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Top-down approach for the direct characterization of low molecular weight heparins using LC-FT-MS

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Abstract

Low molecular heparins (LMWHs) are structurally complex, heterogeneous, polydisperse, and highly negatively charged mixtures of polysaccharides. The direct characterization of LMWH is a major challenge for currently available analytical technologies. Electrospray ionization (ESI) liquid chromatography-mass spectrometry (LC-MS) is a powerful tool for the characterization complex biological samples in the fields of proteomics, metabolomics and glycomics. LC-MS has been applied to the analysis of heparin oligosaccharides, separated by size exclusion, reversed phase ion-pairing chromatography and by chip-based amide hydrophilic interaction chromatography (HILIC). However, there have been limited applications of ESI-LC-MS for the direct characterization of intact LMWHs (top-down analysis) due to their structural complexity, low ionization efficiency and sulfate loss. Here we present a simple and reliable HILIC-Fourier transform (FT)-ESI-MS platform to characterize and compare two currently marketed LMWH products using the top-down approach requiring no special sample preparation steps. This HILIC system relies on cross-linked diol rather than amide chemistry, affording highly resolved chromatographic separations using a relatively high percentage of acetonitrile in the mobile phase, resulting in stable and high efficiency ionization. Bioinformatics software (GlycerSoft 1.0) was used to automatically assign structures within 5-ppm mass accuracy.

Keywords

Low molecular weight heparin Top-down glycomics; Hydrophilic interaction chromatography; High-resolution mass spectrometry

Introduction

Heparin is a polydisperse polysaccharide extracted from animal tissues and is widely used as a clinical blood anticoagulant (Figure 1).[1,2] Heparin's anticoagulant activity results

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primarily from its binding and activation of antithrombin III (AT), a protease inhibitor, causing the inhibition of blood coagulation proteases, including thrombin (Factor IIa) and Factor Xa. Low molecular weight heparins (LMWHs)[3] are derived from heparin by controlled chemical or enzymatic depolymerization.[4,5] In these processes heparin, having an average molecular weight (MW_{avg}) of ~12,000,corresponding to ~40 saccharide units, is broken into smaller polysaccharides, having an MW_{avg} of ~4,000 to ~8,000, corresponding to ~13 to ~26 saccharide units. This reduction in MW_{avg} improves the bioavailability of LMWHs, making them subcutaneously active, increasing their *in vivo* half-life, improving their pharmacology, and altering their activity profile decreasing their thrombin inhibitory activity without markedly altering their Factor Xa inhibitory activity.[3–8] Since their introduction in the 1990s, LMWHs have captured about 70% of the US heparin market.[5,9] The major share of the LMWH market is controlled by Lovenox® (enoxaparin) and a recently approved generic version.[5,9] These products are prepared from the sodium salt of heparin by forming an organic soluble heparin benzethonium salt, converting this salt to the benzyl ester, and treating this ester derivative with base, cleaving the polysaccharide backbone through β-elimination and hydrolyzing the residual benzyl esters (Figure 1). This process affords a polydisperse mixture of LMWH chains having unnatural, unsaturated uronate residues at their non-reducing ends and unnatural 1,6-anhydro amino sugar residues at the reducing ends of some of their polysaccharide chains.[10,11]

A number of methods have been used for the top-down characterization of LMWHs, such as enoxaparin. Proton and carbon nuclear magnetic resonance (NMR) spectroscopy provide the most detailed information of the primary structure of LMWH[12] but NMR lacks both the analytical sensitivity to detect minor structural features, such as the 1,6-anhydro amino sugar residues, and is relatively low throughput. Furthermore, NMR affords only number average molecular weight (M_N), thus, providing limited molecular weight information on this polysaccharide mixture,[13] as both M_N and weight average molecular weight (M_W) are required to calculate polydispersity ($PD = M_W/M_N$).[14,15] Size exclusion chromatography (SEC)[15] and polyacrylamide gel electrophoresis[14] provide this needed molecular weight information on LMWHs, but provide little or no detailed structural information. Bottom-up analysis of LMWHs include disaccharide compositional analysis[16–22] and oligosaccharide mapping.[23,24] In these methods LMWH is further broken down to its constituent disaccharides or oligosaccharides by more complete chemical or enzymatic treatment and these fragments are characterized and quantified using LC,[16,18] capillary electrophoresis (CE),[21,25] polyacrylamide gel electrophoresis,[24,26] or hyphenated techniques,[27] such as LC-MS[27–30] or CE-laser induced fluorescence (LIF).[21] While these methods allow the sensitive detection of minor components, such as 1,6-anhydro amino sugar residues, they result in the loss of much of the information required to understand the primary structure or sequence of the individual polysaccharide chains comprising a LMWH. Furthermore, bottom-up analysis requires extensive sample processing steps by a skilled analyst and is often quite difficult to apply in routine quality control and quality assurance.

