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PAPER IN FOREFRONT

Analysis of heparin oligosaccharides by capillary electrophoresis–negative-ion electrospray ionization mass spectrometry

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Abstract Most hyphenated analytical approaches that rely on liquid chromatography–MS require relatively long separation times, produce incomplete resolution of oligosaccharide mixtures, use eluents that are incompatible with electrospray ionization, or require oligosaccharide derivatization. Here we demonstrate the analysis of heparin oligosaccharides, including disaccharides, ultralow molecular weight heparin, and a low molecular weight heparin, using a novel electrokinetic pump-based CE–MS coupling eletrospray ion source. Reverse polarity CE separation and negative-mode electrospray ionization were optimized using a volatile methanolic ammonium acetate electrolyte and sheath fluid. The online CE hyphenated negative-ion electrospray ionization MS on an LTQ Orbitrap mass spectrometer was useful in disaccharide compositional analysis and bottom-up and top-down analysis of low molecular weight heparin. The application of this CE–MS method to ultralow molecular heparin suggests that a charge state distribution and the low level of sulfate group loss that is achieved make this method useful for online tandem MS analysis of heparins.

Keywords Hyphenated techniques · CE–MS · Heparin · Low molecular weight heparin · Oligosaccharides · Glycosaminoglycan

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Introduction

Glycosaminoglycans (GAGs) are polydisperse, linear, polyanionic polysaccharides that are particularly challenging to analyze [1, 2]. Heparin is a particularly important GAG as it is a widely used clinical anticoagulant [3, 4]. Heparin and the structurally related GAG heparan sulfate have a highly variable sulfated structure comprising a repeating disaccharide unit of → 4) α-D-glucosamine (GlcN) (1 → 4) hexuronic acid (1→, where the GlcN residue can be modified with *N*-acetyl (Ac) or *N*-sulfo (S) groups and 6-*O*-sulfo and/or 3-*O*-sulfo groups and the hexuronic acid can be β-D-glucuronic acid or α-L-iduronic acid

residues that can be modified with 2-*O*-sulfo groups [1, 5]. The analysis of heparin and low molecular weight (LMW) heparins has become increasingly important since the contamination of the heparin supply chain in 2007–8 [6, 7].

Heparin-based drug products are becoming more dependent on mass spectrometry (MS) for high sensitivity, high-throughput analyses. In particular, hyphenated MS methodologies, such as liquid chromatography (LC)–MS [4, 8–13] and more recently capillary electrophoresis (CE)–MS, are taking leading roles in these analyses [14–18]. These methods can provide enhanced resolution for the analysis of complex mixtures of heparin polysaccharide and oligosaccharide chains without loss of detection sensitivity. There are three types of routinely performed heparin and LMW heparin structural analysis that utilize hyphenated MS methodologies, compositional analysis [4, 10, 11], bottom-up analysis [9, 12], and top-down analysis [8, 13]. In the case of bottom-up analysis and compositional analysis, heparin sample is first treated with one or more heparin lyases [4, 9–12] to afford either oligosaccharides or disaccharides for subsequent LC or CE separation and MS analysis. Top-down analysis examines the whole intact GAG chain and has been limited to either small GAG chains, such as bikunin [19], or LMW heparins [8, 13]. Electrophoresis, while offering extremely high-resolution separations, is particularly complicated for online coupling to MS. Sheathless CE–MS interfaces are potentially promising directions; they transfer the post-electrophoresis pre-electrospray reaction directly to the CE–MS system. Online sheathless CE–MS has been successfully applied to the analysis of GAG-derived chondroitin and dermatan sulfate oligosaccharides [17]. Alternatively, the sheathed CE–MS interface can mix a sheath liquid with the capillary output; this has the advantages of allowing CE eluent modification and analyte derivatization before it enters the mass spectrometer. Numerous studies also suggest that negatively charged heparin oligosaccharides represent ideal precursor ions for tandem MS analysis by reducing the level of sequence non-informative fragmentation through sulfate loss [20–22]. However, the negative-mode CE–MS interface, which is more suitable and applicable to online analysis of negatively charged heparins, remains a major technical challenge. The current study relies on a reverse polarity separation with the analyte injected at the cathode (Fig. 1) and a coated sheath capillary interface to promote the electrokinetic pumping of negatively charged heparin oligosaccharides for MS analysis.

