Ultra-performance ion-pairing liquid chromatography with on-line electrospray ion trap mass spectrometry for heparin disaccharide analysis

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ABSTRACT

A high-resolution method for the separation and analysis of disaccharides prepared from heparin and heparan sulfate (HS) using heparin lyases is described. Ultra-performance liquid chromatography in a reverse-phase ion-pairing mode efficiently separates eight heparin/HS disaccharides. The disaccharides can then be detected and quantified using electrospray ionization mass spectrometry. This method is particularly useful in the analysis of small amounts of biological samples, including cells, tissues, and biological fluids, because it provides high sensitivity without being subject to interference from proteins, peptides, and other sample impurities.

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Heparin and heparan sulfate (HS)[1] are structurally related members of the glycosaminoglycan (GAG) family and are widely distributed on cell surfaces, inside cells, and within the extracellular matrix of animal tissues. They play many critical roles in physiological and pathophysiological processes, including cell adhesion, proliferation, motility, and differentiation, viral and bacterial infection, cancer, and inflammation, through their interaction with chemokines, cytokines, and growth factor receptors [1–4]. As pharmaceutical agents, heparin and low-molecular-weight heparin (LMWH) are currently in widespread clinical use as anticoagulants that act by binding and activating the serine protease inhibitor antithrombin [5,6]. There is growing interest in characterizing the fine structure of pharmaceutical heparin [7–10] and in elucidating the interaction of HS with a wide array of proteins, ligands, receptors, and pathogens [11].

Heparin and HS are highly sulfated, acidic linear polysaccharides having closely related structures and consist of alternating 1,4-linked hexuronic acid and D-glucosamine residues with molecular weights ranging from 5 to 70 kDa. The hexuronic acid can be either D-glucuronic acid (GlcA) or L-iduronic acid (IdoA), both of which can be modified with 2-O-sulfo groups. The glucosamine residue can be modified with N-acetyl (GlcNAc) or N-sulfo (GlcNS) groups and can be substituted with 3- and/or 6-O-sulfo groups. HS has a more highly variable structure than heparin, with less sulfo group substitution, and is rich in GlcA and GlcNAc residues [5]. The structural complexity of heparin/HS is attributed to the variable action of sulfotransferases and C5-epimerase in their biosynthesis [12]. In addition, heparin/HS sequence microheterogeneity also depends on species, individual organism, organ, tissue, cell type, environmental conditions, and developmental stage [13]. The structural characterization of the heparin/HS polysaccharides poses significant challenges for analytical chemists due to their high negative charge, polydispersity, and sequence

heterogeneity. Because of the difficulty of analyzing intact heparin/HS, partial depolymerization by either enzymatic or chemical means is often used to obtain disaccharide units or oligosaccharides for detailed structural analysis. During the enzymatic depolymerization process using a heparin lyase, a double bond is introduced as a 4,5-unsaturated uronic acid residue (ΔUA) at the nonreducing end that absorbs at 232 nm [14,15]. Exhaustive heparin lyase digestion and chemical modification has been used to prepare 12 commercially available heparin/HS-derived disaccharides. Eight of these disaccharides can be obtained from the natural polysaccharides (Table 1). Currently, there are no heparin lyases capable of preparing 3-O-sulfo group-containing disaccharides [16]. Several modern techniques, such as high-performance liquid chromatography (HPLC) [17,18], gel permeation chromatography (GPC) [19–21], and capillary electrophoresis (CE) [22,23], have been applied in the analysis of these eight heparin/HS-derived disaccharides as well as other heparin/HS-derived oligosaccharides. Although nuclear magnetic resonance (NMR) spectroscopy is an effective tool for the structural elucidation of heparin, providing monosaccharide composition, linkage position, and sulfation patterns, it requires 10 to 100 µg of sample [10,24,25]. Soft ionization mass spectrometry (MS) and liquid chromatography–mass spectrometry (LC–MS) have become increasingly important for fast screening and detailed sequence analysis of small quantities (sub-µg) of heparin/HS-derived disaccharides and oligosaccharides [26,27]. In particular, reverse-phase ion-pairing (RPIP)–HPLC, using lipophilic ion-pairing reagents, represents a promising method for heparin/HS disaccharide and oligosaccharide analysis. Several studies have also demonstrated the amenability of RPIP–HPLC to on-line MS detection by using volatile ion-pairing reagents, greatly improving the sensitivity and amount of structural information that can be acquired [28–31]. The impact of the concentration of different volatile ion-pairing reagents, such as triethylamine, dibutylamine, tributylamine (TrBA), tripentylamine, tetrapropylamine, and tetrabutylamine, on ionization efficiency has been systematically studied in the analysis of heparosan oligosaccharide (ΔUA[GlcNAc–GlcA]$_n$GlcNAc, n = 2–19) [30] and heparin oligosaccharide (ΔUA2S[GlcNS6S–IdoA2S]$_n$GlcNS6S, n = 0–13) analysis. RPIP–HPLC–MS analysis has also been applied to heparin/HS disaccharide compositional analysis, including the use of RPIP–microflow (Mf)–HPLC with on-line electrospray ionization (ESI)–MS, relying on isotopically enriched disaccharide internal standards [29].

