analytical chemistry

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Capillary Electrophoresis–Mass Spectrometry for the Analysis of Heparin Oligosaccharides and Low Molecular Weight Heparin

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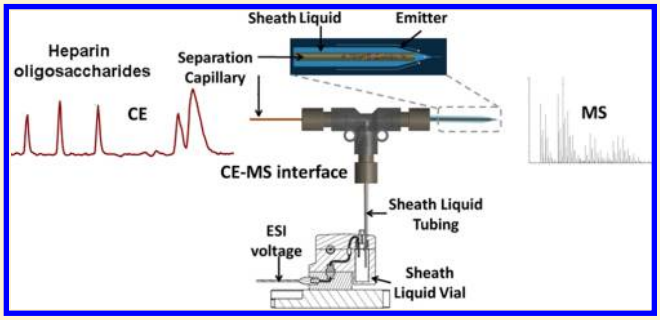
Supporting Information

ABSTRACT: Heparins, highly sulfated, linear polysaccharides also known as glycosaminoglycans, are among the most challenging biopolymers to analyze. Hyphenated techniques in conjunction with mass spectrometry (MS) offer rapid analysis of complex glycosaminoglycan mixtures, providing detailed structural and quantitative data. Previous analytical approaches have often relied on liquid chromatography (LC)–MS, and some have limitations including long separation times, low resolution of oligosaccharide mixtures, incompatibility of eluents, and often require oligosaccharide derivatization. This study examines the analysis of glycosaminoglycan oligosaccharides using a novel electrokinetic pump-based capillary electrophoresis (CE)–MS interface. CE separation and electrospray were optimized using a volatile ammonium bicarbonate electrolyte and a methanol–formic acid sheath fluid. The online analyses of highly sulfated heparin oligosaccharides, ranging from disaccharides to low molecular weight heparins, were performed within a 10 min time frame, offering an opportunity for higher-throughput analysis. Disaccharide compositional analysis as well as top-down analysis of low molecular weight heparin was demonstrated. Using normal polarity CE separation and positive-ion electrospray ionization MS, excellent run-to-run reproducibility (relative standard deviation of 3.6–5.1% for peak area and 0.2–0.4% for peak migration time) and sensitivity (limit of quantification of 2.0–5.9 ng/mL and limit of detection of 0.6–1.8 ng/mL) could be achieved.



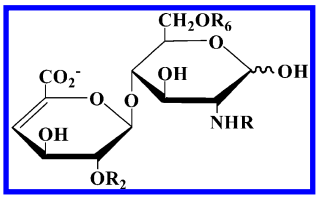
Glycosaminoglycans are a family of linear, polydisperse, anionic polysaccharides responsible for a number of critical biological functions.[1−3] Heparan sulfate is the most structurally complex glycosaminoglycan. It is composed of 20–40 disaccharide repeating units of 1,4-glycosidically linked uronic acid and hexosamine residues. The uronic acid residue in each disaccharide repeating unit can be D-glucuronic acid (GlcA) or L-iduronic acid (IdoA) with or without 2-*O*-sulfo (2S) groups, and the hexosamine residue is D-glucosamine (GlcN) with and *N*-sulfo or *N*-acetyl or free amino group and with or without 3-*O*-sulfo (3S) and 6-*O*-sulfo (6S) groups.[1,4] The structural diversity of heparan sulfate and its polyanionic nature make its analysis quite difficult. A specialized heparan sulfate chain biosynthesized in mast cells, known as heparin, is particularly challenging to analyze because of its high level of sulfation.[5] Heparin is a pharmacologically important glycosaminoglycan as it is used widely as a clinical anticoagulant,[6] and its analysis has become increasingly important since the adulteration of pharmaceutical heparin in 2007–2008 led to a heparin crisis resulting in the death of over one-hundred Americans.[7,8]

Hyphenated techniques, especially those involving sensitive analytical methods such as mass spectrometry (MS), have played an increasingly important role in the analysis of glycosaminoglycans with complex molecular structures, such as heparin. Early studies of glycosaminoglycans by liquid chromatography (LC)–MS required the removal of sulfo groups and the selective chemical derivatization of the resulting oligosaccharides, a cumbersome procedure accompanied by the loss of structural information.[9] The development of electrospray ionization (ESI) facilitated the use of high-performance (HP)LC–MS, which was first described using a high-resolution reversed-phase ion-pairing (RPIP) separation involving a volatile amine on an octadecyl C18 column.[10] While useful in

Received: November 20, 2015
Accepted: December 29, 2015
Published: December 29, 2015

DOI: 10.1021/acs.analchem.5b04405

Table 1. Structure and Molecular Weight of Heparan Sulfate Disaccharides.



