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Ultraperformance 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. Ultraperformance 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, as it provides high sensitivity without being subject to interference from proteins, peptides and other sample impurities.

Keywords

heparin; heparan sulfate; heparin lyase; disaccharide analysis; biological samples; ultraperformance liquid chromatography; mass spectrometry

Introduction

Heparin and heparan sulfate (HS) 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

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differentiation, viral and bacterial infection, cancer, and inflammation, through their interaction with chemokines, cytokines, growth factors receptors [1; 2; 3; 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 a growing interest in characterizing the fine structure of pharmaceutical heparin [7; 8; 9; 10] and in elucidating the interaction of HS with a wide array of proteins, ligands, receptors and pathogens [11].

Heparin/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 either be 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, environment 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 (Δ UA) at the non-reducing 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; 20; 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. While 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 10–100 μ g 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-microgram) 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; 29; 30; 31]. The impact of the concentration of different volatile ion-pairing reagents, such as triethylamine, dibutylamine, tributylamine, tripentylamine, tetrapropylamine and tetrabutylamine, on ionization efficiency have been systematically studied in the analysis of heparosan oligosaccharides (Δ UA[GlcNAc-GlcA]_nGlcNAc, n = 2–19) [30] and heparin oligosaccharide (Δ UA2S [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 ESI-MS, relying on isotopically enriched disaccharide internal standards [29].

Ultra-performance liquid chromatography (UPLC) separations are performed at high pressures (up to 10^8 Pa) using columns packed with 1.7 μ m sorbent particles. This new category of analytical separation science retains the practicality and principles of HPLC while yielding major improvements in chromatographic performance. Compared to 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 tributylamine 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 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 a 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 as five minutes [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 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: Δ UA-GlcNAc, NS: Δ UA-GlcNS, 6S: Δ UA-GlcNAc6S, 2S: Δ UA2S-GlcNAc, NS2S: Δ UA2S-GlcNS, NS6S: Δ UA-GlcNS6S, 2S6S: Δ UA2S-GlcNAc6S, triS: Δ UA2S-GlcNS6S) were obtained from Iduron Co (Manchester, UK.). Actinase E was from Kaken Biochemicals (Tokyo, Japan). Urea, CHAPS and tributylamine (TrBA) were from Sigma Chemical Company (St. Louis, MO, USA). Vivapure MINI Q H spin columns were from Viva science (Edgewood, NJ, USA). Syringe filters (0.22 μ m) and Microcon Centrifugal Filter Units YM-10 were from Millipore Company (Bedford, MA, USA). Cloning, *E. 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 *F. heparinum* were performed in our laboratory as described [35; 36; 37]. Pharmaceutical heparin and low molecular weight heparin (LWMH) 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) 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 previously described [39; 40] and used with some modification. De-fatting involved the 3-step washing of lyophilized cells ($\sim 10^6$ 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% of 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 KD, a molecular weight cut-off (MWCO)) by centrifugation at 12,000 × g, 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, then dialyzed for 4 days against 4 L of double-distilled water using 1000 MW cutoff membranes. After dialysis, the urine sample was concentrated and lyophilized for future use.

Enzymatic Digestion

LMW heparin samples were weighed and the amount GAGs recovered from biological samples were determined by micro-carbazole assay [29] and were then used to prepare a stock solution from which 5 µg of analyte could be taken. The heparin lyase I, II, and III (10 mU each, assayed prior to use) in 5 µL of 25 mM Tris, 500 mM NaCl, 300 mM inidazole buffer (pH 7.4) were added to 5 µg 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 HS/heparin disaccharides were recovered in the flow-through and freeze-dried. The digested GAGs disaccharides were re-dissolved in water to concentration of 50–100 ng/2 µL for LC-MS analysis.

