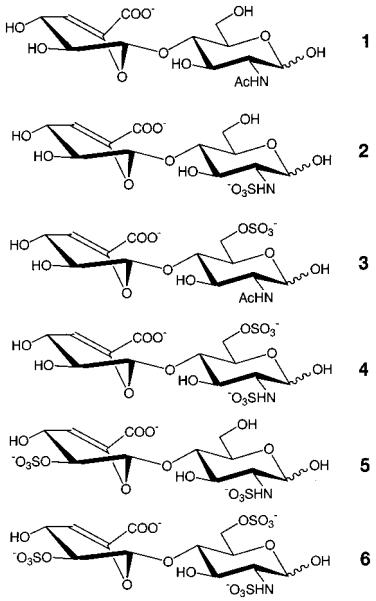

Figure 2. Structures of unsaturated HS disaccharides studied in capillary reverse phase high performance liquid chromatography with various ion-pairing agents.

0−100% solvent B (see Experimental Section for gradient details) containing 1, 3, and 5 mM of tetraalkylammonium agent (Figure 3). Retention time of some disaccharides increased with increasing concentration of the ion-pair reagent because of the higher surface potential at the stationary phase (Figure 3). Resolution of disaccharides gradually improved with increasing ion-pairing reagent concentration as depicted in Figure 3. The disaccharides **4** and **5** were poorly resolved at 1 mM concentration of ion-pairing agents, were partially resolved at 3 mM concentration, and were resolved fully at 5 mM concentration. These disaccharides, **4** and **5**, contain two sulfate groups and differ from each other with regard to the regiospecific positioning of sulfate groups. The peaks corresponding to disaccharides **2** and **3** have better baseline separation at higher concentrations of ion-pairing agents. The disaccharides **2** and **3** have a single sulfate group and they occupy different positions in the glucosamine residue. Though the retention time for disaccharides **1**, **2**, and **3** increased to a small degree at three different concentrations of tetrabutylammonium agent under the same eluant/solvent composition, disaccharides **4**, **5**, and **6** were retained more tightly at higher concentrations reflecting their stronger interaction with the stationary phase.

A solution of neat di- or trialkylamine (Figure 1) does not function as an ion-pair reagent for IP-RP-HPLC due to the lack

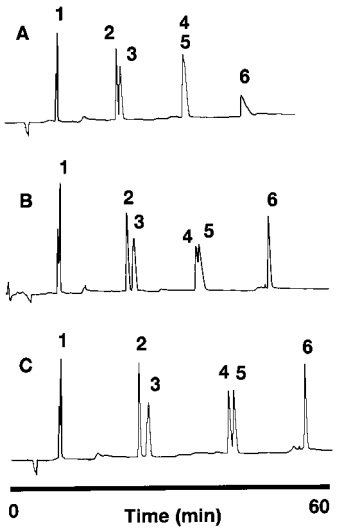

Figure 3. Capillary HPLC separation of unsaturated HS disaccharides with different concentrations of tetrabutylammonium ion as the ion-pairing agent: (A) 1 mM TBA, (B) 3 mM TBA, and (C) 5 mM TBA. Peak labels correspond to disaccharides listed in Figure 2.

of a permanent positive charge on the amphiphile**.** Hence, an acidic compound such as acetic acid is usually added to the mobile phase to protonate the dialkyl- and trialkylamine. In general, acids of higher volatility should offer better detectability for ESI-MS. The different counterions in the ion-pair reagent had only a moderate effect on retention times and chromatographic separation efficiency.[25] However, protonation of alkylamine with an acid has two major effects on solution properties and hence mass spectra quality. First, pH of the solution decreases, and second, conductivity of the solution increases. The addition of acids influences the charge-state distribution of analytes, which can be explained on the basis of solution and gas-phase acid−base equilibria. The more acidic the solution, the more likely acids will donate protons to analyte anions and reduce the charge states of analyte species. An increase in the alkylammonium acetate concentration, which is desirable to obtain better chromatographic performance, entailed a further decrease in signal intensity. Since ESI generates a roughly constant ion current, an increase in the intensity from added acids will reduce the intensity of the analyte ions. Since ions of higher conductivity (such as chloride) will suppress the signal significantly, acetate ions are preferred in the buffer composition used for LC/MS. Therefore acetic acid was used to protonate alkylamines resulting in acetate as the counterion.

Volatile Ion-Pairing Agents. The effect of a panel of volatile ion-pairing agents on retention and resolution of six disaccharides was next studied. These ion-pairing agents are listed in Figure 1. Of all the ion-pairing agents tested, volatile triethylamine, notably, was most widely used in several HPLC-MS analyses of oligonucleotides, but its suitability as an ion-pairing agent for HS analysis was never investigated.[25] Since 5 mM tetrabutylammonium ions provided the best chromatographic resolution, it was decided to keep all the other ion-pairing agents at the same concentration, 5 mM, for the current study and their effects on the resolution of disaccharides were investigated. Although dibutylammonium acetate resolved less satisfactorily the critical disaccharides **4** and **5**, it is important to note that all

(25) Huber, C. G.; Krajete, A. *J. Mass Spectrom.* **2000**, *35*, 870−877.