HS disaccharides	structure	R_2	NR	R_6	mol wt
0S	ΔUA(1,4)GlcNAc	H	Ac	H	379.1115
2S	ΔUA2S(1,4)GlcNAc	SO_3^-	Ac	H	459.0683
6S	ΔUA(1,4)GalNAc6S	H	Ac	SO_3^-	459.0683
NS	ΔUA(1,4)GlcNAc6S	H	SO_3^-	H	417.0577
2S6S	ΔUA2S(1,4)GlcNAc6S	SO_3^-	Ac	SO_3^-	539.0251
NS2S	ΔUA2S(1,4)GlcNS	SO_3^-	SO_3^-	H	497.0145
NS6S	ΔUA(1,4)GlcNS6S	H	SO_3^-	SO_3^-	497.0145
TriS	ΔUA2S(1,4)GlcNS6S	SO_3^-	SO_3^-	SO_3^-	576.9713

separating, detecting, and identifying heparin-derived oligosaccharides, RPIP-LC−MS analysis leaves residual ion-pairing reagent in the mass spectrometer making its use problematic. The use of hydrophilic interaction liquid chromatography (HILIC), pioneered for heparin analysis by Zaia and coworkers,[11−13] eliminated the need for ion-pairing reagent. HILIC−MS, accompanied by oligosaccharide derivatization, still represents the gold standard for bottom-up and top-down analysis of low molecular weight heparins (LMWHs).[12,14] Recently, mechanized column switching methods have facilitated the multiple heart-cutting two-dimensional (2D)-LC separations of LMWHs with MS detection.[15] To date, LC−MS has dominated the area of hyphenated analysis of heparins.

Electrophoresis, a particularly effective method for the high-resolution fractionation of polyanionic biopolymers such as nucleic acids,[16] has recently been applied to the separation of glycosaminoglycans.[17−19] Preparative polyacrylamide gel electrophoresis (PAGE) was used off-line with MS analysis in the first successful sequencing of a glycosaminoglycan.[20] Unfortunately, such PAGE separations require MS-incompatible buffers and contain leached soluble oligoacrylamides that interfere with MS analyses.[21] Capillary electrophoresis (CE) has taken on a prominent role in sequencing nucleic acids because of its high separation efficiency,[17] and CE has also been applied to glycosaminoglycan analysis.[22] The off-line coupling of CE with MS has been successfully applied to the analysis of low molecular weight heparin.[19] While MS-friendly CE buffer systems for the separation of heparin-derived oligosaccharides were designed over a decade ago,[23] the lack of suitable CE−MS interfaces has stymied the development of online CE−MS analysis of heparins. The current study explores the first use of a newly developed interface for the CE−MS analysis of heparin oligosaccharides and LMWH.

EXPERIMENTAL SECTION

Materials. HS disaccharide standards (see Table 1 for structures) were purchased from Iduron, Manchester, U.K. Fully sulfated tetrasaccharide standard was isolated in our laboratory as described (see Supporting Information Figure S1 for structure).[4] Arixtra and Lovenox were purchased from Sanofi, Bridgewater, New Jersey. Ammonium acetate (HPLC grade), calcium chloride (HPLC grade), acetic acid (HPLC grade), and ammonium bicarbonate (HPLC grade) were purchased from Fisher Scientific, Springfield, New Jersey. *Escherichia coli* expression and purification of the recombinant *Flavobacterium heparinum* heparin lyase I, II, III (Enzyme Commission (EC) nos. 4.2.2.7, 4.2.2.X, 4.2.2.8) were performed in our laboratory as previously described.[24]

Sample Preparation. Four HS disaccharide standards, unsulfated (0S, ΔUA(1 → 4)GlcNAc, where ΔUA is 4-deoxy-α-L-*threo*-hex-4-enopyranosyl uronic acid), monosulfated (NS, ΔUA(1 → 4)GlcNS), disulfated (2S6S, ΔUA2S(1 → 4)-GlcNAc6S), and trisulfated (TriS, ΔUA2S(1 → 4)GlcNS6S), and fully sulfated tetrasaccharide standard (ΔUA2S(1 → 4)GlcNS6S(1 → 4)IdoA2S(1 → 4)GlcNS6S), were prepared as stock solutions at a concentration of 1 μg/μL. A five-component solution containing four disaccharides and one tetrasaccharide, each at a final concentration 250 ng/μL, was used in method development studies. A series of the four mixed disaccharide standards were also prepared for method validation by adding aqueous solutions containing varying amounts of each standard affording final concentrations of from 0.005 to 0.5 ng/μL.

Desalted and lyophilized Arixtra and Lovenox were redissolved in distilled water at a concentration of 1 and 10 μg/μL, respectively. Lovenox (100 μg in 10 μL of distilled water) was added to 100 μL of digestion buffer (50 mM ammonium acetate, 2 mM calcium chloride, pH 7.0). Heparin lyase I, II, and III (10 mU each in Tris−HCl buffer, pH 7.0) were added and mixed well. The solution was placed in a water bath at 37 °C overnight. The enzymatic digestion was terminated by removing the enzymes using a 3 kDa molecular weight cutoff (MWCO) spin column. The filtrate containing disaccharides from the completely depolymerized Lovenox was vacuum-centrifuge dried and redissolved in 100 μL of distilled water at a concentration of 1 μg/μL.