Top-down analysis relying on MS detection poses a number of challenges.[31] ESI-MS affords better ionization of highly charged polyanions than matrix assisted laser desorption (MALDI)-MS, which is only effective for the direct analysis of relatively small (<hexasaccharide) heparin oligosaccharides[32] or on intermediate-sized (<decasaccharide) heparin oligosaccharides in complex with cationic peptides.[33] Negative-ion ESI-MS analysis of intact polysaccharides poses its own set of challenges associated with the formation of adducts, sulfate loss, and a large number of charge states. Direct analysis of complex polysaccharide mixtures requires high-resolution Fourier transform (FT)-ion cyclotron resonance (ICR) instruments. For example, the direct analysis of the relatively simple 150-component bikunin polysaccharide by FT-ICR-MS analysis, required multiple ‘Quad-windowed’ mass spectra.[34]

LC-MS analysis of LMWH, first reported nearly 10 years ago, used reversed phase ion pairing (RPIP)-LC to characterize the major chains of a LMWH up to octadecasaccharide in size.[29,35–37] Unfortunately, RPIP-LC has not been widely used because the volatile ion-pairing reagents, required in this analysis, contaminate instruments and the required mobile phase does not provide sufficient sensitivity, resolution, and mass range needed for the thorough characterization of LMWHs.

While HILIC-LC is lower resolution than RPIP-HLPC, HILIC-LC–MS offers several potential advantages for LMWH analysis over RPIP-LC-MS. Separations are fast and HILIC-LC–MS utilizes a mobile phase well-suited for negative ESI-MS with a pH range and buffer salt selectivity suitable for LMWH separation and detection.[30] Current HILIC-LC–MS technology utilizing an amide HILIC support has been successfully applied for LC-MS profiling of heparin oligosaccharide mixtures.[24] Effective use of HILIC-LC–MS for analysis of negatively charged saccharides has been demonstrated using a number of mass spectrometry systems.[30,38,39] Agilent has introduced a novel amide-HILIC HPLC chip that facilitates HILIC LC-MS and allows the introduction of makeup flow to the effluent, eliminating the need to increase spray voltages as aqueous content increases providing a stable spray throughout an LC run even as it reaches 100% aqueous. This chip-based nano-spray amide HILIC LC/MS system has been used to analyze LMWH up to dp18 as isolated size fractions.[40] This system was limited by the mass resolution of the time-of-flight analyzer to resolve patterns of overlapping isotope clusters. In order to analyze LWMH as an unfractionated mixture, we sought to use a high resolution LC-FT-MS system to provide sufficient resolution for LMWH analysis.

In this paper we report the use of a universal diol-based HILIC LC-FT-MS platform to separate and analyze intact LMWH directly. With the new developed bioinformatics software package (GlycReSoft),[41] a quantitative comparison of two commercial LMWH products, Lovenox® and a generic enoxaparin are presented demonstrating very similar profiles for 200–500 components.

EXPERIMENTAL SECTION

Materials

LMWHs, of enoxaparin sodium from Sanofi-Aventis (Lovenox®) or Sandoz (a generic version of Lovenox®), were obtained from hospital pharmacies (3 lots each) and were freeze-dried prior to analysis. Arixtra® ($C_{31}H_{53}N_3O_{49}S_8$, Organon Sanofi-Synthelabo LLC (West Orange, NJ)), a synthetic ultra-LMWH,[9] was obtained from hospital pharmacy and was desalted by dialysis using 1000 molecular weight cut-off membranes (Spectrum Medical, Los Angelis, CA). Stock solutions of LMWHs and ultra-LMWH were prepared at 100 mg/mL in water. Acetonitrile, ammonium acetate, and water were of HPLC grade (Sigma Aldrich, St. Louis, MO).

HILIC LC ESI-LTQ-Orbitrap-FT-MS analysis of LMWH

A Luna HILIC column (2.0 × 150 mm, 200 A, Phenomenex, Torrance, CA) was used to separate the LMWHs. Mobile phase A was 5 mM ammonium acetate prepared with HPLC grade water. Mobile B was 5 mM ammonium acetate prepared in 98% HPLC grade acetonitrile with 2% of HPLC grade water. After injection of 8.0 μL LMWH (1.0 μg/μL) through an Agilent 1200 auto-sampler, HPLC binary pump was used to deliver the gradient from 10%A to 35%A over 40 min at a flow rate of 150 μL/min. The LC column was directly connected online to the standard ESI source of LTQ-Orbitrap XL FT MS (Thermo Fisher Scientific, San-Jose, CA). The source parameters for FT-MS detection were optimized using Arixtra® to minimize the in-source fragmentation and sulfate loss and maximize the signal/noise in the negative-ion mode. The optimized parameters, used to

prevent in-source fragmentation, included a spray voltage of 4.2 kV, a capillary voltage of −40 V, a tube lens voltage of −50 V, a capillary temperature of 275 °C, a sheath flow rate of 30, and an auxiliary gas flow rate of 6. External calibration of mass spectra routinely produced a mass accuracy of better than 3 ppm. All FT mass spectra were acquired at a resolution 60,000 with 400–2000 Da mass range.