Table 1
Structures of eight Δ-disaccharide standards from heparin/HS.
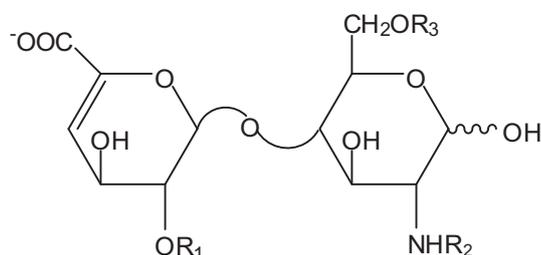
	Heparin/HS disaccharide	R$_1$	R$_2$	R$_3$	Theoretical molecular mass
0S	ΔUA–GlcNAc	H	Ac	H	379.3
NS	ΔUA–GlcNS	H	SO$_3^-$	H	417.3
6S	ΔUA–GlcNAc6S	H	Ac	SO$_3^-$	459.4
2S	ΔUA2S–GlcNAc	SO$_3^-$	Ac	H	459.4
NS6S	ΔUA–GlcNS6S	H	SO$_3^-$	SO$_3^-$	497.4
2SNS	ΔUA2S–GlcNS	SO$_3^-$	SO$_3^-$	H	497.4
2S6S	ΔUA2S–GlcNAc6S	SO$_3^-$	Ac	SO$_3^-$	539.4
TriS	ΔUA2S–GlcNS6S	SO$_3^-$	SO$_3^-$	SO$_3^-$	577.5

Ultra-performance liquid chromatography (UPLC) separations are performed at high pressures (up to 10^8 Pa) using columns packed with 1.7-µm sorbent particles. This new category of analytical separation science retains the practicality and principles of HPLC while yielding major improvements in chromatographic performance. Compared with traditional HPLC analysis, UPLC takes advantage of technological strides made in resolution, peak capacity, sensitivity, efficiency, and speed of analysis [32,33]. Ion-pairing reagents play a complex role of analyte competition in the resolution of heparin/HS disaccharides related to their steric bulk and hydrophobicity [34]. Carefully controlling the TrBA ion-pairing reagent has facilitated the compositional profiling and quantification of heparin/HS by RPIP–UPLC–MS. Rapid, robust, and simple RPIP–UPLC–MS methods have been established for heparin oligosaccharide (from degree of polymerization 6 [dp6] to dp22) analysis using pentylamine as ion-pairing reagent and a unique additive 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), resulting in highly efficient separation with enhanced MS detection [9]. RPIP–UPLC–MS has also been applied to the analysis of unfractionated heparin/HS from various bovine and porcine tissues and pharmaceutical heparins in as little time as 5 min [8].

Although several RPIP–LC–MS techniques have been successfully used in heparin/HS analysis, there are some challenging analytical problems remaining for real biological samples, including shifting retention times, interference with ultraviolet (UV) detection, and the broadening of peaks, due to the deleterious effects from the proteins, salts, and other impurities contained in these samples. In this work, we have developed a rapid, efficient, and repeatable RPIP–UPLC–MS method for heparin/HS disaccharide compositional analysis. This unique method has applicability for analysis of very small amounts of heparin/HS derived from cells, tissues, and urine sample as well as for structural profiling of pharmaceutical heparins.

Materials and methods

Materials

Unsaturated heparin/HS disaccharide standards (0S: ΔUA–GlcNAc; NS: ΔUA–GlcNS; 6S: ΔUA–GlcNAc6S; 2S: ΔUA2S–GlcNAc; NS2S: ΔUA2S–GlcNS; NS6S: ΔUA–GlcNS6S; 2S6S: ΔUA2S–GlcNAc6S; TriS: ΔUA2S–GlcNS6S) were obtained from Iduron (Manchester, UK). Actinase E was obtained from Kaken Biochemicals (Tokyo, Japan). Urea, Chaps, and TrBA were purchased from Sigma Chemical (St. Louis, MO, USA). Vivapure Mini Q H spin columns were purchased from Viva Science (Edgewood, NJ, USA). Syringe filters (0.22 µm) and Microcon Centrifugal Filter Units YM-10 were obtained from Millipore (Bedford, MA, USA). Cloning, *Escherichia coli* expression, and purification of the recombinant heparin lyase I (EC 4.2.2.7), heparin lyase II (no EC assigned), and heparin lyase III (EC 4.2.2.8) from *Flavobacterium heparinum* were performed in our laboratory as described previously [35–37]. Pharmaceutical heparin and LMWH samples were obtained from a variety of commercial suppliers. Chinese hamster ovary (CHO)-S cells were grown in suspension culture on CD–CHO medium supplemented with 2% HT (hypoxanthine/thymidine mixture, Gibco–Invitrogen, Carlsbad, CA, USA) and 8 mM glutamine [38]. Arabian camel intestinal tissue and camel urine were obtained from a slaughterhouse in Egypt. All other chemicals were of HPLC grade.