CE−MS Analysis. The CE separation was performed on a CMP ECE-001 capillary electrophoresis equipped with a CMP EMASS-II CE−MS ion source (CMP Scientific, Brooklyn, NY). Aqueous ammonium bicarbonate (7 mM) was used as background electrolyte in a 50 cm bare separation capillary (360/200 μm o.d. × 50/40 μm i.d.) at 25 °C. The sheath liquid was 0.5% formic acid in 50% aqueous methanol. The sample was injected under 0.2 psi pressure for 5 s. The separation voltage of 30 kV under normal polarity was used. The electrospray voltage at the CE−MS interface was 2.0 kV. The spray emitter was 1.0 mm o.d. × 0.75 mm i.d. borosilicate glass with a 15 μm tip, and the distance from emitter tip to mass

DOI: 10.1021/acs.analchem.5b04405

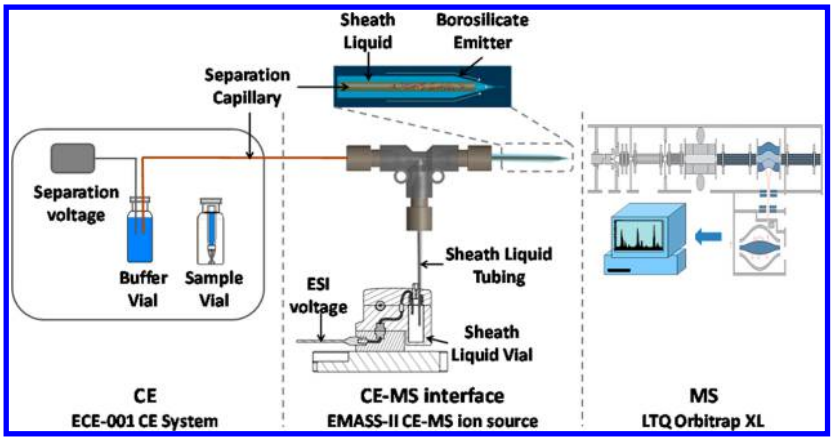


Figure 1. Schematic diagram of CE−MS system.

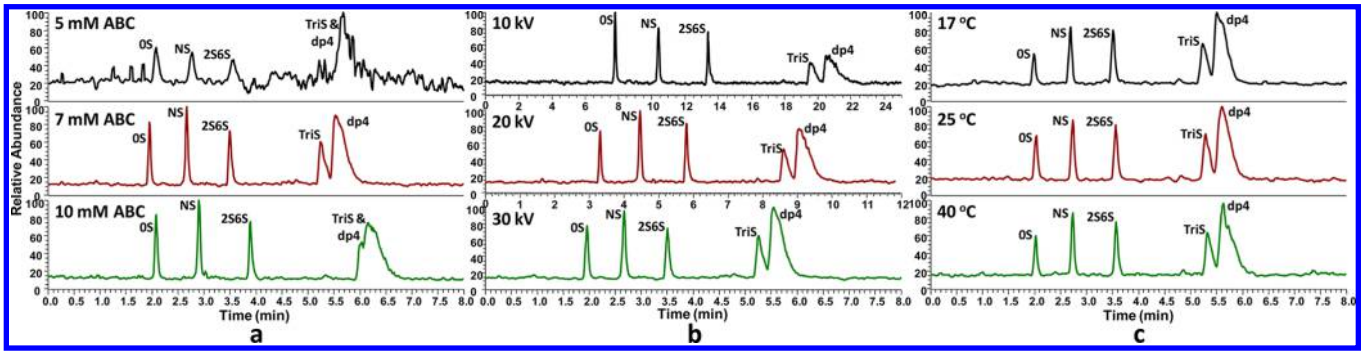


Figure 2. CE−MS analysis of four disaccharide standards and fully sulfated tetrasaccharide (see Figure S1 for structure) standard mixture. Panel a shows the CE−MS analysis with 30 kV separation voltage at 25 °C under different buffer concentrations. Panel b shows the CE−MS analysis using 7 mM ammonium bicarbonate (ABC) at 25 °C with different separation voltages. Panel c shows the CE−MS analysis with 30 kV separation using 7 mM ABC at different temperatures.

spectrometer was adjusted to 2 mm, adjusted with the help of a microscope.