Bioinformatics

Charge deconvolution was performed manually with electronic spreadsheets or auto-processed by DeconTools software (web source from PNNL at OMICS.PNL.GOV). LMWH structural assignment was done by either manual or automatic processing using GlycReSoft 1.0 software developed at Boston University School of Medicine (http://code.google.com/p/glycresoft/downloads/list)[41] For manual interpretation of large oligomers (degree of polymerization (dp)18-dp26), the third isotope peak was used to match the accurate mass of the third isotope peaks in the database because the mono-isotope peak was too low to determine experimentally. For automatic processing, GlycReSoft 1.0 parameters were set as: Minimum Abundance, 1.0; Minimum Number of Scans, 1; Molecular Weight Lower Boundary, 500 Da; Molecular Weight Upper Boundary, 6000 Da; Mass Shift, ammonium; Match Error (E_M), 5.0ppm; Grouping Error (E_G), 80ppm; Adduct Tolerance (E_A), 5.0ppm. For LMWH components identification, theoretical database was generated by GlycReSoft 1.0 using the following parameters: A, ΔHexA = 0 or 1; B, HexA = 0 to 12; C, HexNAc = A+B−1 to A+B+1; D, Ac = 0 to 5; E, SO3 = B to A+B+(C*2)+1-D; Modification, Adduct = ammonium from 0–14. For anhydo-component identification, 1 extra water loss was added to A to generate the theoretical andydro-database and the other parameters are keeping the same. The data from three lots of each LMWH vender were processed and statistical profiling results are presented in Figure 3, 4, and 5. A detailed table is included in the supplemental materials (supplemental Table 2S).

RESULTS AND DISCUSSION

LMWHs are widely used for clinical anticoagulation and have been the subject of regulatory scrutiny by the FDA as new generic versions enter the marketplace.[37,42] The characterization LMWHs poses a number of unique challenges[31] as they contain a large variety of components having different chain lengths, with different numbers of sulfo groups on each chain of given length, different positioning of sulfo groups, and other unique structural features, such as uronic acid C-5 epimers, unsaturated uronic acids and 1,6 anhydro amino sugar residues.[10] Recent progress has been reported on the top-down analysis of the structurally related, chondroitin sulfate polysaccharide chains of bikunin by using preparative electrophoresis[43] to obtain simplified polysaccharide mixtures for FT-MS analysis.[34] However, LMWH chains are larger, considerably more highly sulfated, and much more structurally heterogeneous than the bikunin chondroitin sulfate chains. Most technologies used for LMWH characterization currently rely on bottom-up analysis, such as disaccharide compositional analysis or oligosaccharide mapping, or low-resolution top-down analysis such as SEC or polyacrylamide, providing molecular weight properties but limited information on fine structure.[17,19,20,22–24] RPIP-LC analysis, first successfully applied for the top-down analysis of a LMWH nearly a decade ago,[4,36] had a number of serious limitations. The volatile ion-pairing reagent contaminated instrumentation, the mobile phase composition resulted in low ionization efficiency, and the ion-trap detector employed afforded low-resolution mass spectra and were only capable of handling relatively low charge-states.[35] The successful introduction of amide HILIC for the separation of heparin oligosaccharides[17,24] suggested an approach for eliminating ion-pairing reagent and the application of FT-MS to heparin oligosaccharides[44] and also suggested an approach to improving mass resolution and handling higher charge-states. The major challenges remaining was to optimize the HILIC separation using mobile phase with a high organic

content to ensure a stable ion-spray providing high-sensitivity detection for the routine on-line HILIC LC-FTMS analysis of highly complex heparin oligomer mixtures.

HILIC separation

Luna HILIC-LC, relying on a cross-linked diol solid-phase support instead of the standard amide support, was selected after surveying a number of HILIC chemistry. A relatively high-resolution of separation of the very complex, highly negatively charged mixture of LMWH components was possible using an ESI-MS friendly mobile phase (Figure 2). The diol-based HILIC separation still affords lower resolution than RPIP-HPLC, particularly for oligosaccharides of the same size but having different charge. This HILIC separation is based on chain size and polarity, and affords sufficient separation for an Obritrap FT mass spectrometer to resolve the relatively small number of components eluting at each retention time. The simplification of the polysaccharide mixture had been essential in the successful MS analysis of the structurally less complex chondroitin sulfate chains of bikunin.[33] Most previous work for LMWH MS characterization relies on extensive off-line fractionation and purification either by SAX and/or SEC and generally requires days to weeks to finish a full characterization. On-line HILIC-LC-MS offers a relatively high throughput platform for detailed characterization of LMWH components.