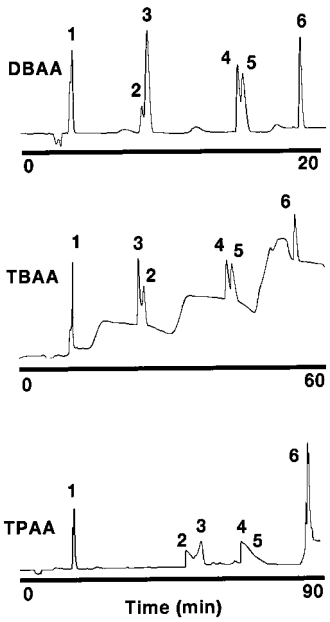

Figure 4. Chromatogram of HS disaccharides under different ion-pairing agents: dibutylammonium acetate (DBAA), tributylammonium acetate (TBAA), and tripentylammonium acetate (TPAA).

six disaccharides were eluted in less than 20 min while holding good overall resolution (Figure 4). The resolution ability of tributylammonium acetate was nearly identical with that of dibutylammonium acetate, but it required greater than 60 min to elute all six disaccharides under the given set of conditions. Tripentylammonium acetate lengthens even further the HPLC run time and in addition disaccharides **4** and **5** containing two sulfate groups were hardly resolved (Figure 4).

Coupling of Capillary IP-RP-HPLC with ESI-MS. To render this chromatographic separation system amenable to ESI-MS, several factors have to be considered that are related to the solution chemistry of the column effluent to be electrosprayed. Higher concentration of an ion-pairing reagent is not suitable for HPLC-ESI-MS because of the poor detectability of the eluted analytes by ESI-MS. Tetrabutyl- and tetrapropylammonium ion, even at 1 mM concentration and at the expense of poor chromatographic separation efficiency, has suppressed the signal intensity in ESI-MS. The strong signal-suppressing effect of the tetraalkylammonium ion was attributed to its nonvolatile nature (hence accumulation of counterions in the microdroplets) and thus results in lowering the ionization efficiency of the dissolved HS saccharides. To find the best compromise for the analysis of oligosaccharide by IP-RP-HPLC-ESI-MS, the influence of the following solution parameters on chromatographic and mass spectrometric performance was investigated independently with IP-RP-HPLC and ESI-MS before their direct coupling for LC/MS analysis: concentration of ion-pair reagent and the structural features of ion-pair reagent.

The disaccharides were analyzed with negative ESI MS under several different ion-pairing reagents and concentrations. Tetrabutylammonium ion suppressed the signal of analytes at all concentrations, 1, 3, and 5 mM. Our efforts to explore tetrapropylammonium reagent also were futile. Since 5 mM tetraalkylammonium ion provided the best chromatographic performance, it was decided to keep the same concentration, 5 mM, for the subsequent experiments with different volatile ion-pairing agents: tributylammonium, tripentylammonium, triethylammonium, and dibutylammonium acetates. Dibutylammonium acetate, among many ion-pairing agents mentioned above, provided the better chromatographic resolution and also required a shorter time. Hence dibutylammonium acetate was chosen for further LC/MS analysis of complex HS-like oligosaccharides.

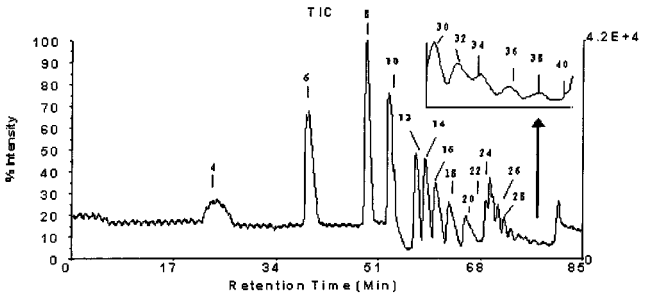

Figure 5. Total ion chromatogram (TIC) from a capillary HPLC-ESI-TOF-MS analysis of a partially digested heparosan. The inset shows the expanded view of TIC representing oligosaccharides higher than triacontasaccharide.

Partial Digestion of Heparosan Polysaccharide. Heparan sulfate can be broken down enzymatically into oligosaccharides. Three distinct types of lyases are known, with their unique substrate specificity.[26,27] These enzymes have been used to study the structure of heparan sulfate and to prepare heparan sulfate derived/like oligosaccharides for the evaluation of biological activity. *Flavobacterium heparinum* heparitinase cleaves −4)-GlcNpAcα(1−4)-GlcApβ(1− linkages through an elimination reaction, yielding 4,5-unsaturated tetra- and higher oligosaccharides as final products (Scheme 1). Heparosan, a high molecular weight, linear polysaccharide is a glycosaminoglycan found in the *E. coli* K5 bacteria.[28] Heparosan was subjected to partial digestion with heparitinase, and analyzed by a newly developed method of high-resolution capillary HPLC coupled to ESI-MS.