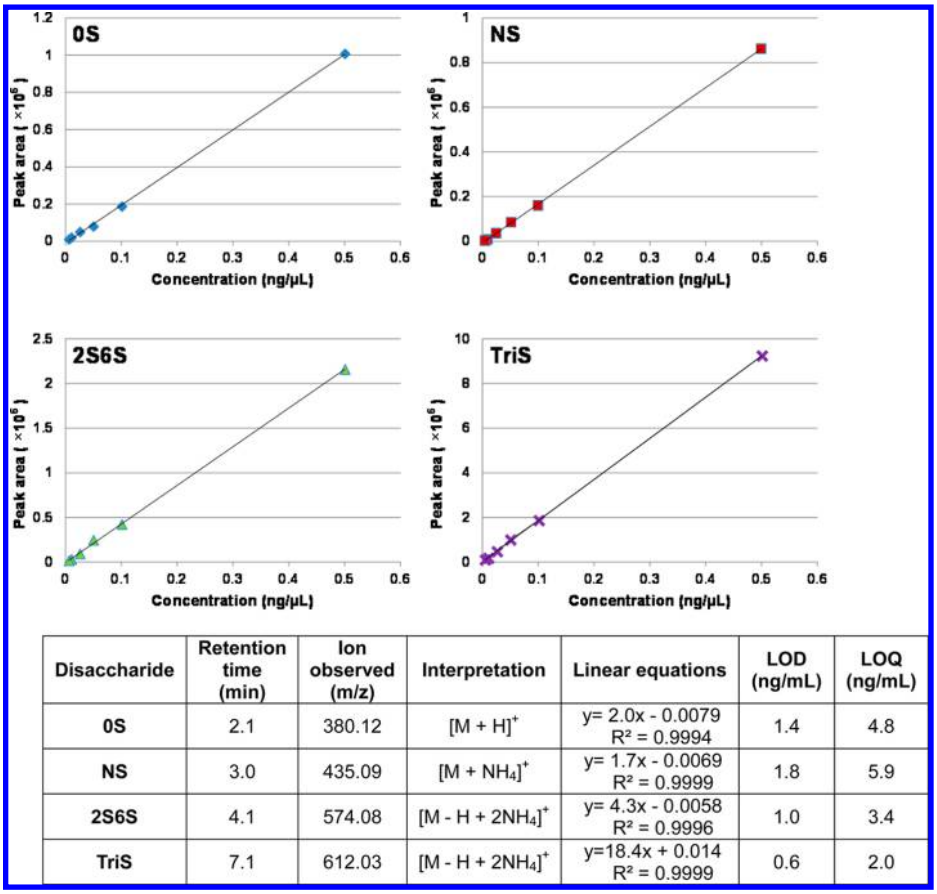
A Thermo Fisher Scientific (San Jose, CA) LTQ-Orbitrap XL was coupled online to the CE. The MS analysis was under positive-ion mode. The capillary voltage was 45 V. The capillary temperature was 200 °C, and the tube lens voltage was 100 V. The Fourier transform (FT) MS resolution was 30 000, and all other parameters were set as default.

Data Analysis. The data analysis was performed on Thermo Xcalibur software.

RESULTS AND DISCUSSION

CE holds exciting promise for glycosaminoglycans (GAG) analysis due to its notable properties, such as high sensitivity, high separation efficiency, and compatibility with various measurement techniques. Furthermore, fabrication simplicity and modification convenience can greatly enrich the capillary systems available for use in the field of bioanalysis. Here, we pioneer the use of an electrokinetic pump-based CE−MS coupling technology[25] for the analysis of heparin oligosaccharides and LMWHs using positive-ion detection mode (Figure 1). In this system, the sheath liquid flow was generated by electroosmosis on the surface of borosilicate glass spray emitter. The ESI voltage was passed through embedded wiring from an external high-voltage power supply to the electrode, which was placed in the sheath liquid vial and kept distant from the spray emitter. The outlet end of the separation capillary was thinned so that it could be inserted within a couple of hundreds of micrometers to the emitter tip.[26] This approach brings some significant advantages over other CE−MS interfacing technologies. First, when the emitter was moved to within a couple of millimeters to the mass spectrometer, the electrical field between the emitter tip and the grounded mass spectrometer entrance generates an electroosmotic flow (EOF) along the inner surface of the emitter. This EOF pumps the sheath liquid at a very low (nanoliters per minute) flow rate.[27] In contrast, the commonly used triple tube sprayer CE−MS interface design uses a sheath liquid flow rate of between 5 and 10 μL/min. Second, since the electrode is kept away from the emitter, electrolysis within the spray emitter is eliminated. This minimizes the formation of bubbles, which is frequently encountered in other interfaces. Third, the combination of eletrokineticly pumped sheath liquid flow and the electrode placement results in a steady and stable electrospray at a very low flow rate. Fourth, a low sheath liquid flow minimizes the dilution of sample analytes as they exit the separation capillary, thus maximizing detection sensitivity. Fifth, the coaxial placement of the electrophoresis capillary and the spray emitter provides critical room for postelectrophoresis pre-electrospray reaction. A well-designed interface has been proven essential in our CE−MS method development for the heparin analysis. By optimizing the chemical composition of background electrolyte and sheath liquid, it is possible to separate the negatively charged heparin analytes by electrophoresis, then turn these into positively charged analytes for MS analysis. This is done by having the sheath liquid entraining the anionic analytes as they exit the electrophoresis capillary, titrating their positive charges, and electrospraying them to exit the emitter tip and into the