Isolation and purification of GAGs from cells, tissue, and urine

Isolation and purification of GAGs from biological sample was described previously [39,40] and used with some modification. De-fatting involved lyophilized cells and media ($\sim$10^6 cells) and lyophilized minced camel intestinal tissue (0.5 mg) with 3 ml of

2:1, 1:1, and 1:2 (v/v) chloroform/methanol. De-fatted samples were individually subjected to proteolysis at 55 °C for 20 h with 10% actinase E (20 mg/ml). After proteolysis, particulates were removed from the resulting solutions by passing each through a syringe filter containing a 0.22-μm membrane. Samples were then passed through Microcon Centrifugal Filter Units YM-10 (10 kDa, a molecular weight cutoff [MWCO]) by centrifugation at 12,000*g* and washing with 15 ml of distilled water to remove peptides. The retentate was collected and lyophilized. Samples were dissolved in 0.5 ml of 8 M urea containing 2% Chaps (pH 8.3). A Vivapure Mini Q H spin column was prepared by equilibrating with 200 μl of 8 M urea containing 2% Chaps (pH 8.3). The clarified filtered samples were loaded onto and run through the Vivapure Mini Q H spin columns under centrifugal force (700*g*). The columns were first washed with 200 μl of 8 M urea containing 2% Chaps at pH 8.3. The columns were then washed five times with 200 μl of 200 mM NaCl. GAGs were released from the spin column by washing three times with 50 μl of 16% NaCl. GAGs were desalted with a YM-10 spin column. The GAGs were lyophilized and stored at room temperature for future use.

The camel urine sample (5 ml) was filtered through a 0.22-μm filter to remove particulates and then dialyzed for 4 days against 4 L of double-distilled water using 1000-MWCO membranes. After dialysis, the urine sample was concentrated and lyophilized for future use.

Enzymatic digestion

LMWH samples were weighed, and the amounts of GAGs recovered from biological samples were determined by microcarbazole assay [29] and then used to prepare a stock solution from which 5 μg of analyte could be taken. Heparin lyases I, II, and III (10 mU each, assayed prior to use) in 5 μl of 25 mM Tris, 500 mM NaCl, and 300 mM imidazole buffer (pH 7.4) were added to 5 μg of GAG sample in 25 μl of distilled water and incubated at 37 °C for 10 h to completely degrade the GAG sample. The products were recovered by centrifugal filtration using a YM-10 microconcentrator, and the heparin/HS disaccharides were recovered in the flow-through and freeze-dried. The digested GAG disaccharides were redissolved in water to a concentration of 50 to 100 ng/2 μl for LC–MS analysis.

RPIP–UPLC–MS

LC–MS analyses were performed on an Agilent 1200 LC/MSD instrument (Agilent Technologies, Wilmington, DE, USA) equipped with a 6300 ion trap and a binary pump followed by a UV detector equipped with a high-pressure cell. The column used was an Acquity UPLC BEH C18 column (2.1 × 150 mm, 1.7 μm, Waters, Milford, MA, USA). Eluent A was water/acetonitrile (85:15, v/v), and eluent B was water/acetonitrile (35:65, v/v). Both eluents contained 12 mM TrBA and 38 mM NH_4OAc with pH adjusted to 6.5 with HOAc. A gradient of solution A for 10 min followed by a linear gradient from 10 to 40 min (0–50% solution B) was used at a flow rate of 100 μl/min for disaccharide analysis. The column effluent entered the source of the ESI–MS for continuous detection by MS. The electrospray interface was set in negative ionization mode with a skimmer potential of −40.0 V, a capillary exit of −40.0 V, and a source temperature of 350 °C to obtain the maximum abundance of the ions in a full-scan spectrum (200–1500 Da). Nitrogen (8 L/min, 40 psi) was used as a drying and nebulizing gas.

Calibration

Quantification analysis of heparin/HS disaccharides was performed using calibration curves constructed by separation of increasing amounts of unsaturated heparin/HS disaccharide standards (2, 5, 10, 15, 20, 30, 50, and 100 ng per disaccharide). Linearity was assessed based on the amount of disaccharide and peak intensity in MS. All analyses were performed in triplicate.

Results and discussion

The structural complexity of heparin/HS is related to sequence heterogeneity, molecular weight properties, charge density, and chain flexibility. Heparin and HS consist of various disaccharide units, and their chain length variability results from either biosynthetic variability or differences in the level of endoglucuronidase (heparanase) processing in different tissues and different species. The quantitative disaccharide composition of heparin/HS is believed to have a direct relationship with important biological functions [13]. Moreover, heparin and LMWH, currently used as pharmaceutical drugs to control blood coagulation, were contaminated in 2008, leading to a health crisis receiving considerable attention. Therefore, rapid, sensitive, and reliable methods for quantitative analysis of heparin/HS structural composition are needed.

RPIP–UPLC–MS method

RPIP–HPLC, an increasingly popular method for the analysis of heparin/HS disaccharides and oligosaccharides, relies on lipophilic ion-pairing reagents that play a critical role as mobile-phase modifiers, aiding in the retention and resolution of charged species on hydrophobic stationary phases [41,42]. In RPIP–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. The application of volatile ion-pairing reagents, such as hexylamine (HXA) and TrBA, allowed the development of on-line separation and structural identification of heparin/HS-derived oligosaccharides and disaccharides by RPIP–HPLC–MS.