RPIP-UPLC-MS

LC-MS analyses were performed on an Agilent 1200 LC/MSD instrument (Agilent Technologies, Inc. Wilmington, DE) 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 NH₄OAc 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 (0–100% solution B) was used at 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, 100 ng/per each disaccharide). Linearity was assessed based on amount of disaccharide and peak intensity in mass spectrometry. 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/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 in different species. The quantitative disaccharide composition of HS/heparin is believed to have a direct relationship to 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 CS/DS disaccharides by using HXA as ion-pairing reagent and HFIP as an organic modifier in the mobile phase [43]. Heparin/HS-derived disaccharides Δ UA-GlcNAc6S (6S) and Δ UA2S-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 tradeoff between 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 7.00 was successfully used for compositional profiling and quantification of heparin/HS by RPIP-UPLC- 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 disaccharides analysis was explored by using a 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 post-column addition of 5 μ L/min acetonitrile to improve TrBA evaporation in the ion source [29].

In the current experiments, we relied on a 1.7 μ m particle size Acquity UPLC BEH C18 column and 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) (Figure 1). TIC and EIC detection exhibited higher sensitivity and a flatter baseline than UV_{232 nm} 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 efficiency of

ionization of the eight different disaccharides 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 (Figure 2). Peaks were observed at m/z 377.8 (Δ UA-GlcNAc), 415.7 (Δ UA-GlcNS), 457.7 (Δ UA-GlcNAc6S) and (Δ UA2S-GlcNAc), 495.6 (Δ UA-GlcNS6S) and (Δ UA2S-GlcNS), 537.7 (Δ UA2S-GlcNAc6S) and 575.6 (Δ UA2S-GlcNS6S), respectively. 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 Δ UA-GlcNAc6S and Δ UA2S-GlcNAc had been resolved [29]. Interestingly, the sensitivity of detection is similar with and without additional post-column acetonitrile addition. This may result from the high pressure of the UPLC column (10^8 Pa) and the high flow rate (100 μ L/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 the linearity of disaccharide amount and peak intensity using EIC detection. Samples 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 (Figure 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, while monosulfated disaccharide NS and trisulfated disaccharide Tri S 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 μ L, 20 ng/5 μ L, and 20 ng/8 μ L were analyzed at injection volumes of 2 μ L, 5 μ L and 8 μ L, respectively. Very similar peak areas and identical retention times were obtained, 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 OS, NS, 6S, 2S, NS6S, NS2S, 2S6S and TriS, respectively) and M2 (10, 15, 20, 25, 30, 35, 40 and 50 ng of OS, 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 low molecular-weight heparin (LMWH) is prepared from the controlled chemical or enzymatic depolymerization of pharmaceutical heparin [7; 10]. Three pharmaceutical heparins and one LMWH were analyzed RPIP-UPLC-MS. The EIC of disaccharides analysis of these samples

are presented in Figure 4. Eight common heparin/HS disaccharides were detected in all samples, their quantity was calculated by the linear equation shown in Figure 3, and their disaccharide compositions are given in Table 3. Analyses show, as expected [8; 9; 10], that the major disaccharide in heparin and LMWH is Tri S, but its content ranges from 67.3–84.9%, suggesting that manufacturing processes and/or source material have an impact disaccharide composition.

Quantitative analysis of HS/heparin disaccharides 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. Quantitative disaccharide compositional analysis is one of the most important ways to characterize the structures of HS/heparin 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 had 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 RPLC peak resolution and enhance detection sensitivity using fluorescence [46; 47]. Nevertheless, since 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 an RPIP-HPLC-MS method greatly increases confidence in peak assignment, resulting in improved qualitative disaccharide compositional analysis since 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].