DOI: 10.1021/acs.analchem.5b04405



Disaccharide	Retention time (min)	Ion observed (m/z)	Interpretation	Linear equations	LOD (ng/mL)	LOQ (ng/mL)
0S	2.1	380.12	$[M + H]^+$	y= 2.0x - 0.0079 R^2 = 0.9994	1.4	4.8
NS	3.0	435.09	$[M + NH_4]^+$	y= 1.7x - 0.0069 R^2 = 0.9999	1.8	5.9
2S6S	4.1	574.08	$[M - H + 2NH_4]^+$	y= 4.3x - 0.0058 R^2 = 0.9996	1.0	3.4
TriS	7.1	612.03	$[M - H + 2NH_4]^+$	y=18.4x + 0.014 R^2 = 0.9999	0.6	2.0

Figure 3. Quantitative compositional analysis of four disaccharide standards. Retention time, major ion, linear equation, LOD, and LOQ of four disaccharide standards are listed in the table at the bottom of the figure.

mass spectrometer. In this regard, the advantage of having sheath liquid is that it provides opportunities for method development based on postseparation reactions. Although sheathless CE−MS interfaces points to a potentially promising direction,[27−29] transferring this postelectrophoresis pre-electrospray reaction directly to sheathless CE−MS system still represents a major technical challenge. Despite these challenges, online sheathless CE−MS has been successfully applied to the analysis of chondroitin sulfate and dermatan sulfate derived oligosaccharides.[18]

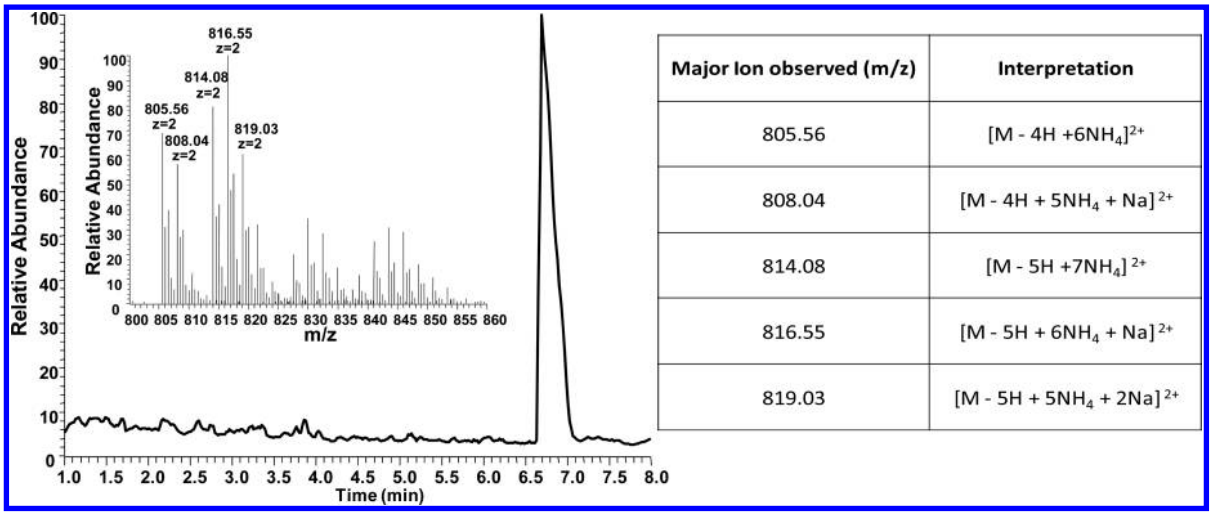
CE separation systems generally have large footprints. This has required a capillary length of at least 70 cm in order to reach the mass spectrometer. In the current study we used an ECE-001 CE system specifically designed for this CE−MS interface (Figure 1). The capillary inlet end was placed right next to the capillary exit on the unit, thus allowing the use of separation capillary as short as ∼50 cm. When running electrophoresis at 30 kV, a 50 cm capillary generates an electrical field of 600 V/cm, affording very high separation efficiency and requiring very short running time for CE−MS analysis.

Next, three key elements of CE−MS analysis were optimized: buffer concentration, separation voltage, and temperature (Figure 2). Our previous studies showed that volatile ammonium bicarbonate worked well in both CE and MS environment.[23] Under normal polarity mode, in basic ammonium bicarbonate electrolyte, sample was injected at the anode and detected at the cathode. Negatively charged species are prevented from migrating out of the end of capillary at the anode by the dominant EOF that moves all species toward the cathode. Thus, unsulfated disaccharides migrate fastest followed by monosulfated, disulfated, and finally trisulfated disaccharides counter to their electrophoretic mobility.