Experimental

Reagents

Heparin/heparan sulfate disaccharide standards were purchased from Iduron, Manchester, UK. Arixtra® and Lovenox® were purchased from Sanofi, Bridgewater, New Jersey. HPLC-grade ammonium acetate, calcium chloride, acetic acid, and methanol were purchased from Fisher Scientific, Springfield, New Jersey. Bare separation capillary (360/150 μm

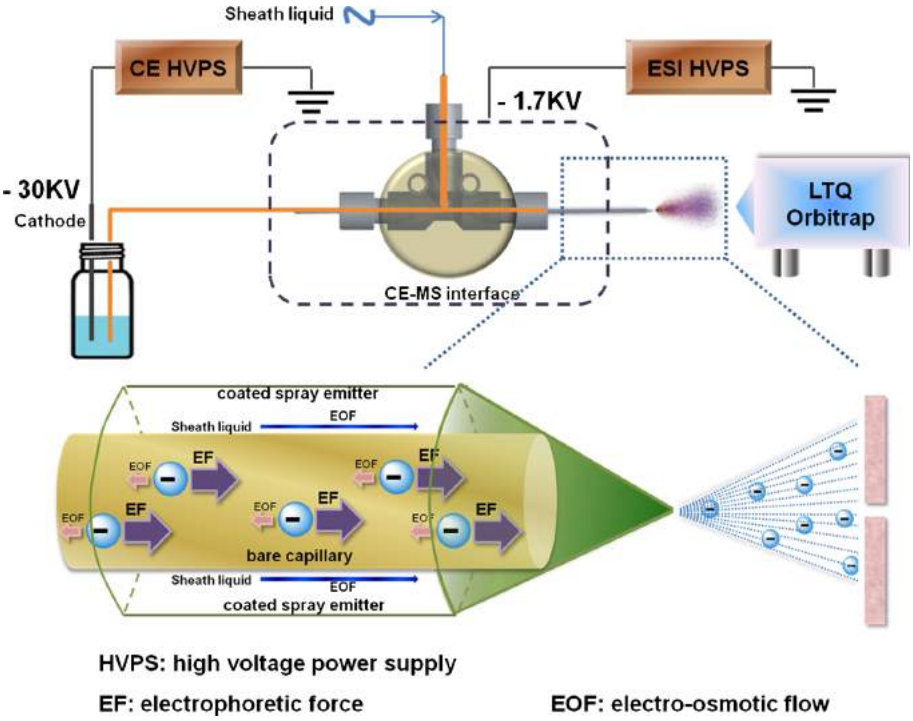


Fig. 1 Schematic representation of negative-mode CE–MS system. A reverse polarity separation under a dominant electrophoretic force (*EF*) and low electroosmotic flow (*EOF*) is used to move analyte down a bare separation capillary. The end of the separation capillary is capped with a protein-coated spray emitter sheath capillary with sheath liquid pumped by EOF, mixing with separation flow and affording a stable electrospray of negatively charged analyte that is introduced into an LTQ Orbitrap for MS analysis

OD × 50 μm ID × 50 cm) and coated spray emitter (1.0 mm OD × 0.75 mm ID, E-BS-CC1-750-1000-10u-B20) were obtained from CMP Scientific, Brooklyn, NY. *Escherichia coli* expression and purification of the recombinant *Flavobacterium heparinum* heparin lyase I, II, III (Enzyme Commission (EC) numbers 4.2.2.7, 4.2.2.×, 4.2.2.8) were performed in our laboratory as previously described [23].

Sample preparation

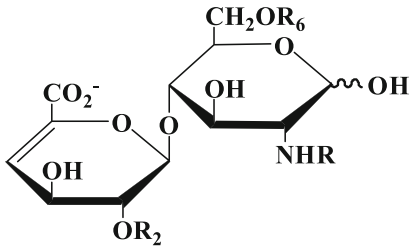
Eight heparin/heparan sulfate disaccharide standards, namely unsulfated (0S, ΔUA(1 → 4)GlcNAc, where ΔUA is 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid), monosulfated (2S, ΔUA2S(1 → 4)GlcNAc; 6S, ΔUA(1 → 4)GlcNAc6S; NS, ΔUA(1 → 4)GlcNS), disulfated (2S6S, ΔUA2S(1 → 4)GlcNAc6S; NS6S, ΔUA(1 → 4)GlcNS6S; NS2S, ΔUA2S(1 → 4)GlcNS), and trisulfated (TriS, ΔUA2S(1 → 4)GlcNS6S) (Table 1), were prepared as stock solutions with a concentration of 1 μg/μL. A standard mixture solution containing eight disaccharides (each with a final concentration of 100 μg/mL) was used in method development studies.