LC/MS Analysis of HS-like Oligosaccharides. The analysis of HS precursor oligosaccharides, obtained from the partial digestion of heparosan polymer by IP-RP-HPLC coupled to ESI-MS, was undertaken to determine the greater applicability of the developed methodology. With an eluent containing 5 mM dibutylammonium acetate and a stepwise gradient of solvent B, all oligosaccharides were separated. Adduction with alkali and alkaline earth metal ions has always been a major problem in negative-ion ESI-MS of oligosaccharides. Whereas on-line cation exchange enables the trapping of cations on a cation exchanger of relatively high affinity for the cations, removal of cations during IP-RP-HPLC has to take place in the mobile phase through competition of an excess of dibutylammonium ions with alkali or alkaline earth metal cations for the negative charges at the sugar−carboxylate backbone. The total ion chromatogram of oligosaccharides, eluting from capillary HPLC, is shown in Figure 5. The eluent was diverted from the mass spectrometer for 20 min at the flow rate of 5 μL/min because of the presence of buffer salts in the digestion buffer of 100 μL volume and the mass spectra of resolved oligosaccharides are shown in Figure 6. The bottom panel in Figure 6 of a given

(26) Linhardt, R. J.; Turnbull, J. E.; Wang, H. M.; Loganathan, D.; Gallagher, J. T. *Biochemistry* **1990**, *29*, 2611−2617.
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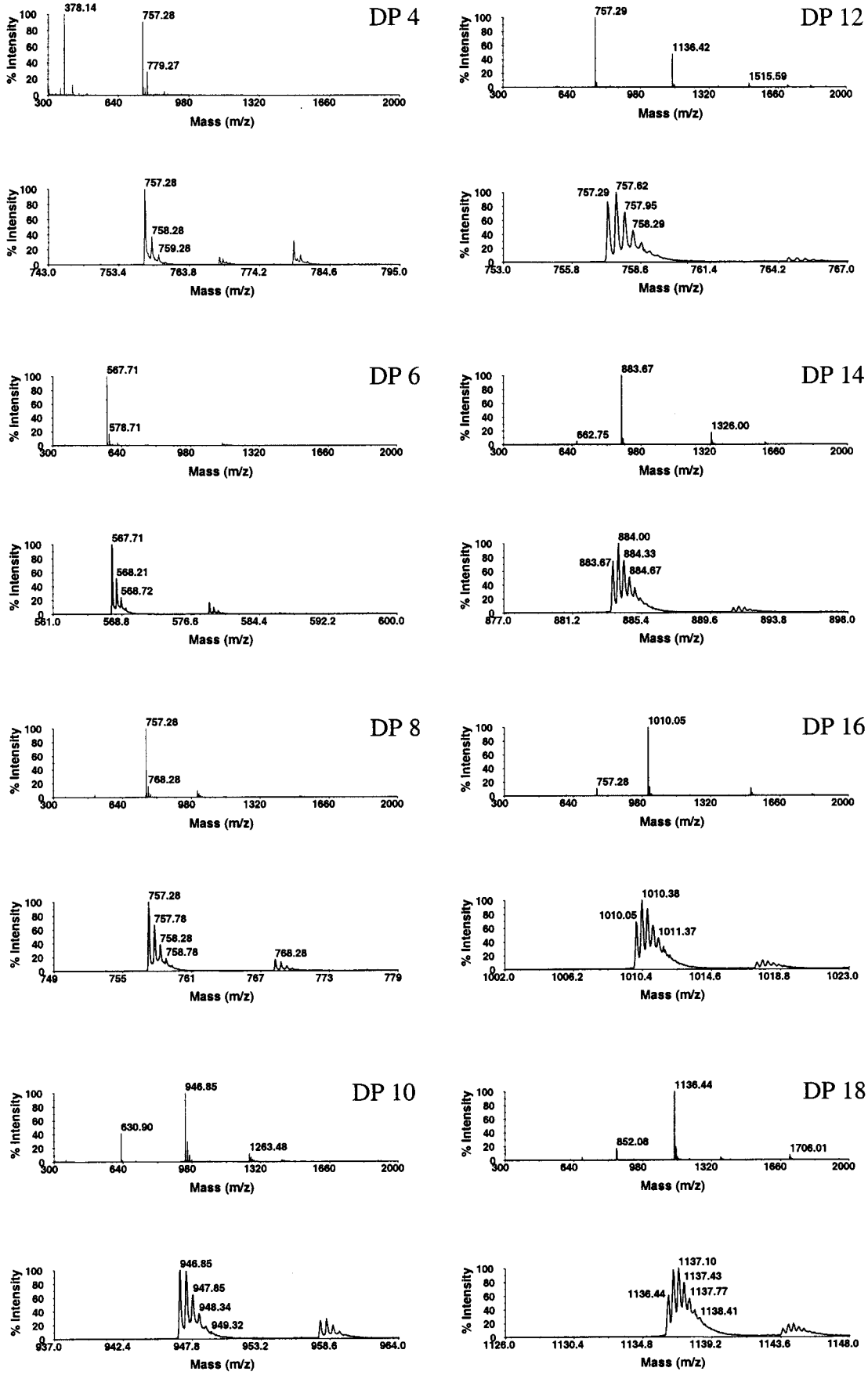
(28) Vann, W. F.; Schmidt, M. A.; Jann, B.; Jann, K. *Eur. J. Biochem.* **1981**, *116*, 359−364.