Various concentrations of ammonium bicarbonate running buffer were examined (Figure 2a and Supporting Information Figure S4), and we found that at too low of electrolyte concentration, 2 mM, the EOF was insufficient to move the analyte to the cathode for detection and no peaks were observed. At higher electrolyte concentrations, 5−30 mM, peaks corresponding to analytes were detected, but their migration times increased. On the basis of the results of this study an optimum ammonium bicarbonate concentration of 7 mM was selected, as this electrolyte showed optimal resolution with minimal analysis time. Next, the separation voltage, another crucial parameter for the oligosaccharide separation, was examined (Figure 2b). Separation time gradually decreased with an increase in voltage resulting from the complex interplay between EOF and electrophoresis as the dominant separation force. Finally, the impact of temperature was examined, and baseline resolution for the four different disaccharides was achieved at room temperature (Figure 2c). Thus, 7 mM ammonium bicarbonate at 30 kV and 25 °C were selected as the optimal parameters for heparin oligosaccharide separation.

On the basis of these optimal conditions the analytical reproducibility of the CE separation was examined (Supporting Information Figure S5). The relative standard deviation (RSD) for peak area was 3.6−5.1%, and the RSD for peak migration time was 0.2−0.4%. These low RSDs demonstrate that a

DOI: 10.1021/acs.analchem.5b04405



Major Ion observed (m/z)	Interpretation
805.56	$[M - 4H + 6NH_4]^{2+}$
808.04	$[M - 4H + 5NH_4 + Na]^{2+}$
814.08	$[M - 5H + 7NH_4]^{2+}$
816.55	$[M - 5H + 6NH_4 + Na]^{2+}$
819.03	$[M - 5H + 5NH_4 + 2Na]^{2+}$

Figure 4. CE−MS analysis of Arixtra. The figure at the left is the TIC, and the inset shows the major ions observed in the peak at 6.8 min. The interpretations are listed in the table on the right.

reliable CE separation had been established. Different concentrations of heparin disaccharide standards were next applied in the testing system based on the optimal assay conditions to determine the linearity and sensitivity of the method (Figure 3). The mass signal response was different for different disaccharide concentrations. The peak area linearly increased as the concentrations of disaccharides increased from 0.005 to 0.5 ng/μL. The limits of detection (LODs) ranged from 0.6 to 1.8 ng/mL for the different disaccharides tested. The limits of quantification (LOQs) were estimated from the measurement at a signal-to-noise of 10:1 and ranged from 2.0 to 5.9 ng/mL for the different disaccharides tested. Calibration curves and the LOQ values for each disaccharide are provided in the table shown Figure 3. It is noteworthy that the sample injection volume was only 20 nL; this means picogram amounts of disaccharide can be analyzed by CE−MS. Moreover, the method sensitivity is ~1000-fold higher than that of ultraviolet detection (Supporting Information Figure S6). Thus, CE−MS affords a highly sensitive method of heparin disaccharide analysis over a wide range of concentrations.

Next, we examined a synthetic heparin pentasaccharide, Arixtra, which is not amenable to ultraviolet detection because of the lack of a nonreducing terminal ΔUA residue. Arixtra, a homogeneous, chemically synthesized ultralow molecular weight heparin (ULMWH) (see Supporting Information Figure S2 for structure), is a clinically used antithrombotic drug. As expected for a pharmaceutical product, a single sharp peak was observed at 6.8 min in CE−MS analysis (Figure 4). The top left spectrum and the table in Figure 4 show the five major ions observed for this CE peak. The high-resolution MS results could be easily interpreted as doubly charged ions having a different number of ammonium and sodium adducts.

Lovenox, a complex, polycomponent LMWH (see Supporting Information Figure S3 for a generalized structure), is commonly used as an anticoagulant drug formulated served in 0.9% sodium chloride. The salt was removed from the formulated drug by dialysis (MWCO 1 kDa) to avoid its adverse effect on MS analysis. Next, the bottom-up and the top-down analysis of Lovenox were attempted using CE−MS. In the bottom-up analysis, Lovenox sample was first depolymerized by heparin lyase I, II, and III to afford primarily disaccharide products. CE−MS analysis of heparin lyase-depolymerized Lovenox is shown in Figure 5a. All disaccharides could be separated within 6 min (Table 1). Resistant