Desalted and lyophilized Arixtra® and Lovenox® were redissolved in distilled water at a concentration of 2 μg/μL and 1 μg/μL, respectively. Lovenox® (100 μg in 10 μL of distilled water) was added to 100 μL digestion buffer (50 mM NH_4Ac, 2 mM $CaCl_2$, 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 an incubator at 37 °C overnight. The enzymatic digestion was terminated by removing the enzymes using a 3-kDa molecular weight cutoff (MWCO) spin-column (Millipore, Bedford, MA, USA). The filtrate containing disaccharides from the completely depolymerized Lovenox® was lyophilized 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 instrument equipped with a CMP EMASS-II CE–MS ion source (CMP Scientific, Brooklyn, NY). A solution of 10 mM ammonium acetate (AA) in 80 % aqueous methanol (pH ca. 7.5) was used as background electrolyte and applied in the bare separation capillary (360/150 μm OD × 50 μm ID) at 25 °C.

Table 1 Structures of heparin/heparan sulfate disaccharide standards



HS Disaccharides	Structure	R_2	NR	R_6	Molecular Weight
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)GlcNAc6S	H	Ac	SO_3^-	459.0683
NS	ΔUA(1,4)GlcNS	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

The sheath liquid was 10 mM AA in 80 % aqueous methanol. The sample was injected under 0.2-psi pressure for 5 s. The applied CE separation voltage was 30 kV with reversed polarity. The electrospray voltage at the CE–MS interface [24, 25] was −1.7 kV. The spray emitters were 1.0 mm OD × 0.75 mm ID, borosilicate glass with a 10-μm tip, which were coated by CMP using proprietary methods (E-BS-CC1-750-1000-10u-B20, CMP Scientific, Corp.). The distance from emitter tip to mass spectrometer was adjusted to 4 mm with the help of a microscope camera.

A Thermo Fisher Scientific (San Jose, California) LTQ-Orbitrap XL was coupled online to the CE system. The MS analysis was under negative-ion mode. The capillary voltage was −43 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 and bioinformatics

The data analysis was performed on Thermo Xcalibur software. Automatic intact chain processing was done as published before [8, 26] and oligosaccharides up to dodecasaccharides are focused on.

Results and discussion

CE–MS interface

A reverse polarity separation of heparin oligosaccharides used an uncoated capillary with 10 mM aqueous ammonium acetate buffer containing 80 % methanol (Fig. 1). The presence of 80 % methanol in the separation capillary reduces solute–wall