DP 4
% Intensity
Mass (m/z)
378.14
757.28
779.27
757.28
758.28
759.28
DP 12
757.29
1136.42
1515.59
757.29
757.62
757.95
758.29
DP 6
567.71
578.71
567.71
568.21
568.72
DP 14
883.67
662.75
1326.00
884.00
884.33
883.67
884.67
DP 8
757.28
768.28
757.28
757.78
758.28
758.78
768.28
DP 16
1010.05
757.28
1010.38
1010.05
1011.37
DP 10
946.85
630.90
1263.48
946.85
947.85
948.34
949.32
DP 18
1136.44
852.06
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1136.44
1137.77
1138.41

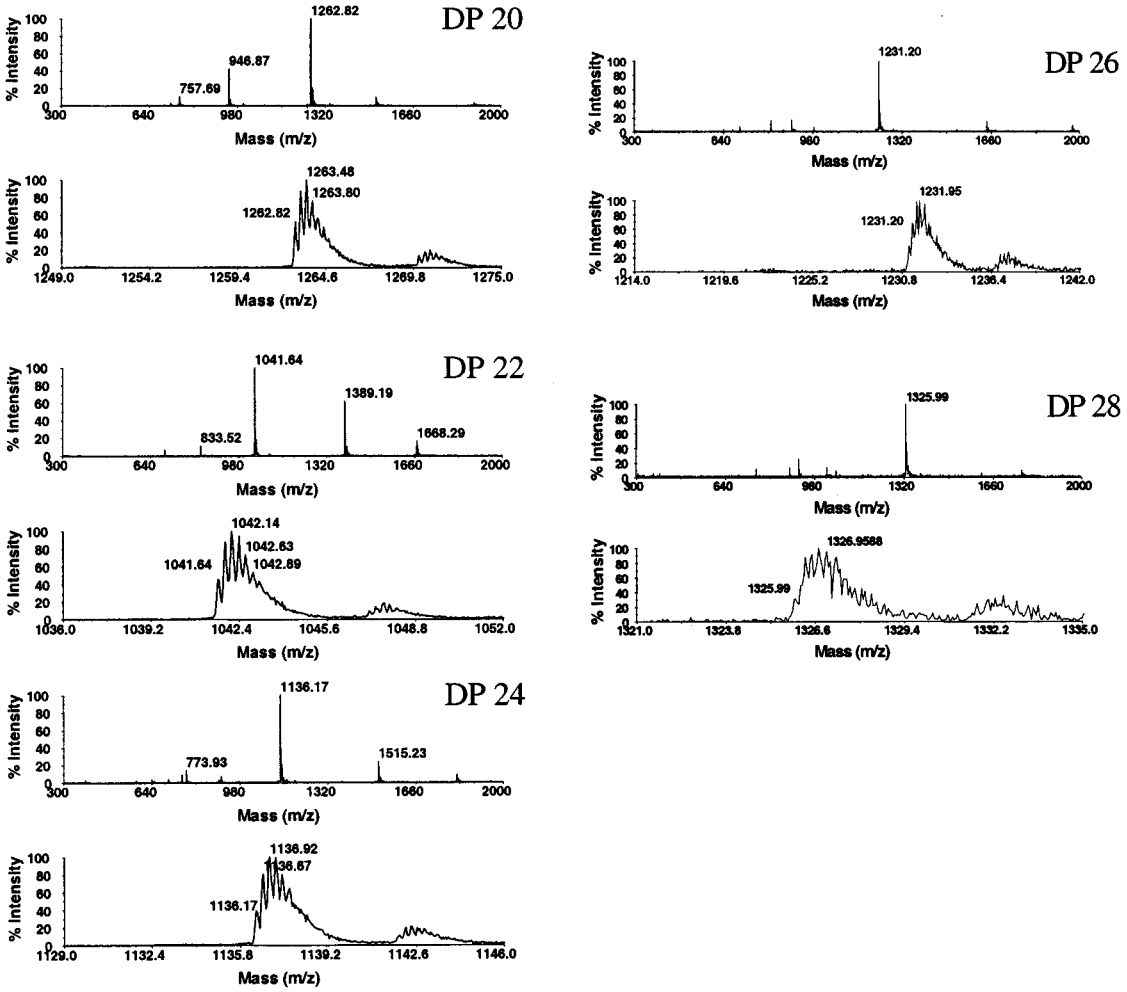

Figure 6. Electrospray spectra of the heparosan oligosaccharides ranging in size from tetrasaccharide (DP = 4) to octaicosaccharide (DP = 28). The top panel shows the mass spectrum of each oligosaccharide and the bottom panel shows the isotope cluster of parent oligosaccharide ion illustrating its resolution.