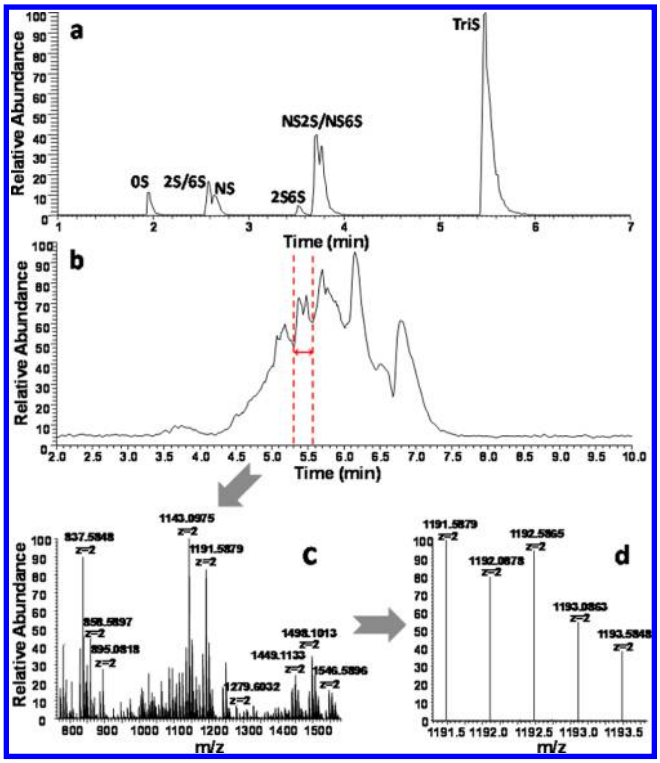


Figure 5. CE−MS analysis of Lovenox. Panel a shows the extracted ion chromatograms (EICs) of disaccharides derived from completely digested Lovenox. Panel b shows the TIC of CE−MS analysis of intact Lovenox. Panel c shows the mass spectrum corresponding to the peak labeled in panel b. Panel d shows a zoom-in of the spectrum of the isotopic peaks of m/z = 1191.5897, which corresponds to an octasaccharide with 11 sulfo groups.

tetrasaccharides, likely containing 3-*O*-sulfoglucosamine residues, corresponding to fragments of the antithrombin III binding site,[4] were observed (Table S1 in Supporting Information). In addition, tetrasaccharides and disaccharides containing 1,6-anhydrosugars at the reducing ends, an artifact of conditions used to prepare Lovenox, could also be detected.

DOI: 10.1021/acs.analchem.5b04405

Table 2. Major Low Molecular Weight Heparin Chains Identified by CE-MS.

no.	oligosaccharide size	substitution composition	1,6-anhydro	m/z monoisotopic	major ions obsvd	exptl MW_{mono}	theor MW_{mono}	mass accuracy (ppm)
1	dp3	3S,0Ac	no	805.0883	$[M - 2H + 3NH_4]^+$	753.0009	753.0034	−3.3
2	dp3	4S,0Ac	no	885.0451	$[M - 2H + 3NH_4]^+$	832.9577	832.9602	−3.0
3	dp4	5S,0Ac	no	580.5651	$[M - 3H + 5NH_4]^{2+}$	1073.9819	1073.9858	−3.6
4	dp4	6S,0Ac	no	629.0559	$[M - 4H + 6NH_4]^{2+}$	1153.937	1153.9426	−4.9
5	dp5	6S,0Ac	no	708.5594	$[M - 3H + 5NH_4]^{2+}$	1329.9705	1329.9747	−3.2
6	dp5	7S,0Ac	no	757.0508	$[M - 4H + 6NH_4]^{2+}$	1409.9268	1409.9315	−3.3
7	dp6	7S,1Ac	no	858.5900	$[M - 4H + 6NH_4]^{2+}$	1613.0052	1613.0109	−3.5
8	dp6	8S,0Ac	yes	877.0724	$[M - 5H + 7NH_4]^{2+}$	1632.9435	1632.9465	−1.8
9	dp8	10S,0Ac	no	1143.0977	$[M - 6H + 8NH_4]^{2+}$	2147.9676	2147.9716	−1.9
10	dp8	11S,0Ac	no	1191.5897	$[M - 7H + 9NH_4]^{2+}$	2227.9251	2227.9284	−1.5
11	dp10	14S,0Ac	yes	1496.6055	$[M - 10H + 12NH_4]^{2+}$	2786.8772	2786.8891	−4.3
12	dp10	14S,0Ac	no	1514.1250	$[M - 11H + 13NH_4]^{2+}$	2804.8897	2804.8997	−3.6
13	dp12	16S,0Ac	no	1186.7762	$[M - 12H + 15NH_4]^{3+}$	3301.9074	3301.9142	−2.1
14	dp12	17S,0Ac	no	1213.4277	$[M - 12H + 15NH_4]^{3+}$	3381.8619	3381.871	−2.7
15	dp14	19S,0Ac	no	1390.4538	$[M - 14H + 17NH_4]^{3+}$	3878.8872	3878.8855	0.4
16	dp14	19S,1Ac	no	1404.4595	$[M - 14H + 17NH_4]^{3+}$	3920.9043	3920.8961	2.1
17	dp16	20S,1Ac	yes	1543.1453	$[M - 15H + 18NH_4]^{3+}$	4319.9352	4319.9432	−1.9
18	dp16	21S,1Ac	no	1581.4792	$[M - 16H + 19NH_4]^{3+}$	4417.9088	4417.9106	−0.4
19	dp16	22S,0Ac	no	1599.8004	$[M - 17H + 20NH_4]^{3+}$	4455.8475	4455.8568	−2.1
20	dp18	24S,1Ac	no	1785.1510	$[M - 18H + 21NH_4]^{3+}$	4994.8710	4994.8819	−2.2

The elution order follows those of the standard disaccharides examined with those having a greater number of sulfo groups and disaccharides having an *N*-sulfo group eluting later. These results suggest that the disaccharide building block composition could be easily profiled by CE−MS. The improved sensitivity of the CE−MS method might be useful for detecting minor differences between different types of LMWHs, different manufactures, or even different batches from the same manufacturer.[14,30,31] Further method development will certainly be needed in order to apply this CE−MS for such purposes.