FT-mass spectra

The source parameters of the Obritrap were optimized to obtain stable spray, reduced in-source fragmentation, and reduced sulfate loss. The Luna HILIC LC was primarily responsible for platform stability and reproducibility, and this front-end separation should be transferable to any other high-resolution MS instruments without special hardware requirements. For glycomics LC-MS, high-resolution and high mass accuracy MS is necessary to define glycan *m/z* values and charge states accurately; thus deconvolution is an essential data processing step. In addition, glycans as a compound class range from neutral to acidic, and the use of negative-ion MS is often recommended. DeconTools is an open-source software package designed for automated processing of high-resolution mass spectral data. High resolution MS is needed for resolving highly complex and highly charged components with molecular weights from 1000 Da up to 10000 Da. High mass accuracy (usually less than 5 ppm is preferred) is also important for unambiguous structure assignment.

The Luna-HILIC LC FTMS platform is robust and easy to use, providing high-quality data for the routine profiling of the commercial LMWHs up to dp18 using DeconTools and GlycerSoft bioinformatics tools. For larger oligomers including dp20-dp30, the complexity of isotopic clusters became very complex due to the presence of multiple ammonium adducts, making deconvolution using DeconTools impossible. Interpretation of the data using a manual approach allowed the identification of a few additional structures with multiple ammonium adducts (Table 1 and Table 1S). Manual identification, however, was not reproducible from run to run for these large oligomers. The difficulty in manual interpretation is likely due to the high degree of sulfation of heparin oligomers, since for non-sulfated heparosan or hyaluronic acid chains of up to dp30 and dp45, were easily be identified without ammonium adduction using Luna HILIC LC FTMS platform (data not shown). Therefore, in the current study, a comparison between LMWH sources was made for oligosaccharides up to dp18.

The major components identified in the LMWH, enoxaparin, were even-numbered chains ranging from tetrasaccharides (dp4) up to polysaccharides of dp26. These components contain from 2 to 3-sulfo groups/disaccharide and 0–3 *N*-acetyl groups/disaccharide. The sulfo group/disaccharide ratio was lowest (~2) for the dp values between 10 and 16

consistent with the presence of the low sulfate domain structure, containing one GlcNAc and one GlcA and one GlcNS3S/6S, making up the AT-binding site and responsible for most of the anticoagulant activity associated with heparin and LMWH. The major structures identified in LMWH are summarized in supplemental Table 1S. From the sulfate number, we can see these are neither random nor uniformly distributed. In the smaller oligomers (dp4 and dp6), there are almost no GlcNAc residues detected and the most abundant structures have sulfation levels between 4–6 for dp4 and 6–9 for dp6. From dp8 to dp18, structures that contain a single GlcNAc begin to dominate, and from dp20 to dp26, the dominant structures contain 1–2 GlcNAc residues. The relative abundance of different sulfation levels in each dp grouping show a statistical distribution with the highest abundance species in the middle. On-line HILIC LC-FTMS allows detection over a dp range covering ~90% of the components present in the LMWH, enoxaparin, providing a detailed characterization platform for the analysis of this LMWH product.

On-line HILIC LC-FTMS also provides structural information on the minor components present in LMWH. Odd-numbered oligosaccharide and polysaccharide chains were also identified. These can either result from the chains arising from the reducing end of the heparin starting material or from pealing of the low molecular weight heparin under the basic depolymerization conditions (Figure 1). It is unclear whether these odd-numbered chains show any differences from even-numbered chains in their pharamacology. After the approval process for the innovator drug, Lovenox®, but prior to the approval of generic enoxaparin, minor (~16%)[11] components containing 1,6-anhydro aminosugars (*N*-sulfo-1,6-anhydro-β-D-glucosamine or *N*-sulfo-1,6-anhydro-β-D-mannosamine) residues were discovered at their reducing ends of certain chains.[10,11] These residues result from base-catalyzed cyclization reaction as well as a 2-epimerization reaction.[11,45] On-line HILIC LC-FTMS identifies the expected amounts of these structures in dp4, dp6, dp8, and higher components (Figure 3). The presence and appropriate amount of these 1,6-anhydro process by-products are required for the approval of generic versions of Lovenox®.[11,42]

Comparison of two commercial LMWHs

On-line HILIC LC-FTMS was next used to compare three lots of Lovenox® with three lots of generic enoxaparin. Both products are marketed as sterile water solutions in syringes that were freeze-dried to a powder, weighed and directly used in our analysis. Both products show small low lot-to-lot variability and no significant differences between these two products were evident from our HILIC LC-FTMS analysis (Figure 3, 4 and 5).