pair of mass spectra of each oligosaccharide shows the expanded views of the signals for the most abundant charge state of each oligosaccharide ranging in size from DP 4 to DP 28. The charge state was calculated from the mass difference between two monoisotopic signals $[M - nH]^{n-}$ and $[(M - nH) + (1/n)]^{n-}$. Since monoisotopic masses are not resolved very well for oligosaccharides from triacontasaccharide (DP = 30) to tetracontasaccharide (DP = 40), only average masses are obtained and they are shown in Figure 7. However, the average mass was sufficient for assignment of HS-like oligosaccharide compositions of 30- to 40-mers eluting from a C-18 column. Table 1 summarizes the mass spectral results obtained for all the HS-like oligosaccharides. Oligosaccharide ions are detected as $[M - nH]^{n-}$ ions with no adduction by the dibutylammonium (DBA) cation. It is evident that adduction of oligosaccharides with calcium ions or other alkaline earth or alkali metal ions was efficiently reduced during IP-RP-HPLC with 5 mM dibutylammonium acetate as eluent. The intensities of the monosodium adducts were negligible with respect to the total ion intensity of all charge states. The presence of small amounts of sodium and potassium adducts was highly advantageous at times for the determination of the charge state of a signal, especially when only one charge state could be observed due to charge-state reduction with dibutylammonium acetate. Although excessive adduction is undesirable because the signal intensity is distributed among many species resulting in a low signal-to-noise ratio for the detected species and particularly accurate mass determinations using the signals of higher charge states would be hampered because of the overlapping of adduct signals, cation adduction would be advantageous when it occurs in a controlled or limited manner. The doubly charged state was the most abundant for oligosaccharides up to decasaccharides, except tetrasaccharide, whereas the triply charged molecular ion was observed as the most abundant charge state for each oligosaccharide ranging from DP = 12 to 20. Quadruple charge state as the abundant charge state for oligosaccharides ranging from DP = 22 to 34 and the abundant quintuplet charge state for oligosaccharides with DP = 36, 38, and 40 were observed. The result obtained is significant, in that the samples used were generated by HS lyases with no purification step entering the LC/MS system.

Enzymatic Modification of Antithrombin III Binding Pentasaccharide. Heparin, more sulfated than HS glycosaminoglycan, is a widely used anticoagulant drug and elicits its effect through specific binding with antithrombin III (AT III), which specifically recognizes the following structure: −GlcNS/Ac(6S)-GlcA-GlcNS(3S±6S)-IdoA(2S)-GlcNS(6S)−, contained within the polymer. AT III binding to the above structure triggers a conformational change that results in the acceleration of the biochemical cascade. A synthetic AT III binding pentasaccharide

Table 1. Molecular Masses of HS-like Oligosaccharides Produced by the Partial Digestion of Heparosan

			mass (daltons)[a]	
dp	*m/z*	obsd charge sate	calcd	theor
4	0757.28	1−		
	0378.14	2−	0758.28	0758.22
6	0567.71	2−	1137.42	1137.33
8	0757.28	2−	1516.56	1516.45
10	0946.85	2−	1516.56	
	0630.90	3−	1895.70	1895.56
12	1136.42	2−	2274.87	2274.67
	0757.29	3−	2274.87	
	1515.59	3−(dimer)	2274.87	
14	0883.67	3−	2654.01	2653.78
	1326.00	4−(dimer)	2654.01	
16	1010.05	3−	2654.01	
	0757.28	4−	3033.15	3032.89
18	1136.44	3−	3033.15	
	0852.08	4−	3412.32	3412.00
20	1262.82	3−	3791.46	3791.11
	0946.87	4−	3791.46	
22	1389.19	3−	3791.46	
	1041.64	4−	4170.56	4170.23
24	1515.23	3−	4170.56	
	1136.17	4−	4548.68	4549.34
26	1231.20	4−	4928.80	4928.45
28	1325.99	4−	5307.96	5307.56
30	1421.54	4−	5690.16	5689.82
32	1516.53	4−	6070.12	6069.14
34	1611.41	4−	6449.64	
	1288.69	5−	6448.45	6448.46
36	1706.14	4−	6828.56	
	1365.09	5−	6830.40	6827.78
38	1440.34	5−	7206.70	7207.10
40	1516.27	5−	7586.35	7586.42

[a] Monoiotopic mass was calculated for oligosaccharides up to octaicosaccharide (dp = 28) and for triacontasaccharide and higher oligosaccharides up to tetracontasaccharide, only average mass was calculated due to the poor resolution of isotopic clusters of the parent ion.

(pentasaccharide 2) is under clinical trials as an alternative anticoagulant to animal-derived heparin polymer (Scheme 2).[29,30] The detailed mass spectrometric studies were earlier carried out on pentasaccharide and its sequence.[31,32] Intensive biochemical and biophysical studies suggest that the 3-*O*-sulfate group of *N*-sulfoglucosamine residue C and the 6-*O*-sulfate group of nonreducing terminal glucosamine residue A are critical for binding to AT III.[33−36] Indeed, we have recently demonstrated that 3-OST-1 sulfotransferase isoform catalyzes the rate-limiting biosynthetic reaction leading to cellular production or cell free generation of anticoagulant heparan sulfate besides other 6-OST sulfotransferases.[17] The 3-OST1 enzyme recognizes a specific precursor structure, corresponding to the antithrombin-binding site devoid of just the 3-*O*-sulfate (pentasaccharide **III**), and adds this rare substituent to complete the formation of anticoagulant heparan sulfate.[37] To evaluate the biosynthetic pathway of anticoagulant HS, it is necessary to prepare and resolve anticoagulant pentasaccharide and its precursor structures to homogeneity. These pentasaccharides closely resemble each other, and are highly charged, making them difficult to be characterized. Pentasaccharide **II** was prepared by regioselective sulfation of pentasaccharide **III** with PAPS as a sulfate donor, catalyzed by baculovirus cloned and expressed 3-OST1 sulfotransferase. Pentasaccharide **II** was then treated with commercially available recombinant glucosamine-6-sulfatase (EC 3.1.6.14), a lysosomal hydrolase enzyme whose deficiency is implicated in Sanfilippo D syndrome in humans, to selectively remove the 6-*O*-sulfate group from the terminal *N*-sulfoglucosamine residue at the nonreducing end (residue A), yielding pentasaccharide **I**.[38] Similarly pentasaccharide **IV** was prepared from pentasaccharide **III** by selective removal of the 6-*O*-sulfate group from the terminal glucosamine residue and then was treated with PAP^{34}S, prepared by a slightly modified procedure, to regioselectively re-sulfate at the same 6-*O*-position of the terminal glucosamine residue at the nonreducing end to obtain pentasaccharide **V** (Scheme 2). We explored then the newly developed LC/MS system toward resolving these pentasaccharides.