Chinese hamster ovary (CHO) cells are widely used in the biotechnology/biopharmaceutical industry for the production of recombinant therapeutic proteins. They are accepted by the 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 the 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 digested with heparin lyase I, II, and III. The heparin/HS disaccharide composition of the CHO-S cell sample analyzed by this RPIP-UPLC-MS is presented in Figure 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 Tri S 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, had previously been 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, disaccharides analysis was performed (Figure 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 GAGs from camel intestine were nearly equally divided between 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 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 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 while traditional 5 μ m C18 columns can cleanly resolve eight heparin/HS disaccharides standards (Figure S1), in the analysis of biological samples, shifting retention times and peak broadening result from interfering from proteins (such as heparinases). Thus, the Δ UA-GlcNAc6S (6S) and Δ UA2S-GlcNAc (2S) disaccharides could not be completely resolved, leading to difficulties in quantification of these disaccharides on conventional 5 μ m columns (Figure S2). The retention times of GAGs from all 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 cell, tissues and biological fluids. Moreover various injection volumes provided similar analyses (data not shown). RPIP-UPLC-MS also provides higher sensitivity than traditional 5 μ m C18 columns. The small amount of disaccharides (NS2S, NS6S and Tri S) present in the compositional analysis of the CHO-cell and camel urine samples were not detected (Figure S3) when using 5 μ m C18 column. Furthermore, the 1.7 μ m column requires a shorter equilibration time than the 5 μ m column. Therefore, compared to the conventional 5 μ m RP-HPLC column, the UPLC 1.7 μ m 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. GAG structure 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. Herein we present 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Hacker U, Nybakken K, Perrimon N. Heparan sulphate proteoglycans: the sweet side of development. *Nat Rev Mol Cell Biol.* 2005; 6:530–541. [PubMed: 16072037]
2. Tumova S, Woods A, Couchman JR. Heparan sulfate proteoglycans on the cell surface: versatile coordinators of cellular functions. *Int J Biochem Cell Biol.* 2000; 32:269–288. [PubMed: 10716625]
3. Petitou M, Herault JP, Bernat A, Driguez PA, Duchaussoy P, Lormeau JC, Herbert JM. Synthesis of thrombin-inhibiting heparin mimetics without side effects. *Nature.* 1999; 398:417–422. [PubMed: 10201371]
4. Wang L, Fuster M, Sriramaraio P, Esko JD. Endothelial heparan sulfate deficiency impairs L-selectin- and chemokine-mediated neutrophil trafficking during inflammatory responses. *Nat Immunol.* 2005; 6:902–910. [PubMed: 16056228]