In our laboratory, a RPIP–UPLC separation coupled to ESI–MS has been applied successfully for analysis of chondroitin sulfate (CS)/dermatan sulfate (DS) disaccharides by using HXA as an ion-pairing reagent and HFIP as an organic modifier in the mobile phase [43]. Heparin/HS-derived disaccharides ΔUA–GlcNAc6S (6S) and ΔUA2S–GlcNAc (2S) could not be separated by using this solvent system. TrBA is the most commonly used ion-pairing agent in RPIP–HPLC–MS analysis of heparin/HS-derived oligosaccharides and disaccharides. The effect of TrBA concentration on ionization and separation efficiency has been well studied [30]. There is a trade-off in choosing a concentration of the ion-pairing agent that is sufficient to separate highly sulfated disaccharides but low enough so as not to compromise MS sensitivity. Eluent buffer containing 5 mM TrBA and 50 mM ammonium acetate at pH 7.0 was successfully used for compositional profiling and quantification of heparin/HS by RPIP–UPLC–quadrupole time-of-flight (QTOF)–MS [9]. A buffer containing a higher concentration of 15 mM TrBA and 50 mM ammonium acetate was developed to separate heparin/HS-derived oligosaccharides [28]. In our previous work, a quantification method of heparin/HS disaccharide analysis was explored by using an RPIP–Mf–HPLC–MS method and 12 mM TrBA/50 mM ammonium acetate ion-pairing agent. The ionization efficiency and detection sensitivity using TrBA was much lower than with HXA, requiring a postcolumn addition of 5 μl/min acetonitrile to improve TrBA evaporation in the ion source [29].

In the current experiments, we relied on a 1.7-μm particle size Acquity UPLC BEH C18 column and a 12-mM TrBA/50 mM ammonium acetate solvent system for separation of the heparin/HS

disaccharides in Table 1. These eight disaccharides were baseline separated and detected by total ion chromatography (TIC), high-pressure UV spectroscopy (232 nm), and extracted ion chromatography (EIC) (Fig. 1). TIC and EIC detection exhibited higher sensitivity and a flatter baseline than UV_{232nm} detection. EIC detection,

obtained by extracting the exact mass of analytes, is particularly useful for real biological samples that contain residual proteins, peptides, and other UV-active impurities [8,29,40]. However, accurate quantification of analytes by EIC is difficult because of the different efficiencies of ionization of the eight different disaccharides
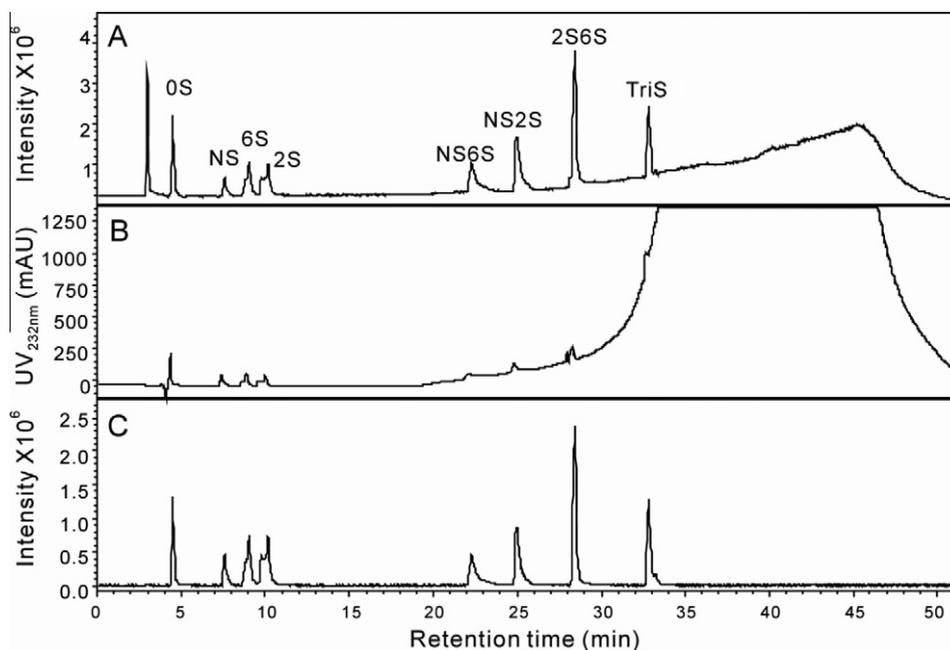