The top-down analysis of Lovenox was next undertaken using the total ion chromatogram for intact chain mapping (Figure 5b). Compared to the previous top-down analysis of Lovenox using HILIC-LC−MS and IPRP-LC−MS, CE−MS achieves good separations and reduces the analysis time from >1 h to 8 min, making higher-throughput analysis possible. Another benefit of CE−MS is that analytes that cannot afford positively charged ions are also unable to migrate to ion source. Thus, the spectra obtained on LMWHs are relatively clean (Figure 5c). The mass-to-charge ratio (m/z) and the charge status of major ions were clearly identifiable. The isotopic peaks of analytes containing sulfur were also observed (Figure 5d).

With the help of high-resolution MS, 20 major LMWH chains, ranging from degree of polymerization (dp) of 3−18, were identified (Table 2). The ions observed were interpreted by matching their m/z to the equation of $m/z = [M - nH + (n + z)NH_4]^{z+}$ (where n is an integer). The analyte is injected as the sodium salt form and in the ammonium bicarbonate eluent is nearly completely ion-exchanged into the ammonium salt form. Let us consider two species being analyzed in Table 2, no. 1 (M is the protonated trisaccharide with three sulfo and one carboxyl groups) and no. 20 (M is the protonated octadecasaccharide with 24 sulfo groups and 9 carboxyl groups). Thus, at the end of the separation, the neutral trisaccharide no. 1 and octadecasaccharide no. 20 would carry 4 ammoniums and 33 ammoniums, respectively. The introduction of the sheath fluid containing formic acid strips away one ammonium from trisaccharide no. 1 and adds two protons, affording $[M - 2H + 3NH_4]^+$, and similarly strips away 12 ammoniums from octadecasaccharide no. 20 and adds 15 protons, affording $[M - 18H + 21NH_4]^+$. The major characteristics of the chains identified, such as substitution composition (number of sulfo, *N*-acetyl, or *N*-sulfo groups) and 1,6-anhydro groups at the chain's reducing ends were easily identified (Table S1). As shown in Figure 5b, the ion m/z = 1191.5897 corresponds to an octasaccharide with 11 sulfo groups, no *N*-acetyl, and no 1,6-anhydro structure. The mass accuracy of all components identified was excellent (<5 ppm) as listed in Table 2.

In summary, compared with previous methods for glycosaminoglycan analysis, CE−MS shows four main advantages. First, this method combines the technical strengths of CE and MS allowing an integrated system with high resolution and sensitivity. Second, the label-free strategy not only greatly simplifies sample processing, but also removes undesired sample loss resulting from low-efficiency labeling reactions. Third, the absence of an ion-pairing reagent, which causes spectrometer contamination and inhibits ionization, results in MS analysis that shows excellent analytical performance with high sensitivity and high signal-to-noise ratios. Sample analysis is rapid (<10 min) and requires very small quantities of running buffer (<10 μL). Thus, CE−MS offers a potentially high-throughput, cost-effective means of glycosaminoglycan analysis and on-site biomolecular investigation. The current study relied on the positive mode because an uncoated capillary sheath emitter tip is used. In the future, the use of coated emitter tips might facilitate the use of negative mode.

The future of CE−MS interfacing technologies will likely require both further instrument development and further method development. There is strong demand for rapid and ultrasensitive analysis. Very short capillaries, online sample enrichment, fast turnaround cycle, as well as vigorous method development should make CE−MS an excellent method for high-throughput analysis. Although this high-end CE−MS platform presented still needs to be evaluated for validated use

DOI: 10.1021/acs.analchem.5b04405

in heparin analysis, the prospects for this new hyphenated approach in glycosaminoglycan analysis is bright.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b04405.

Structures of fully sulfated tetrasaccharide standards, Arixtra, and Lovenox (Figures S1−S3), buffer conditions, reproducibility, and UV detection studies (Figures S4−S6), identification of 1,6-anhydrooligosaccharides and 3-*O*-sulfo group containing tetrasaccharides observed in CE−MS analysis of heparin lyase-treated Lovenox (Table S1) (PDF)

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Notes

The authors declare the following competing financial interest(s): The authors X.S., L.L., F.Z., L.C., and R.J.L. declare no competing financial interest. Q.X. is an employee of CMP Scientific and Beijing Proteomics Front selling CE−MS ion sources.

■ ACKNOWLEDGMENTS

The work was supported by Grants from the National Institutes of Health in the form of Grants HL125371, GM38060, GM090127, HL096972, and HL10172. X.S. was supported by the Shandong University Scholarship Program.

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