Bioinformatics for spectral processing

Online LC-MS has been proven to be a powerful tool for glycomics profiling because of the advantages of combining high resolution, robust separation and sensitive detection without labeling. However, manual interpretation of the LC-MS data is incredibly time consuming. Without the help of bioinformatics tools, it is not practical to routinely profile structures from complex LC-MS datasets. Here we use a newly developed software package called GlycReSoft.[41] This GlycReSoft software package enables the rapid extraction of heparin compositions and abundances from LC/MS data. In the first step, the raw data from HILIC LC-FTMS was deconvoluted using DeconTools. In the second step, the output of DeconTools was processed by GlycResoft to generate the matched structures with quantitative information, matching mass accuracy, adducts number and a confidence score. The results of analysis of two different commercialized LMWHs are shown in Figures 3, 4 and 5 for comparison of different components. The quantification dynamic range was more than a thousand. As the LMWH chain length increased to more than dp18, profiling became very difficult. This was due to low signal to noise, dramatically increased ammonium adduction, and a charge number and mono isotope that were hard to resolve and detect.

Conclusions

On-line HILIC LC-FTMS offers a promising new method for the top-down analysis of LMWHs. Several challenges remain for its routine application by the pharmaceutical industry. These include: 1. The extension of the range of this method to larger chain sizes so the 100% coverage of LMHWH products; 2. Improvement of the HILIC-LC separation to allow the determination of even very minor components in this complex product; 3. The application of this technology to other LMWHs made by different processes and having different molecular weight properties and structural features; 4. The application of MS/MS to the components detected by HILIC LC-FTMS and the application of this analysis using other mass spectrometers, including high resolution FT-ICR instruments; and 5. Finding a way to effectively reduce the ammonium adduction required for the determination of larger and more highly sulfated oligomers. Finally, the extension of on-line HILIC LC-FTMS, beyond the realm of pharmaceutical analysis, to diagnostic applications and to address fundamental biological questions about glycosaminoglycans is currently under active investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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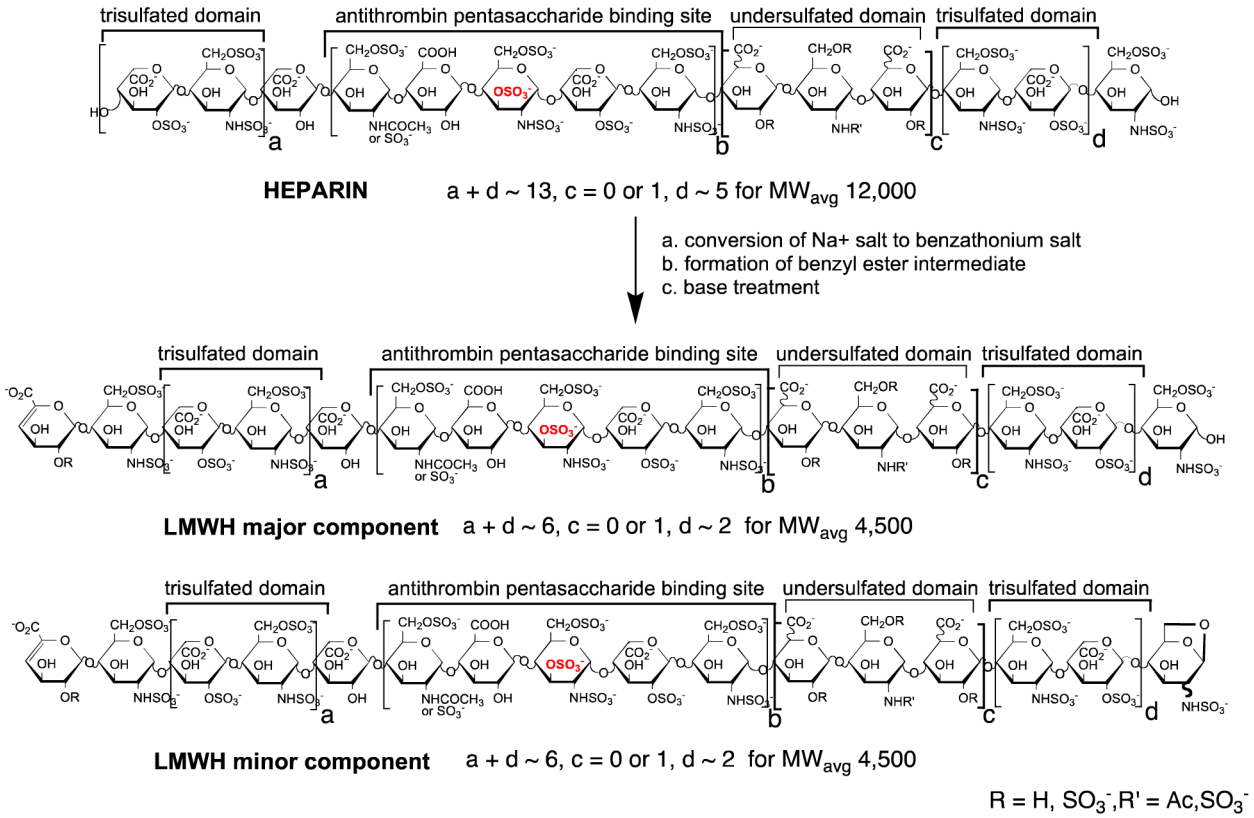

Figure 1.