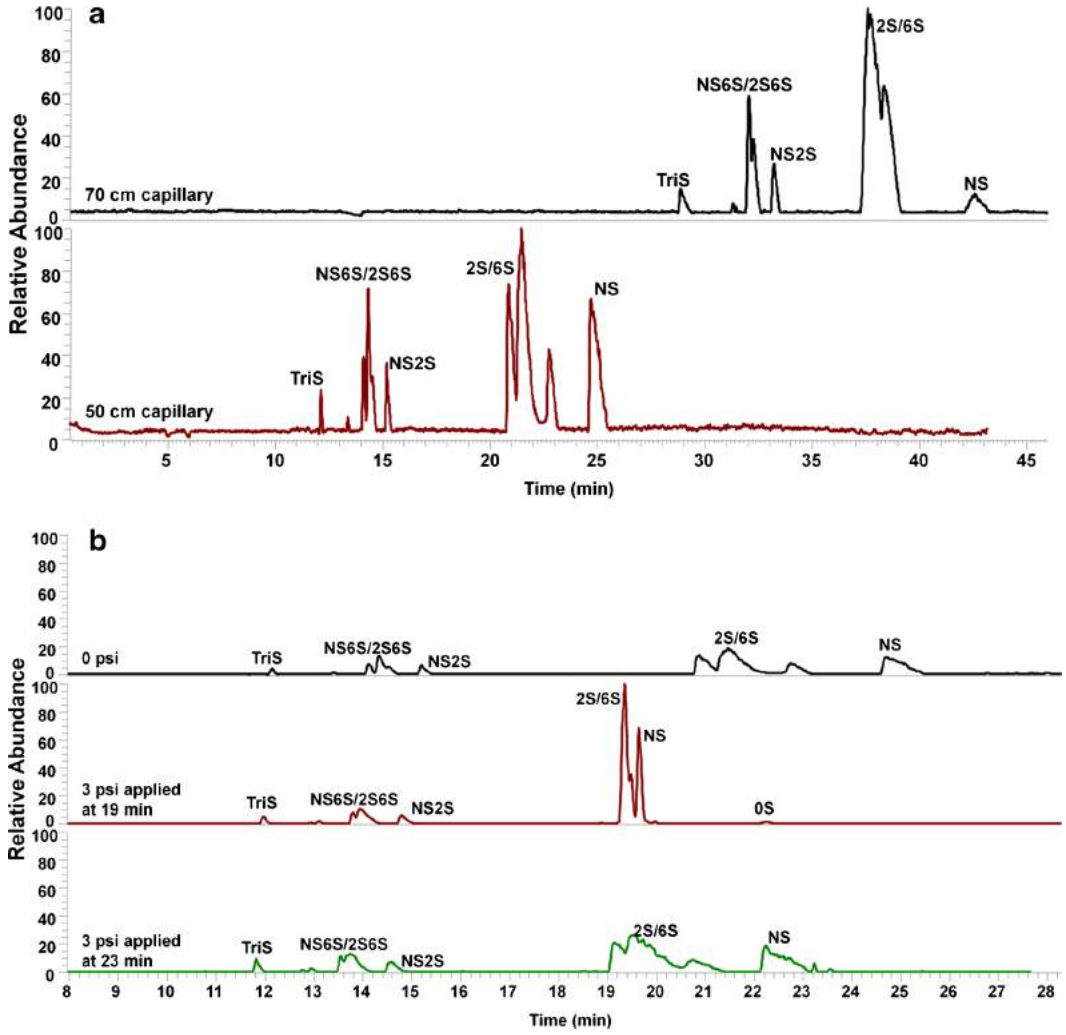


Fig. 2 Optimization of (**a**) capillary length and (**b**) pressure on CE–MS analysis of 8 disaccharide standards. Background electrolyte and sheath liquid, 10 mM ammonium acetate (AA) in 80 % aqueous methanol; separation voltage, −30 kV; electrospray voltage, −1.7 kV

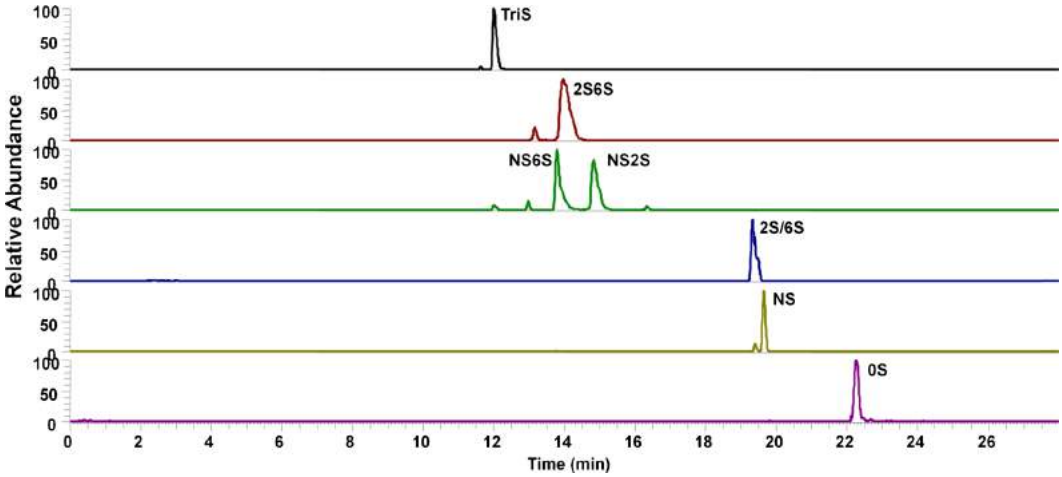
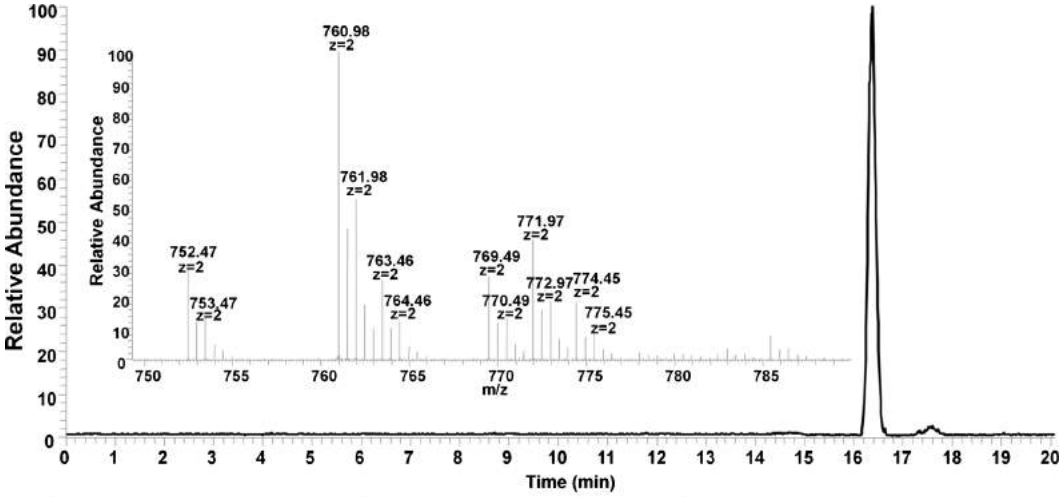