LC/MS of Pentasaccharides. Pentasaccharide **II** and Pentasaccharide **III** differ from one another only by the presence (**II**) or absence (**III**) of a 3-*O* sulfate on the internal glucosamine residue C (Scheme 2). Pentsacchride **III** differs from pentasaccharide **IV** by the presence of critical 6-*O*-sulfate on the nonreducing terminal glucosamine residue A. Thus pentasaccharides **II**, **III**, and **IV** have 8 sulfate groups, 7 sulfate groups, and 6 sulfate groups, respectively. A mixture containing an equimolar amount of pentasaccharides **II**, **III**, and **IV** was prepared and injected into the capillary HPLC system. Figure 8 shows the TIC of the pentasaccharide mix in which three peaks, A, B, and C, corresponding to three pentasaccharides, were resolved very well within a short time period of 10 min. Peaks A, B, and C were assigned to pentasaccharide **IV**, **III**, and **II**, respectively, deduced from mass spectral values of each peak (Figure 9). The doubly charged ions were observed as the most abundant ions along with triple and quadruple charge states as minor ions. Results were summarized in Table 2. This indicates that the least sulfated pentasaccharide **IV** elutes from the column ahead, whereas the most sulfated pentasaccharide **II**, containing intact ATIII binding structure, elutes last and pentasaccharide **III** containing 7 sulfate groups elutes between the least and the most sulfated pentasaccharide. Thus these pentasaccharides can be resolvable by capillary reverse phase columns based on the degree of sulfation. Next, the ability of the LC/MS system to resolve the pentasaccharides **I** and **V** was undertaken. Pentasaccharides **I** and **V** both contain the same number of sulfate groups, seven. However, they differ from each other by the sulfation pattern. Resolving such oligosaccharide mixtures would undoubtedly lead to greater confidence about the homogeneity of any prepared sample for mass spectral analysis. Figure 10 shows the resolution of these pentasaccharides **V** and **I**, separated from each other by 0.6 min, corresponding to peak **A** and **B**, respectively. Mass spectra of these

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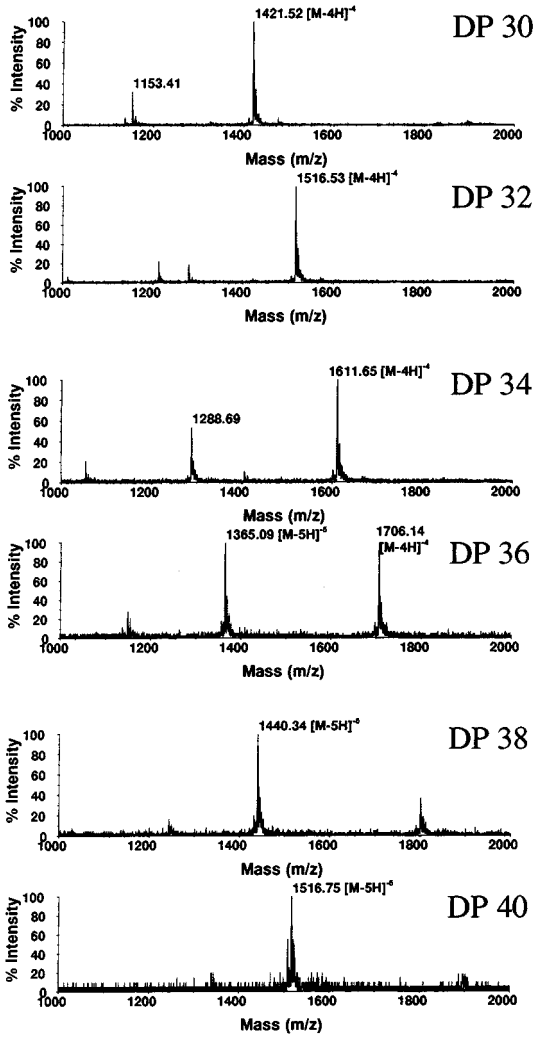

Figure 7. Electrospray mass spectra of oligosaccharides ranging in size from triacontasaccharide (DP = 30) to tetracontasaccharide (DP = 40).