Synthesis and structure of LMWH (enoxaparin). It is produced by optimized cleavage of the of Heparin from porcine intestinal mucosa is converted to its benzathonium salt, then to its benzyl ester and is chemically β-eliminated by alkaline treatment affording enoxaparin as a sodium salt with a mean molecular weight of 4000–4500 Da with approximately 90% of the oligosaccharides are within the range of 2000–8000 Da. The structures of a major and a minor enoxaparin chain are shown.

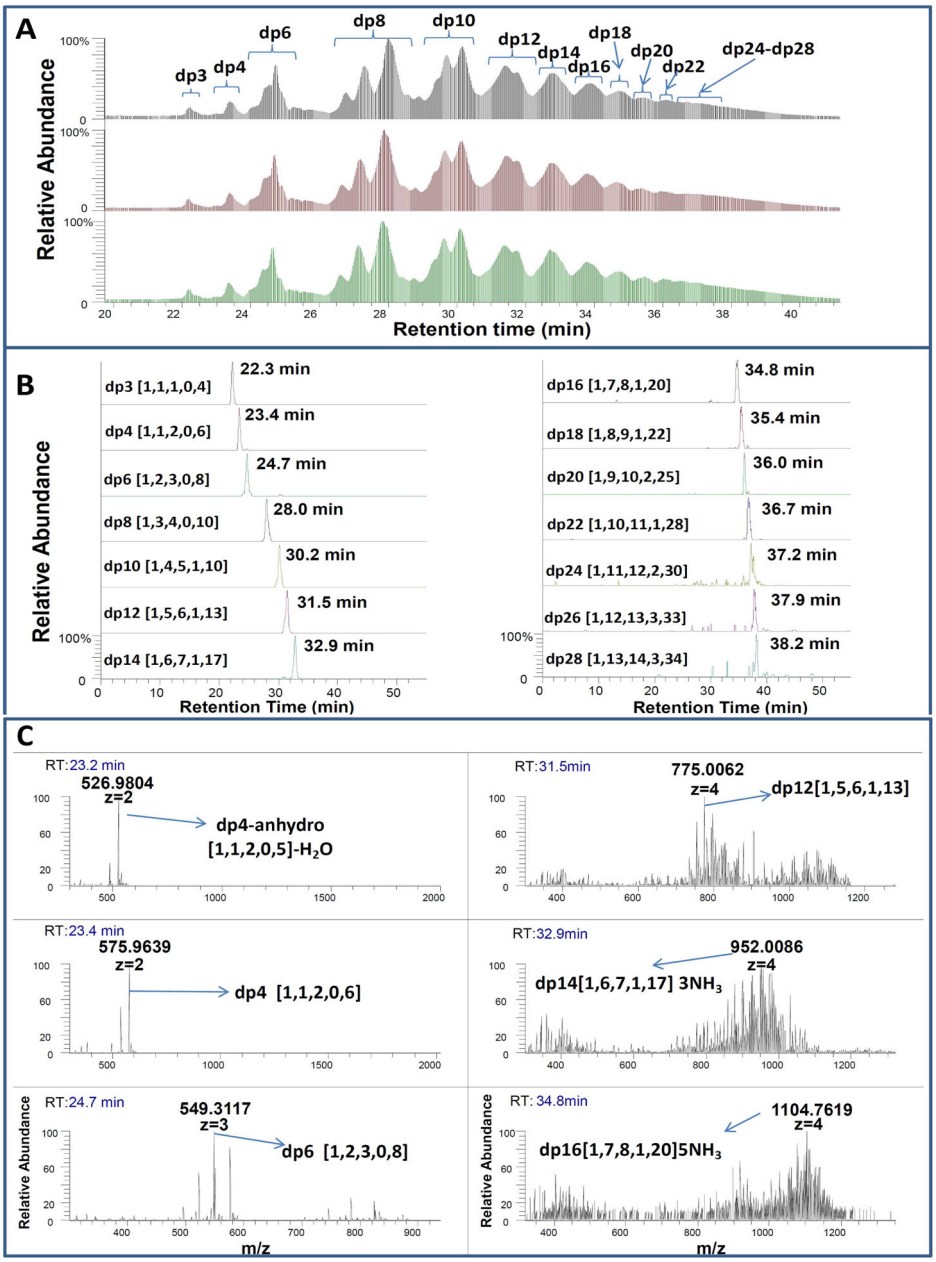

Figure 2.