5. Capila I, Linhardt RJ. Heparin-protein interactions. *Angew Chem Int Ed Engl.* 2002; 41:391–412. [PubMed: 12491369]
6. Muszbek L, Bereczky Z, Kovacs B, Komaromi I. Review: Antithrombin deficiency and its laboratory diagnosis. *Clin Chem Lab Med.* 2010
7. Guerrini M, Beccati D, Shriver Z, Naggi A, Viswanathan K, Bisio A, Capila I, Lansing JC, Guglieri S, Fraser B, Al-Hakim A, Gunay NS, Zhang Z, Robinson L, Buhse L, Nasr M, Woodcock J, Langer R, Venkataraman G, Linhardt RJ, Casu B, Torri G, Sasisekharan R. Oversulfated chondroitin sulfate is a contaminant in heparin associated with adverse clinical events. *Nat Biotechnol.* 2008; 26:669–675. [PubMed: 18437154]
8. Brustkern AM, Buhse LF, Nasr M, Al-Hakim A, Keire DA. Characterization of currently marketed heparin products: reversed-phase ion-pairing liquid chromatography mass spectrometry of heparin digests. *Anal Chem.* 2010; 82:9865–9870. [PubMed: 21069966]
9. Korir AK, Lintiacio JF, Gutierrez SM, Larive CK. Ultraperformance ion-pair liquid chromatography coupled to electrospray time-of-flight mass spectrometry for compositional profiling and quantification of heparin and heparan sulfate. *Anal Chem.* 2008; 80:1297–1306. [PubMed: 18215021]
10. Zhang Z, Li B, Suwan J, Zhang F, Wang Z, Liu H, Mulloy B, Linhardt RJ. Analysis of pharmaceutical heparins and potential contaminants using (1)H-NMR and PAGE. *J Pharm Sci.* 2009; 98:4017–4026. [PubMed: 19642166]
11. Bernfield M, Gotte M, Park PW, Reizes O, Fitzgerald ML, Lincecum J, Zako M. Functions of cell surface heparan sulfate proteoglycans. *Annu Rev Biochem.* 1999; 68:729–777. [PubMed: 10872465]
12. Martin JG, Gupta M, Xu Y, Akella S, Liu J, Dordick JS, Linhardt RJ. Toward an artificial Golgi: redesigning the biological activities of heparan sulfate on a digital microfluidic chip. *J Am Chem Soc.* 2009; 131:11041–11048. [PubMed: 19591465]
13. Rabenstein DL. Heparin and heparan sulfate: structure and function. *Nat Prod Rep.* 2002; 19:312–331. [PubMed: 12137280]
14. Jandik KA, Kruep D, Cartier M, Linhardt RJ. Accelerated stability studies of heparin. *J Pharm Sci.* 1996; 85:45–51. [PubMed: 8926582]
15. Cohen DM, Linhardt RJ. Randomness in the heparin polymer: computer simulations of alternative action patterns of heparin lyase. *Biopolymers.* 1990; 30:733–741. [PubMed: 2275975]
16. Xiao Z, Tappen BR, Ly M, Zhao W, Canova LP, Guan H, Linhardt RJ. Heparin mapping using heparin lyases and the generation of a novel low molecular weight heparin. *J Med Chem.* 2011; 54:603–610. [PubMed: 21166465]
17. Rice KG, Kim YS, Grant AC, Merchant ZM, Linhardt RJ. High-performance liquid chromatographic separation of heparin-derived oligosaccharides. *Anal Biochem.* 1985; 150:325–331. [PubMed: 4091260]
18. Yamada S, Sakamoto K, Tsuda H, Yoshida K, Sugiura M, Sugahara K. Structural studies of octasaccharides derived from the low-sulfated repeating disaccharide region and octasaccharide serines derived from the protein linkage region of porcine intestinal heparin. *Biochem.* 1999; 38:838–847. [PubMed: 9888825]