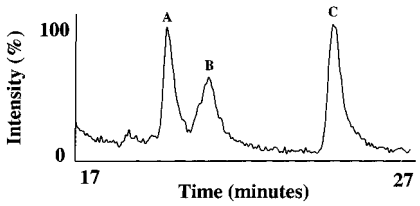

Figure 8. Total ion chromatogram from LC/ESI-MS analysis of pentasaccharides II, III, and IV having different degrees of sulfation. Peaks A, B, and C correspond to pentasaccharides IV, III, and II, respectively.

pentasaccharides whose molecular weights differ by 2 daltons were shown in Figure 11. The observed abundant molecular ion for pentasaccharide **V** was 842.71 corresponding to $[M + 2(DBA) - 4H]^{2-}$ and that for pentasaccharide **I** was 841.71 corresponding to $[M + 2(DBA) - 4H]^{2-}$. Although DBA adductions were not observed in triple and quadruple charge states except for pentsaccharide **II**, adductions with DBA were always observed for these pentasaccharides in the abundant double charge state. The results are summarized in Table 2. Note that these pentasaccharides, **I** and **V**, would have the same molecular weight if pentasaccharide **V** was otherwise sulfated with regular PAPS instead of PAP^{34}S as a sulfate donor, catalyzed by 6-OST1 sulfotransferase. Thus, our current studies demonstrated not only the ability of LC/MS to resolve HS

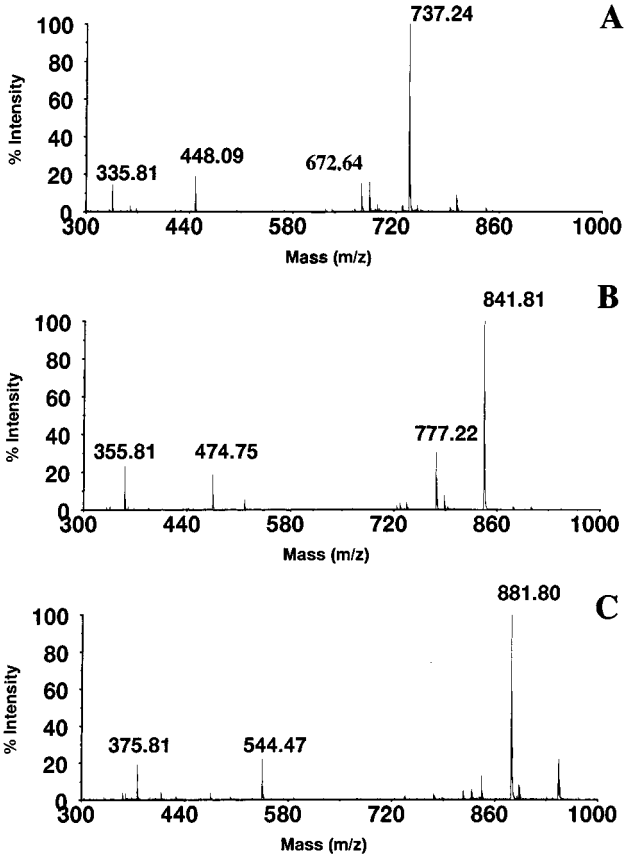

Figure 9. ESI mass spectrm of pentasaccharide IV (A), pentasaccharide III (B), and pentasaccharide II (C) having different degrees of sulfation. The parent ion of pentasaccharide IV does not have adduction with the ion-pairing agent (DBA). Both pentsaccharides III and II form adduction with DBA in the parent ion. Results are summarized in Table 2.

Table 2. Molecular Masses of of AT III Binding Pentasaccharide Variants

pentasaccharide	obsd *m/z*	charge state	quasi molecular ion[a]
I	474.70	3−	$[M - 3H]^{3-}$
	777.13	2−	$[M + 1(DBA) - 3H]^{2-}$
	788.13	2−	$[M + 1(DBA) + Na - 4H]^{2-}$
	841.71	2−	$[M + 2(DBA) - 4H]^{2-}$
II	375.81	4−	$[M - 4H]^{4-}$
	544.47	3−	$[M + 1(DBA) - 4H]^{3-}$
	881.80	2−	$[M + 2(DBA) - 4H]^{2-}$
III	355.81	4−	$[M - 4H]^{4-}$
	474.75	3−	$[M - 3H]^{3-}$
	777.22	2−	$[M + 1(DBA) - 3H]^{2-}$
	841.81	2−	$[M + 2(DBA) - 4H]^{2-}$
IV	335.80	4−	$[M - 4H]^{4-}$
	448.09	3−	$[M - 3H]^{3-}$
	672.64	2−	$[M - 2H]^{2-}$
	737.24	2−	$[M + 1(DBA) - 3H]^{2-}$
V[b]	356.27	4−	$[M - 4H]^{4-}$
	475.37	3−	$[M - 3H]^{3-}$
	778.12	2−	$[M + 1(DBA) - 3H]^{2-}$
	842.70	2−	$[M + 2(DBA) - 4H]^{2-}$

[a] All parent ions were observed in adduction with the ion-pairing agent, dibutylammonium acetate (DBA). [b] Pentasaccharide V labeled with ^{34}S containing sulfate as a mass spectral probe to distinguish it from pentasaccharide I in LC/MS characterization. See Figures 10 and 11 for details.