A. Total ion chromatogram TIC of intact LMWH (triplicate) using ESI HILIC LC-FT MS. Heparin oligomer dp3-dp28 can be separated and FT MS resolved by HILIC LC MS corresponded to the size and charge. B. Selected extracted ion chromatograms (EIC) for the major LMWH structures from dp3 to dp28 demonstrated the highly efficient separation of LMWH and sensitive detection using this HILIC LC-FT MS platform. C. Selected mass spectra and identified major structures. Table 1S in the supplemental materials showed the detailed identification information.

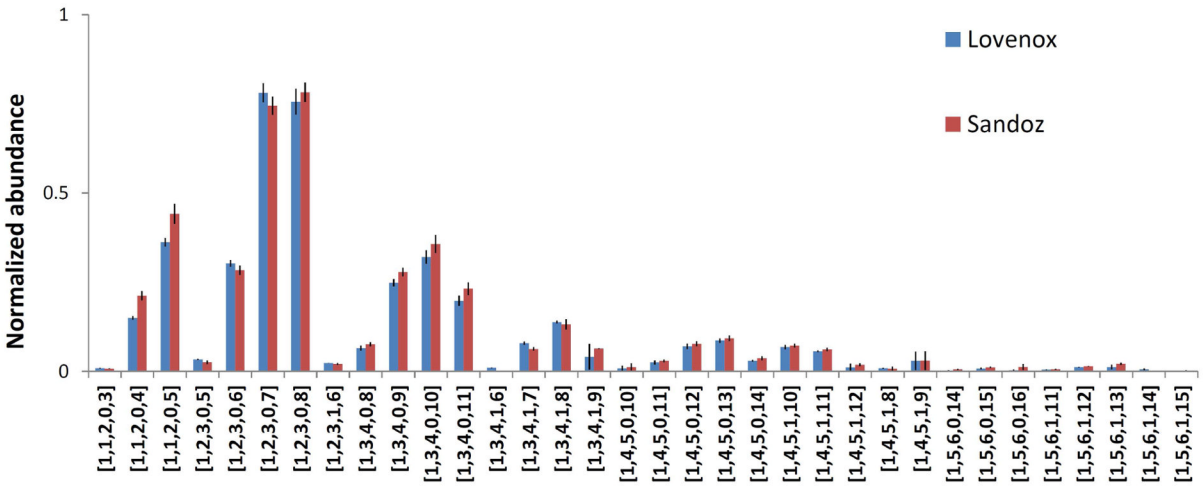

Figure 3.

Quantitative comparison of identified anhydro-heparin oligosaccharides from two commercialized LMWH products. Oligosaccharide compositions are given as [ΔHexA, HexA, GlcN, Ac, SO_3].