Fig.1. RPIP–UPLC chromatograms of eight heparin/HS-derived disaccharide standards with TIC (A), UV_{232nm} (B), and EIC (C) detection.
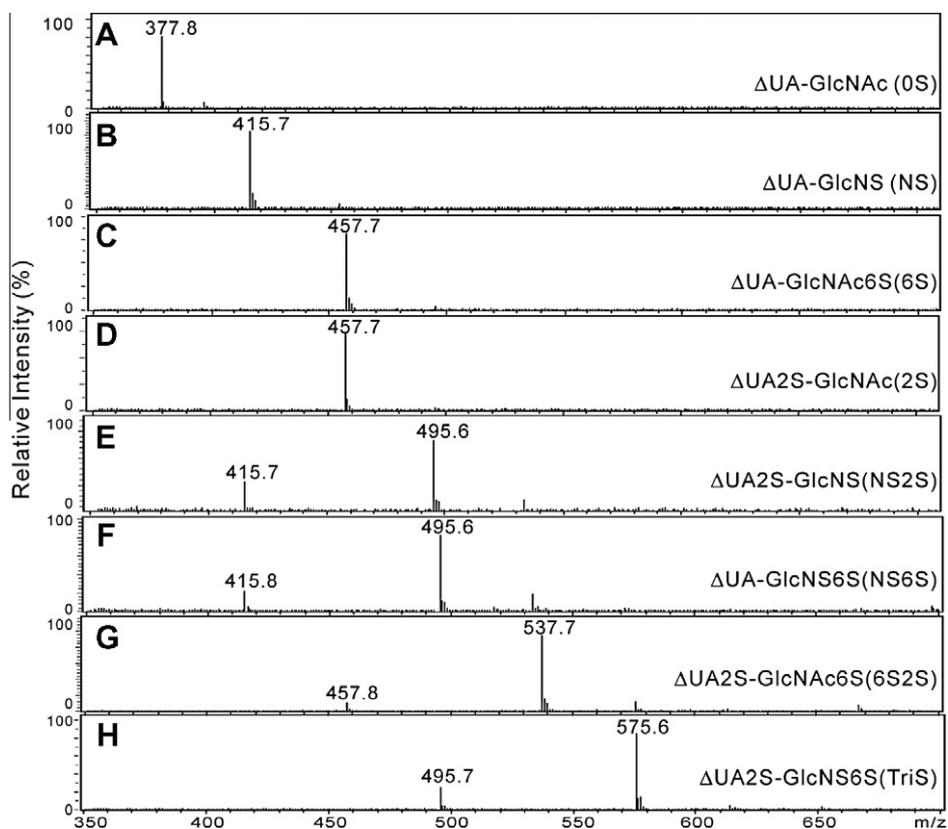

Fig.2. Mass spectra of heparin/HS-derived disaccharide: (A) ΔUA–GlcNAc; (B) ΔUA–GlcNS; (C) ΔUA–GlcNAc6S; (D) ΔUA2S–GlcNAc; (E) ΔUA–GlcNS6S; (F) ΔUA2S–GlcNS; (G) ΔUA2S–GlcNAc6S; and (H) ΔUA2S–GlcNS6S.

in the electrospray ion source. UPLC–MS has been used in pharmaceutical analysis and metabolite studies and has been shown to be a powerful technique, dramatically improving peak resolution, sensitivity, and speed of analysis [9,44]. In addition to UPLC providing excellent separation of heparin/HS disaccharides, ESI–MS detection affords the mass of each disaccharide analyte (Fig. 2). Peaks were observed at *m/z* 377.8 (ΔUA–GlcNAc), 415.7(ΔUA–GlcNS), 457.7 (ΔUA–GlcNAc6S and ΔUA2S–GlcNAc), 495.6 (ΔUA–GlcNS6S and ΔUA2S–GlcNS), 537.7 (ΔUA2S–GlcNAc6S), and 575.6 (ΔUA2S–GlcNS6S). Because sulfo groups are relatively labile, minor peaks corresponding to desulfonation at *m/z* 415.7, 415.8, 457.8, and 495.7 could also be observed in the mass spectra of di- and trisulfated disaccharides. No multiply charged ions were observed even for highly charged disaccharides because the high concentration of TrBA ion-pairing reagent avoids multiple ionizations. RPIP–UPLC–MS can separate and identify the eight natural heparin/HS disaccharides based on both retention times and their masses. The separation observed in EIC showed double peaks, demonstrating that the α- and β-anomeric forms present in ΔUA–GlcNAc6S and ΔUA2S–GlcNAc had been resolved [29]. Interestingly, the sensitivity of detection is similar with and without additional postcolumn acetonitrile addition. This may result from the high pressure of the UPLC column (10^8 Pa) and the high flow rate (100 μl/min), resulting in more efficient solvent spraying and increased efficiency of TrBA and ammonium acetate evaporation.

Quantification analysis of heparin/HS disaccharide

Equal-mass mixtures of eight disaccharide standards (2, 5, 10, 20, 50, and 100 ng per disaccharide) were analyzed by RPIP–UPLC–MS to test the sensitivity and linearity of disaccharide amount and peak intensity using EIC detection. Sample amounts from 2 to 100 ng produced increasing peak intensities and decreasing noise from which integrated peak areas could be accurately calculated. The integrated disaccharide peak areas measured by EIC detection showed excellent linearity when plotted as a function of their amounts (Fig. 3). The different slopes of these curves reflect the different ionization efficiencies for the corresponding disaccharides in an ESI source. In particular, disulfated disaccharide 2S6S showed a greater capacity to ionize, whereas monosulfated disaccharide NS and trisulfated disaccharide TriS were found to have a lower ionization efficiency under these experimental conditions. We also analyzed larger amounts of a mixture of the eight disaccharide standards (200 and 500 ng); EIC detection still showed excellent linearity of peak area as a function of disaccharide amount. Furthermore, the effect of different injection volumes on the quantification was also investigated. Three samples with concentrations of 20 ng/2 μl, 20 ng/5 μl, and 20 ng/8 μl were analyzed at injection volumes of 2, 5, and 8 μl, respectively. Very similar peak areas and identical retention times were obtained,
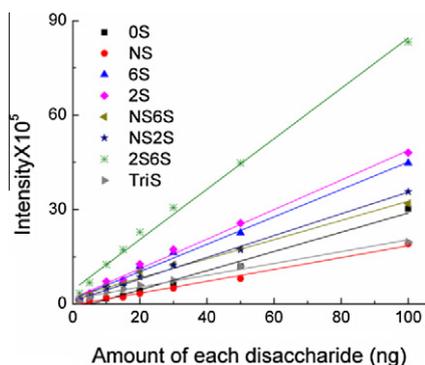
Table 2
Quantification analysis of heparin/HS disaccharide mixtures containing known amounts of disaccharides.