Fig. 3 Extracted ion electropherograms (XIE) of 8 disaccharide standards under optimal experimental conditions. Background electrolyte and sheath liquid, 10 mM ammonium acetate (AA) in 80 % aqueous methanol; separation voltage, −30 kV; electrospray voltage, −1.7 kV; separation pressure (3 psi) was applied at 19 min

interaction. In 10 mM ammonium acetate buffer containing 80 % methanol, the bare fused silica wall is negatively charged with a diffuse layer of cations adjacent to the capillary wall silanol anions. When a reverse polarity electric field is applied, the cations in the diffuse layer migrate towards cathode. When these cations are hydrated, this migration drags solvent with them. When these cations are surrounded by mostly methanol, it is hard to drag solvent with the migration. This phenomenon is mainly due to the viscosity of methanol as well as the weakened interaction between the anions on the surface silanol and the cations in the diffuse layer. The length of the capillary (Fig. 2a) and applied pressure (Fig. 2b) were



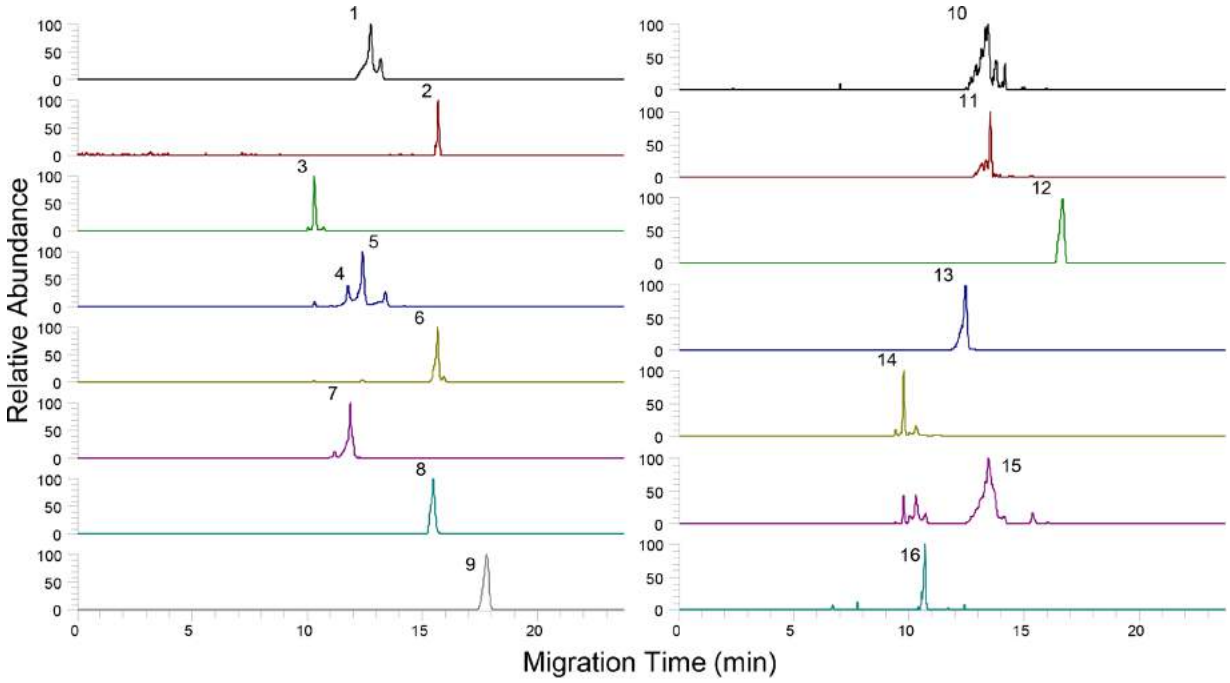
Major ion observed (m/z)	Interpretation
752.47	$[M-2H]^{2-}$
760.98	$[M-3H+NH_4]^{2-}$
763.46	$[M-3H+Na]^{2-}$
769.49	$[M-4H+2NH_4]^{2-}$
771.97	$[M-4H+Na+NH_4]^{2-}$
774.45	$[M-4H+2Na]^{2-}$