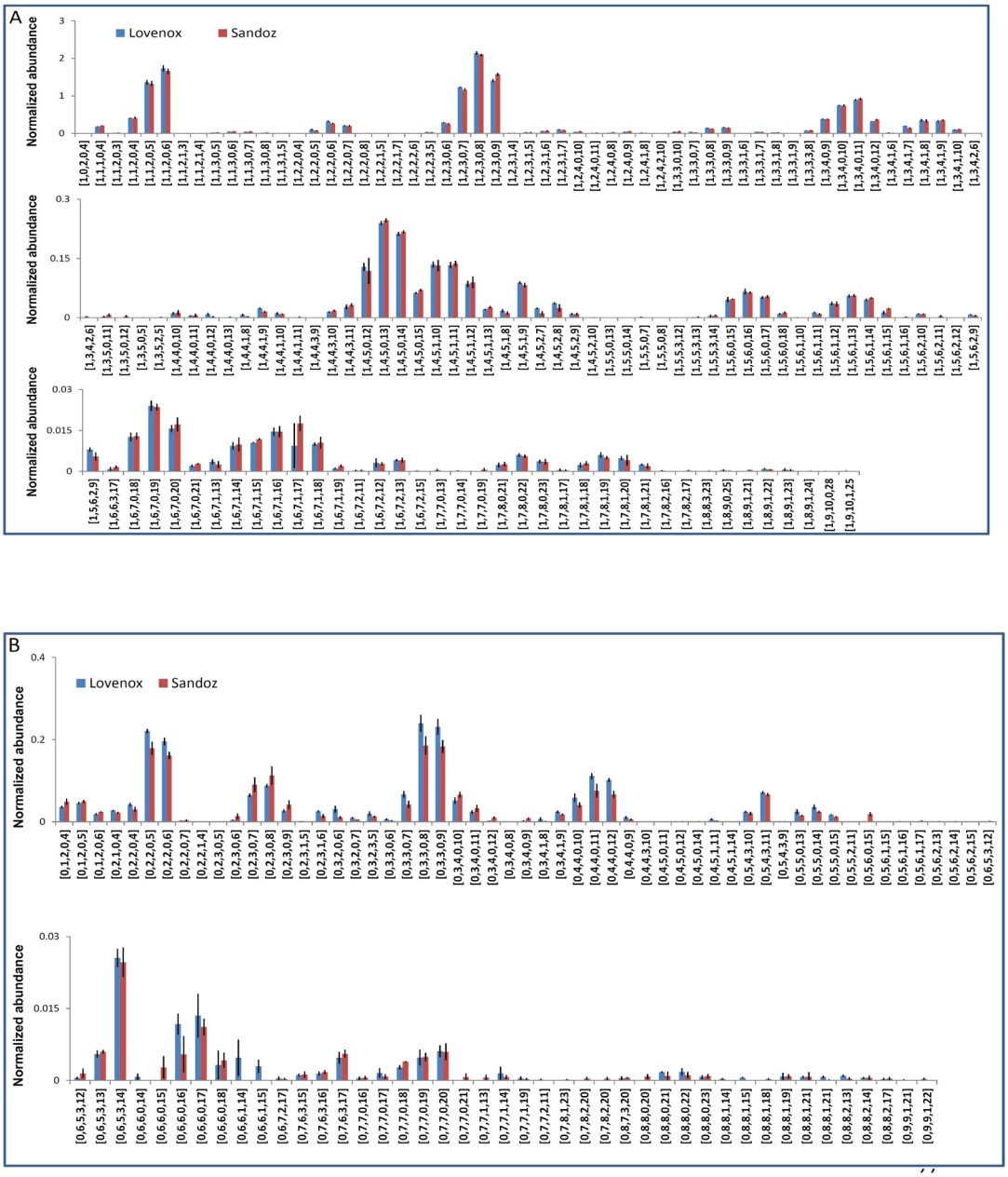

Figure 4.

Quantitative comparison of identified heparin oligosaccharides from two commercialized LMWH products. Oligosaccharide compositions are given as A. [ΔHexA = 1, HexA, GlcN, Ac, SO_3] or B. [ΔHexA = 0, HexA, GlcN, Ac, SO_3].

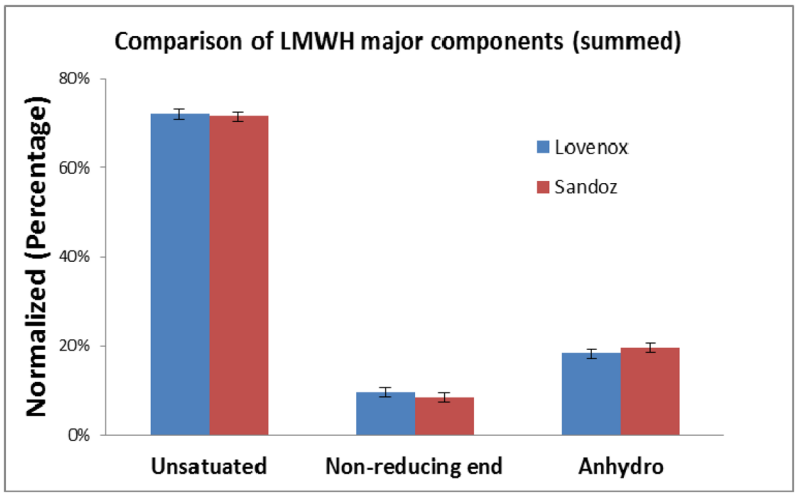

Figure 5.
Relative amount (percentage) of identified 3 major LMWH components.

Table 1

Summarized information for identified LMWH major (even-saccharide number with unsaturated uronate residues at the non-reducing end) structures (dp18 to dp26 structures were manual identified but cannot be quantified because of highly ammonium adducts and very low signal)

Polysaccharide number	Major structures [ΔHexA, HexA, GlcN, Ac, SO3]			
dp4	[1,1,2,0,4–6]	-	-	-
dp6	[1,2,3,0,6–9]	-	-	-
dp8	1,3,4,0,9–12]	[1,3,4,1,7–10]	-	-
dp10	[1,4,5,0,12–15]	[1,4,5,1,8–12]	-	-
dp12	[1,5,6,0,15–18]	[1,5,6,1,11–16]	-	-
dp14	[1,6,7,0,17–21]	[1,6,7,1,13–19]	-	-
dp16	[1,7,8,0,21–23]	1,7,8,1,17–21]	-	-
dp18	[1,8,9,0,21–24]	[1,8,9,1,21–24]	-	-
dp20	[1,9,10,0,26–29]	[1,9,10,1,25–30]	[1,9,10,2,25–30]	-
dp22	-	[1,10,11,1,26–31]	[1,10,11,2,26–32]	-
dp24	-	[1,11,12,1,27–33]	[1,11,12,2,27–32]	-
dp26	-	[1,12,13,1,28–35]	[1,12,13,2,27–33]	[1,12,13,3,27–33]