	M1 (ng)		M2 (ng)	
	Known amount	Calculated amount	Known amount	Calculated amount
0S	50	49.6 ± 0.5	10	9.3 ± 0.3
NS	40	38.3 ± 0.8	15	14.2 ± 0.7
6S	35	34.1 ± 1.1	20	21.3 ± 0.5
2S	30	28.7 ± 0.6	25	25.2 ± 0.7
NS6S	25	25.6 ± 0.3	30	29.5 ± 0.5
NS2S	20	19.1 ± 1.2	35	34.6 ± 0.8
2S6S	15	14.3 ± 0.6	40	39.4 ± 0.4
TriS	10	8.5 ± 1.0	50	48.9 ± 1.4

demonstrating that this UPLC method possesses both outstanding repeatability and quantification. Two known disaccharide mixture samples, M1 (50, 40, 35, 30, 25, 20, 15, and 10 ng of 0S, NS, 6S, 2S, NS6S, NS2S, 2S6S, and TriS, respectively) and M2 (10, 15, 20, 25, 30, 35, 40, and 50 ng of 0S, NS, 6S, 2S, NS6S, NS2S, 2S6S, and TriS, respectively) were further analyzed by this method. The results showed that the calculated amounts were consistent with the known amounts (Table 2). Therefore, the combination of UPLC separation and EIC detection is a fast, sensitive, and reliable method for qualitative and quantitative analysis of heparin/HS disaccharide composition. It is particularly useful for real biological samples that contain proteins and other UV-active impurities.

Analysis of pharmaceutical heparin and LMWH samples

Pharmaceutical heparin is primarily prepared from porcine intestines, and LMWH is prepared from the controlled chemical or enzymatic depolymerization of pharmaceutical heparin [7,10]. Three pharmaceutical heparins and one LMWH were analyzed by RPIP–UPLC–MS. The EICs of disaccharide analysis of these samples are presented in Fig. 4. Eight common heparin/HS disaccharides were detected in all samples, their quantity was calculated by the linear equation shown in Fig. 3, and their disaccharide compositions are given in Table 3. Analyses show, as expected [8–10], that the major disaccharide in heparin and LMWH is TriS, but its content ranges from 67.3% to 84.9%, suggesting that manufacturing processes and/or source material have an impact on disaccharide composition.

Quantitative analysis of heparin/HS disaccharide composition from biological sources

The biological activities of GAGs are intimately related to their structural diversity and ability to interact with many cell surface and extracellular proteins, thereby modifying their behavior.

0S	$y = 0.3504x - 1.6531, R^2 = 0.986$
NS	$y = 0.1886x - 0.3593, R^2 = 0.992$
6S	$y = 0.4335x + 1.5366, R^2 = 0.994$
2S	$y = 0.4695x - 1.8045, R^2 = 0.995$
NS6S	$y = 0.3051x + 2.1267, R^2 = 0.987$
NS2S	$y = 0.3459x + 0.9620, R^2 = 0.996$
2S6S	$y = 0.8002x + 4.4734, R^2 = 0.995$
TriS	$y = 0.1881x + 1.5742, R^2 = 0.986$

Fig.3. Curves and linear equations of intensity as a function of amount of each disaccharide.
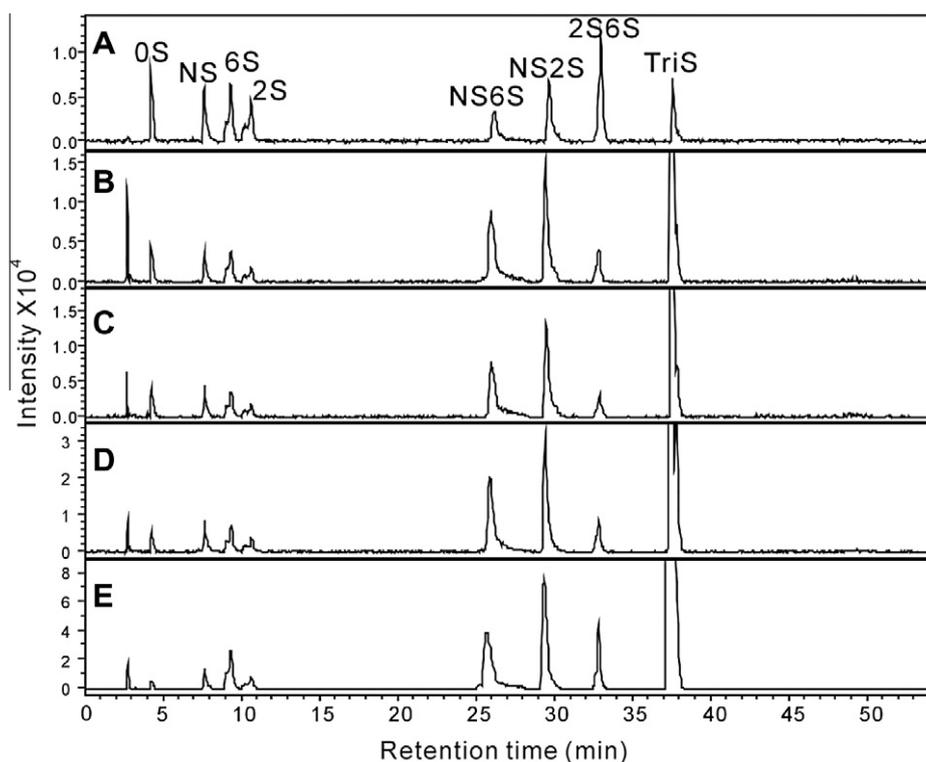
Fig.4. EIC of disaccharide analysis of four commercial heparin samples. (A) disaccharide standards; (B) heparin 1; (C) heparin 2; (D) heparin 3; and (E) LMWH heparin.