Fig. 4 Negative-mode CE–MS analysis of Arixtra®. The *inset* shows the major ions observed in the peak at 16.4 min. The interpretations are listed in the *table* below

investigated by using a mixture of unsulfated, monosulfated, disulfated, and trisulfated (TriS) heparin/heparan sulfate disaccharides to provide the optimal disaccharide separation. On the basis of the results, the optimum capillary length and pressure were 50 cm and 3 psi applied at 19 min, respectively. Under reversed polarity, the dominant electrophoretic force (EF) with a low electroosmotic flow (EOF) moves analyte down the bare separation capillary with the most highly negatively charged analyte, TriS, moving fastest. The end of the separation capillary is capped with a protein-coated spray emitter sheath capillary with sheath liquid pumped by EOF, mixing with separation flow and affording a stable electrospray (see Electronic Supplementary Material (ESM) Fig. S2) of negatively charged analyte that is introduced into the LTQ Orbitrap for MS analysis (ESM Fig. S1).

CE–MS of heparin/heparan sulfate disaccharides

Eight heparin/heparan sulfate disaccharides were separated under optimized conditions and analyzed by negative-ion MS. Their extracted ion electropherograms (XIEs) are shown in Fig. 3. The peaks were identified



Compond #	Name	RT (min)	Structure	Theoretical MW_{mono}	Major m/z observed	Charges state	Error (ppm)
1	Δdp3(3OS,1NS)	12.73	ΔUA2S-GlcNS6S-IdoA2S	832.9602	415.4710	-2	-3.05
2	Δdp3(1OS)	15.67	ΔUA-GlcNAc-IdoA (1OS)	635.1004	634.0912	-1	-2.16
3	ΔIS	10.28	ΔUA2S-GlcNS6S	576.9713	575.9604	-1	-5.32
4	ΔIIS	11.76	ΔUA-GlcNS6S	497.0145	496.0043	-1	-4.77
5	ΔIIIS	12.39	ΔUA2S-GlcNS	497.0145	496.0044	-1	-4.57
6	ΔIVS	15.64	ΔUA-GlcNS	417.0577	416.0488	-1	-2.57
7	ΔIA	11.86	ΔUA2S-GlcNAc6S	539.0251	538.0144	-1	-5.32
8	ΔIIA	15.45	ΔUA-GlcNAc6S	459.0683	458.0587	-1	-3.86
	ΔIIIA		ΔUA2S-GlcNAc				
9	ΔIVA	17.77	ΔUA-GlcNAc	379.1115	378.1024	-1	-3.35
10	ΔIIIS-IIS_{glu}	13.43	ΔUA2S-GlcNS-GlcA-GlcNS3S	1073.9859	535.9827	-2	-4.51
11	ΔIIA-IIS_{glu}	13.54	ΔUA-GlcNAc6S-GlcA-GlcNS3S6S	1036.0396	517.0098	-2	-4.19
12	Linkage region	16.66	ΔUA-Gal-Gal-Xyl-O-Ser_{ox}	690.1855	689.1741	-1	-5.17
13	1,6-anhydro ΔIS-IS^{epi}	12.44	ΔUA2S-GlcNS6S-IdoA2S-ManNS-1,6-anhydro	1055.9753	526.9775	-2	-4.39
14	1,6-anhydro ΔIS	9.76	ΔUA2S-GlcNS-1,6-anhydro	479.0040	477.9940	-1	-4.53
15	1,6-anhydro ΔIIS	13.44	ΔUA-GlcNS-1,6-anhydro	399.0471	398.0378	-1	-3.68
16	dp2(2OS, 1NS) NRE	10.68	IdoA2S-GlcNS6S	594.9819	593.9720	-1	-3.48

Fig. 5 Extracted ion chromatography of building blocks of LMWH detected in CE–MS. (Compositions are shown in the *table*)

using standards. The observed charge states and mass/charge (m/z) ratios of these eight disaccharides are provided in the table in Fig. 5. CE–MS shows sufficient resolution of the eight disaccharide components for disaccharide compositional analysis of heparins and LMW heparins. The results suggest that online analysis of heparins can be achieved through the negative-mode CE–MS platform.