19. Chuang WL, McAllister H, Rabenstein L. Chromatographic methods for product-profile analysis and isolation of oligosaccharides produced by heparinase-catalyzed depolymerization of heparin. *J Chromatogr A*. 2001; 932:65–74. [PubMed: 11695869]
20. Hileman RE, Smith AE, Toida T, Linhardt RJ. Preparation and structure of heparin lyase-derived heparan sulfate oligosaccharides. *Glycobiology*. 1997; 7:231–239. [PubMed: 9134430]
21. Pervin A, Gallo C, Jandik KA, Han XJ, Linhardt RJ. Preparation and structural characterization of large heparin-derived oligosaccharides. *Glycobiology*. 1995; 5:83–95. [PubMed: 7772871]
22. Volpi N, Maccari F, Linhardt RJ. Quantitative capillary electrophoresis determination of oversulfated chondroitin sulfate as a contaminant in heparin preparations. *Anal Biochem*. 2009; 388:140–145. [PubMed: 19232311]
23. Eldridge SL, Higgins LA, Dickey BJ, Larive CK. Insights into the capillary electrophoresis separation of heparin disaccharides from nuclear magnetic resonance, pKa, and electrophoretic mobility measurements. *Anal Chem*. 2009; 81:7406–7415. [PubMed: 19653663]
24. Bigler P, Brenneisen R. Improved impurity fingerprinting of heparin by high resolution (1)H NMR spectroscopy. *J Pharm Biomed Anal*. 2009; 49:1060–1064. [PubMed: 19211212]
25. Chai W, Hounsell EF, Bauer CJ, Lawson AM. Characterisation by LSI-MS and ¹H NMR spectroscopy of tetra-, hexa-, and octa-saccharides of porcine intestinal heparin. *Carbohydr Res*. 1995; 269:139–156. [PubMed: 7773986]
26. Chai W, Luo J, Lim CK, Lawson AM. Characterization of heparin oligosaccharide mixtures as ammonium salts using electrospray mass spectrometry. *Anal Chem*. 1998; 70:2060–2066. [PubMed: 9608845]
27. Zaia J, Costello CE. Compositional analysis of glycosaminoglycans by electrospray mass spectrometry. *Anal Chem*. 2001; 73:233–239. [PubMed: 11199971]
28. Thanawiroon C, Rice KG, Toida T, Linhardt RJ. Liquid chromatography/mass spectrometry sequencing approach for highly sulfated heparin-derived oligosaccharides. *J Biol Chem*. 2004; 279:2608–2615. [PubMed: 14610083]
29. Zhang Z, Xie J, Liu H, Liu J, Linhardt RJ. Quantification of heparan sulfate disaccharides using ion-pairing reversed-phase microflow high-performance liquid chromatography with electrospray ionization trap mass spectrometry. *Anal Chem*. 2009; 81:4349–4355. [PubMed: 19402671]
30. Kuberan B, Lech M, Zhang L, Wu ZL, Beeler DL, Rosenberg RD. Analysis of heparan sulfate oligosaccharides with ion pair-reverse phase capillary high performance liquid chromatography-microelectrospray ionization time-of-flight mass spectrometry. *J Am Chem Soc*. 2002; 124:8707–8718. [PubMed: 12121115]
31. Volpi N, Linhardt RJ. High-performance liquid chromatography-mass spectrometry for mapping and sequencing glycosaminoglycan-derived oligosaccharides. *Nat Protoc*. 2010; 5:993–1004. [PubMed: 20448545]
32. Swartz ME. UPLC™: An Introduction and Review. *J Liq Chromatogr Related Technol*. 2005; 28:1253–1263.
33. MacNair JE, Lewis KC, Jorgenson JW. Ultrahigh-pressure reversed-phase liquid chromatography in packed capillary columns. *Anal Chem*. 1997; 69:983–989. [PubMed: 9075400]
34. Jones CJ, Membreno N, Larive CK. Insights into the mechanism of separation of heparin and heparan sulfate disaccharides by reverse-phase ion-pair chromatography. *J Chromatogr A*. 2010; 1217:479–488. [PubMed: 20004903]
35. Yoshida E, Arakawa S, Matsunaga T, Toriumi S, Tokuyama S, Morikawa K, Tahara Y. Cloning, sequencing, and expression of the gene from bacillus circulans that codes for a heparinase that degrades both heparin and heparan sulfate. *Biosci Biotechnol Biochem*. 2002; 66:1873–1879. [PubMed: 12400686]
36. Shaya D, Tocilj A, Li Y, Myette J, Venkataraman G, Sasisekharan R, Cygler M. Crystal structure of heparinase II from *Pedobacter heparinus* and its complex with a disaccharide product. *J Biol Chem*. 2006; 281:15525–15535. [PubMed: 16565082]
37. Godavarti R, Davis M, Venkataraman G, Cooney C, Langer R, Sasisekharan R. Heparinase III from *Flavobacterium heparinum*: cloning and recombinant expression in *Escherichia coli*. *Biochem Biophys Res Commun*. 1996; 225:751–758. [PubMed: 8780685]