Table 3
Heparin/HS disaccharide composition analysis of GAG samples from biological sources.

Sample source	Heparin/HS disaccharides composition (%)							
	0S	NS	6S	2S	NS6S	NS2S	6S2S	TriS
CHO-S cells	12.6	21.5	54.3	2.2	–	3.6	2.4	3.4
Camel intestine	13.9	19.3	19.0	1.1	9.2	19.8	1.5	16.3
Camel urine	19.1	9.4	64.2	–	1.1	2.0	2.3	1.9
Heparin 1	2.3	2.5	3.1	1.7	12.5	8.9	1.7	67.3
Heparin 2	2.2	2.6	2.8	1.9	10.9	8.4	1.4	69.8
Heparin 3	0.8	1.3	1.4	1.0	6.8	5.3	0.9	82.5
LMWH	0.3	0.4	1.6	0.7	4.9	5.1	2.1	84.9

Quantitative disaccharide compositional analysis is one of the most important ways to characterize the structures of heparin/HS and has a direct relationship with their important biological functions, including viral and bacterial infection and entry, angiogenesis, inflammation, cancer, and development [45]. With the increasing interest in structural characterization of GAGs from novel species and, in comparison, GAGs from different tissues and cells, several analytical techniques have been developed for compositional profiling of GAGs from biological sources. Our laboratory previously demonstrated the quantitative recovery of GAGs from cells, tissues, and biological fluids by de-fatting, proteolysis, urea/Chaps extraction, strong anion exchange (SAX) spin column, and desalting steps [39,40]. However, many biological GAG samples are much less abundant, making detection and quantitative compositional analysis problematic. Several techniques relying on fluorescent derivatization of disaccharides have been used to increase SAX column or reversed-phase HPLC peak resolution and enhance detection sensitivity using fluorescence [46,47]. Nevertheless, because the actual identification of the disaccharides depends on determining the retention time relative to standards, spurious contaminant peaks can confuse interpretation of chromatograms. Therefore, the development of a RPIP–HPLC–MS method greatly increases confidence in peak assignment, resulting in improved qualitative disaccharide compositional analysis because peak identification relies on both chromatographic retention time and mass of each disaccharide [48]. In addition, technologically advanced UPLC columns afford greater analytical speed, peak resolution, and repeatability [8,9].

CHO cells are widely used in the biotechnology/biopharmaceutical industry for the production of recombinant therapeutic proteins. They are accepted by the US Food and Drug Administration (FDA) as a host for the production of human therapeutics, and protocols exist for removal of host cell proteins, nucleic acids, and viral contaminants. CHO cells are known to biosynthesize HS but not heparin [49,50]. Current research in our laboratory is aimed at metabolic engineering of the HS biosynthetic pathway to produce secreted CHO cell heparin. To these ends, we undertook to test our new analytical method on GAGs extracted and purified from CHO-S cells and conditioned media that were then digested with heparin lyases I, II, and III. The heparin/HS disaccharide composition of the CHO-S cell sample analyzed by this RPIP–UPLC–MS is presented in Fig. 5B. The 6S (54.3%) was the major disaccharide, followed by the NS (21.5%) and the 0S (12.6%). Minor amounts of 2S, NS2S, 6S2S, and TriS were also detected.