CE–MS of Arixtra®

Arixtra® is an ultralow molecular weight (ULMW) heparin, a chemically synthesized octasulfated pentasaccharide (ESM Fig. S3), which is used clinically as a specific anti-Factor Xa agent [27, 28]. Arixtra® poses a specific challenge for MS analysis as it is highly charged and prone, under many MS conditions, to lose sulfo groups (SO_3^-). Under specific MS

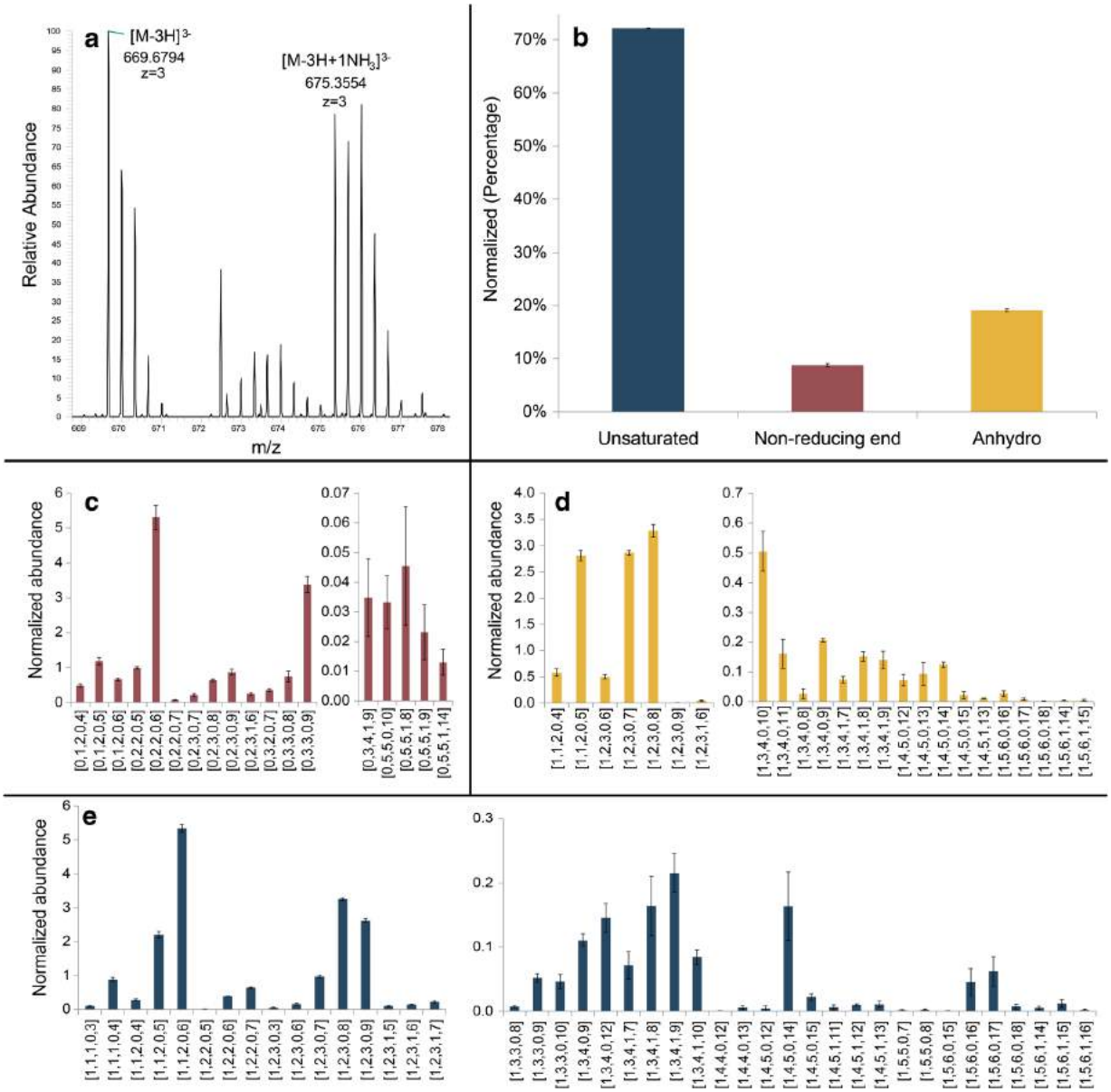


Fig. 6 Intact chain characterization of LMWH (Lovenox®). Oligosaccharide compositions are given as [ΔHexA, HexA, GlcN, Ac, SO_3]. **a** NH_3 adduction in CE–MS (1,6-anhydro dp8, 1Ac, 8SO_3. $M = 2012.06$). **b** Relative amount (percentage) of identified 3 major components of LMWH. Compositional characterization of oligosaccharides with **c** saturated non-reducing end, **d** 1,6-anhydro reducing end, **e** unsaturated non-reducing end