oligosaccharides based on sulfation pattern but also the advantage of introducing the 6-*O* ^{34}SO4 group as a mass spectral probe to identify each peak corresponding to its respective pentasaccharide. A pair of HS-like oligosaccharides of composition $(HexA\text{-}GlcN)_m$ and $(HexA\text{-}GlcN)_{2m}$ containing unsaturated

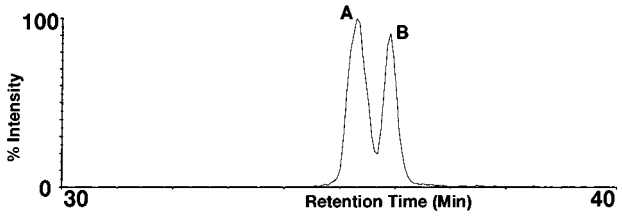

Figure 10. Total ion chromatogram of pentasaccharide V (peak A) and pentasaccharide I (peak B), having the same number of sulfate groups, resolved based on the sulfation pattern.

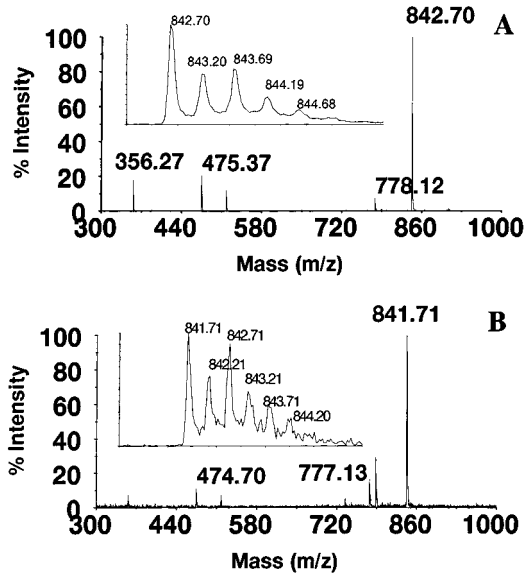

Figure 11. Electrospray mass spectrum of pentasaccharide V (panel A) and of pentasaccharide I (panel B). Pentasaccharide V contains a 6-O-^{34}SO4 group as a mass spectral probe to be distinguished from its isomer pentasaccharide I and hence its molecular mass is 2 daltons more. Isotope clusters of the parent ion of each compound are illustrated as insets in each panel. Results are summarized in Table 2.

uronic acid at the nonreducing end have no mass difference between two oligosaccharides of single and double charge states. As *m* (the number of disaccharide units) increases, the complexity of the isotopic clusters also increases. So it is very important to resolve the oligosaccharides based on charge, size, hydrophobic, or sulfation pattern into as much homogeneity as possible before entering the mass spectrometry. It is also important to note that no fragmentation is observed under current LC/MS experimental conditions. This result has implications for the analysis of HS oligosaccharides of larger sizes by electrospray MS that would definitely lead to determining the alignment of critical functional groups necessary for elucidating structure−function relationships.

Conclusions

In this work we have demonstrated the utility of IP-RP-capillary HPLC-ESI-TOF-MS for HS oligosaccharide analysis. Microcapillary HPLC columns with integrated electrospray emitters provide efficient temporal separation and ionization of oligosaccharide species, while MS operation in external ion accumulation mode maintains chromatographic resolution during mass spectral acquisitions. It is concluded that solution parameters have opposite effects on the performance of IP-RP-HPLC and negative-ion ESI-MS. Therefore, a compromise has to be found to enable the efficient on-line coupling of IP-HPLC to ESI-MS in the analysis of HS. Using capillary columns packed with C18 particles and gradients of methanol in 5 mM dibutylammonium acetate, HS precursor oligosaccharides up to at least tetracontasaccharide (40-mer) were separated. Alkali cation adduction in the separated oligosaccharides is efficiently reduced, allowing highly accurate mass determination. To date, these are the largest nonsulfated HS precursor oligosaccharides to be characterized by LC/MS. Finally, the current methodology was applied in characterizing biologically important AT III binding pentasaccharide and its precursors, which differ from each other by sulfation pattern and/or degree of sulfation. All of these pentasaccharides were well-resolved and characterized by the LC/MS system with the use of $^{34}SO_4$ as a mass spectral probe. Though the biological functions of heparan sulfate are well-known over several decades, only a few heparan sulfate structures attributable to their functions have been solved thus far. It is primarily due to the difficulties involved in isolating pure HS active oligosaccharides for characterization. Besides, several HS sequences may well be capable of binding to a given protein to exert a specific biological function or event.[1,2] In vitro modification of HS-inactive precursors lacking critical functional groups and converting them into HS-active structures with $^{34}SO_4$ or $^{33}SO_4$ as stable isotopes catalyzed by a specific cloned sulfotransferase would allow one to position or align the critical functional groups necessary for biological functions. This newly developed methodology should assist chemical synthesis of HS fragments of biological significance in terms of purification and rapid characterization, and thus expedite the synthesis of biologically significant HS oligosaccharides. Our findings should undoubtedly pave the way in deciphering multiple functional arrangements, ascribed to many biological activities, which are predictably embedded in a single large chaotic, yet well-organized HS polysaccharide chain.

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