Restrictions on the use of porcine heparin in the Middle East have led us to examine heparin/HS from other mammalian species as a source of pharmaceutical heparin. Camels, a unique genus native to Asia that is also used as a food source, were previously shown to contain a highly sulfated HS or heparin in their lung tissue [51]. On this basis, we decided to examine the HS or heparin content of camel intestinal tissue and urine. After extraction and purification of heparin/HS from both tissue and urine, followed by digestion with heparin lyases I, II, and III, disaccharide analysis was performed (Fig. 5C and D), affording disaccharide compositions presented in Table 3. The results showed that eight heparin/HS disaccharides were detected in GAGs from the camel intestine sample. The major heparin/HS disaccharides detected in
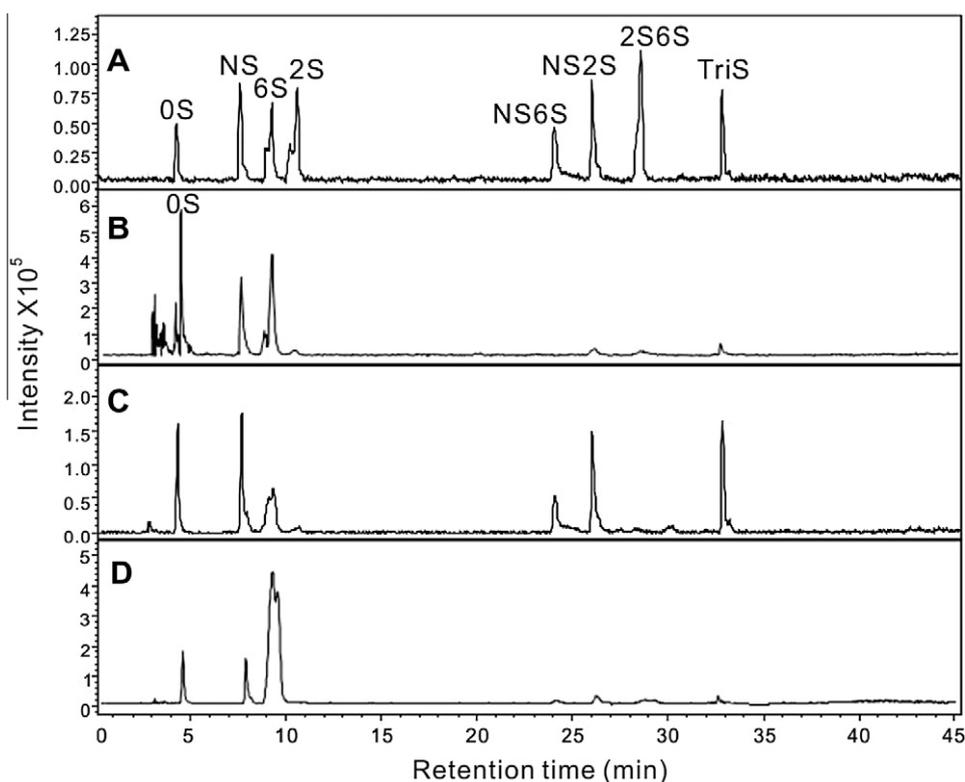
Fig.5. EIC of disaccharide analysis of GAGs from different biological sources: (A) disaccharide standards; (B) CHO cell; (C) camel intestine; and (D) camel urine.

GAGs from camel intestine were nearly equally divided among the NS2S, NS, and 6S, but substantial amounts of TriS, 0S, and NS6S were also present, with minor amounts of 2S and 6S2S unambiguously detected by high-sensitivity RPIP–UPLC–MS. This analysis is consistent with the presence of highly sulfated HS or heparin, potentially having anticoagulant activity. Camel urine contained all of the disaccharides with the exception of 2S; the 6S (64.2%) was the major component, but considerable quantities of 0S (19.1%) and NS (9.4%) were also observed. Thus, camel urine contains primarily HS.

Several RPIP–LC–MS techniques have been successfully used in heparin/HS analysis, but there are still challenging analytical issues remaining for real biological samples, including shifting retention times, interference with UV detection, and the broadening of peaks, due to the deleterious effects from the proteins, salts, and other impurities contained in these samples. Our research shows that although traditional 5-μm C18 columns can cleanly resolve eight heparin/HS disaccharide standards (see Fig. S1 in Supplementary Material), in the analysis of biological samples, shifting retention times and peak broadening result from interference from proteins (e.g., heparinases). Thus, the ΔUA–GlcNAc6S (6S) and ΔUA2S–GlcNAc (2S) disaccharides could not be completely resolved, leading to difficulties in quantifying these disaccharides on conventional 5-μm columns (Fig. S2). The retention times of GAGs from all of the various biological sources examined were completely consistent with those of the disaccharide standards by UPLC column analysis without being subject to interference from proteins, peptides, and other impurities common to cells, tissues, and biological fluids. Moreover, various injection volumes provided similar analyses (data not shown). RPIP–UPLC–MS also provides higher sensitivity than traditional 5-μm C18 columns. The small amount of disaccharides (NS2S, NS6S, and TriS) present in the compositional analysis of the CHO cell and camel urine samples were not detected (Fig. S3) when using the 5-μm C18 column.

Furthermore, the 1.7-μm column requires a shorter equilibration time than the 5-μm column. Therefore, compared with the conventional 5-μm reverse-phase HPLC column, the UPLC 1.7-μm column has significant technological advantages, providing a rapid, sensitive, and reliable method for the quantitative analysis of small amounts of heparin/HS present in biological samples.

Conclusions

Significant diversity in structures of GAGs among different organisms might indicate their evolutionary and functional distinction. The structure of GAGs is typically assessed by analyzing their disaccharide units obtained from chemical and enzymatic depolymerization of the complex chain. There have been problems and challenges for qualitative and quantitative determination of GAGs in biological samples due to their low natural abundance and the presence of interfering impurities. Here we have presented a new method for the rapid and reliable disaccharide analysis of heparin/HS. This method has been applied to both pharmaceutical heparin and heparin/HS extracted and purified from cells, tissues, and biological fluids.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2011.04.003.

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