conditions, parent ions can be generated at an optimal charge state to allow the application of tandem MS using collision-induced dissociation (CID) with a low loss in sulfo groups and an enhanced number of sequence-informative fragments [29, 30]. Thus, we selected Arixtra® as a CE–MS analyte to observe the quality of MS analysis and the distribution of charge states. The XIE and the MS of Arixtra® and the distribution of charge states under CE–MS analysis are presented in Fig. 4. The intact molecular ion is observed as a doubly charged ion, along with ammonium and sodium exchange with protons. On the basis of the quality of these data, future work will investigate the application of CID tandem MS analysis for sequencing oligosaccharides eluting from the capillary.

CE–MS of LMWH building blocks

Figure 5 shows extracted ion electrophoresis of building blocks of LMW heparin, Lovenox®, produced by treatment with heparin lyases I, II, and III, separated by CE, and determined using bottom-up analysis with MS detection. This analysis was highly reproducible (ESM Fig. S6) and the composition is shown in the combined table of Fig. 5. CE–MS could be completed within 20 min and 17 building blocks including modified structures were determined. All components except ΔIIA and ΔIIIA could be separated in extracted ion electropherograms. A signature building block of all enoxaparins, including Lovenox®, is oligosaccharides containing process artifacts, 1,6-anhydro oligosaccharides (ESM Fig. S4), which are observed as disaccharide and tetrasaccharides [10]. In addition to normally encountered building blocks, a linkage region tetrasaccharide was also identified [9]. Moreover, tetrasaccharides containing a 3-*O*-sulfo group that are remnants of the antithrombin pentasaccharide binding site, such as the one present within the structure of Arixtra®, can also be determined by CE–MS. Thus, CE–MS can be used for the rapid bottom-up analysis of LMW heparins.

CE–MS of LMWH intact chains

Finally, CE–MS was applied for the reproducible (ESM Fig. S7) top-down analysis of the LMW heparin Lovenox® (Fig. 6). Eighty-four oligosaccharides, ranging from disaccharides (degree of polymerization (dp) 2) to dodecasaccharides (dp 12) could be detected. These experiments were performed in triplicate to determine the standard deviation of the relative quantity of each component chain measured. Ions with ammonium adduction are also observed and are shown in Fig. 6a. CE–MS analysis could be completed in less than 30 min with a lower level of ammonium adduction than observed using hydrophilic interaction liquid chromatography (HILIC)–LC–MS [9], which results in less false positives, thereby simplifying the data processing.

Conclusions

The results obtained demonstrate the utility of CE with reverse polarity separation and a coated sheath capillary interface to promote the electrokinetic pumping of negatively charged heparin oligosaccharides for negative-ion MS analysis on an LTQ Orbitrap. This CE–MS method was useful for disaccharide compositional analysis, bottom-up analysis, and top-down analysis. The results obtained were comparable to or better than those obtained when these analyses were performed using LC–MS [4, 8, 9]. Moreover, when compared to our previously published, normal polarity CE hyphenated positive-ion MS, the use of reverse polarity CE hyphenated negative-ion MS should facilitate its application for CID tandem MS to determine the sequence of chains within LMW heparins or their lyase-treated products.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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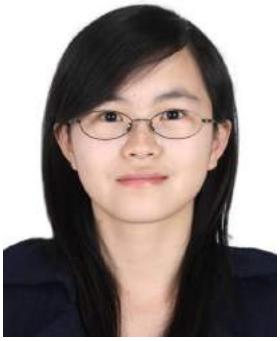

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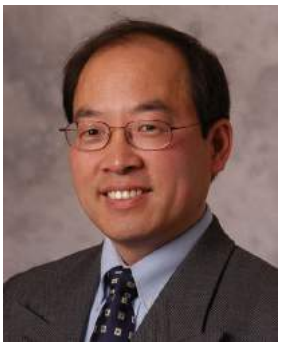

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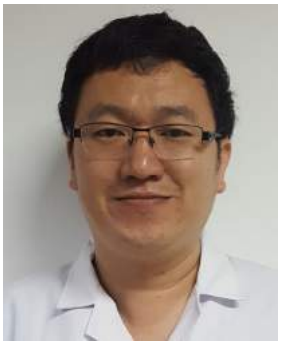

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