38. Li B, Ryan PW, Ray BH, Leister KJ, Sirimuthu NM, Ryder AG. Rapid characterization and quality control of complex cell culture media solutions using raman spectroscopy and chemometrics. *Biotechnol Bioeng*. 2010; 107:290–301. [PubMed: 20506397]
39. Zhang F, Sun P, Munoz E, Chi L, Sakai S, Toida T, Zhang H, Mousa S, Linhardt RJ. Microscale isolation and analysis of heparin from plasma using an anion-exchange spin column. *Anal Biochem*. 2006; 353:284–286. [PubMed: 16529709]
40. Li B, Liu H, Zhang Z, Stansfield HE, Dordick JS, Linhardt RJ. Analysis of glycosaminoglycans in stem cell glycomics. *Methods Mol Biol*. 2011; 690:285–300. [PubMed: 21043000]
41. Cecchi T, Passamonti P. Retention mechanism for ion-pair chromatography with chaotropic reagents. *J Chromatogr A*. 2009; 1216:1789–1797. [PubMed: 18973902]
42. Cecchi T, Pucciarelli F, Passamonti P. Extended thermodynamic approach to ion interaction chromatography. *Anal Chem*. 2001; 73:2632–2639. [PubMed: 11403310]
43. Solakyildirim K, Zhang Z, Linhardt RJ. Ultraperformance liquid chromatography with electrospray ionization ion trap mass spectrometry for chondroitin disaccharide analysis. *Anal Biochem*. 2010; 397:24–28. [PubMed: 19769936]
44. Wren SA, Tchelitcheff P. UPLC/MS for the identification of beta-blockers. *J Pharm Biomed Anal*. 2006; 40:571–580. [PubMed: 16413161]
45. Linhardt RJ. 2003 Claude S. Hudson Award address in carbohydrate chemistry. Heparin: structure and activity. *J Med Chem*. 2003; 46:2551–2564. [PubMed: 12801218]
46. Yamada S, Morimoto H, Fujisawa T, Sugahara K. Glycosaminoglycans in *Hydra magnipapillata* (Hydrozoa, Cnidaria): demonstration of chondroitin in the developing nematocyst, the sting organelle, and structural characterization of glycosaminoglycans. *Glycobiology*. 2007; 17:886–894. [PubMed: 17513885]
47. Deakin JA, Lyon M. A simplified and sensitive fluorescent method for disaccharide analysis of both heparan sulfate and chondroitin/dermatan sulfates from biological samples. *Glycobiology*. 2008; 18:483–491. [PubMed: 18378523]
48. Lawrence R, Olson SK, Steele RE, Wang L, Warrior R, Cummings RD, Esko JD. Evolutionary differences in glycosaminoglycan fine structure detected by quantitative glycan reductive isotope labeling. *J Biol Chem*. 2008; 283:33674–84. [PubMed: 18818196]
49. Cuellar K, Chuong H, Hubbell SM, Hinsdale ME. Biosynthesis of chondroitin and heparan sulfate in chinese hamster ovary cells depends on xylosyltransferase II. *J Biol Chem*. 2007; 282:5195–5200. [PubMed: 17189266]
50. Garud DR, Tran VM, Victor XV, Koketsu M, Kuberan B. Inhibition of heparan sulfate and chondroitin sulfate proteoglycan biosynthesis. *J Biol Chem*. 2008; 283:28881–28887. [PubMed: 18708345]
51. Warda M, Gouda EM, Toida T, Chi L, Linhardt RJ. Isolation and characterization of raw heparin from dromedary intestine: evaluation of a new source of pharmaceutical heparin. *Comp Biochem Physiol C Toxicol Pharmacol*. 2003; 136:357–365. [PubMed: 15012907]

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) Δ UA-GlcNAc; (B) Δ UA-GlcNS; (C) Δ UA-GlcNAc6S; (D) Δ UA2S-GlcNAc; (E) Δ UA-GlcNS6S; (F) Δ UA2S-GlcNS; (G) Δ UA2S-GlcNAc6S; and (H) Δ UA2S-GlcNS6S.

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.

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.

Table 1

The structures of the eight Δ-disaccharide standards from HS/heparin.

	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	
Tri S	ΔUA2S-GlcNS6S	SO ₃ ⁻	SO ₃ ⁻	SO ₃ ⁻	577.5	

Table 2

Quantification analysis of heparin/HS disaccharide mixtures containing known amounts of disaccharides

Disaccharides (ng)	M1		M2	
	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
Tri S	10	8.5±1.0	50	48.9±1.4

Table 3

Heparin/HS disaccharide composition analysis of GAGs samples from biological sources.

Sample source	Heparin/HS disaccharides composition (%)								
	OS	NS	6S	2S	NS6S	NS2S	6S2S	Tri S	
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	