Comprehensive Identification and Quantitation of Basic Building Blocks for Low-Molecular Weight Heparin

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Ⓢ *Supporting Information*

ABSTRACT: Low-molecular weight heparins (LMWHs) are widely used anticoagulant drugs. They inherit the heterogeneous backbone sequences of the parent heparin, while the chemical depolymerization process modifies the nonreducing end (NRE) and reducing end (RE) of their sugar chains. Some side reactions may also occur and increase the structural complexity of LMWHs. It is important to precisely characterize the structures of LMWHs, especially their chemical modifications, to ensure drug quality and safety. Compositional analysis provides a powerful approach to reveal the building blocks that make up the LMWHs, which are the mutual consequence of the heparin starting materials and the manufacturing process. Here, we introduce a comprehensive

analytical method to recover the most basic building blocks of LMWHs. A strategy of combining both enzymatic digestion and oxidative degradation of LMWH was used to make the NRE, RE, and backbone structures differentiable from one another. Satisfactory separation, identification, and quantitation were achieved by coupling hydrophilic interaction chromatography with a triple quadrupole mass spectrometer operating under the multiple reaction monitoring mode. After enzymatic digestion, over 30 species were detected, with both natural and chemically modified heparin basic building blocks. Two novel structures, including a trisaccharide containing two glucosamine residues and a tetrasaccharide containing a 3-O-sulfated uronic acid residue, were discovered. Reduced and oxidatively degraded samples were analyzed to provide the complementary information on both termini of LMWHs. The reproducibility of this method was evaluated, and enoxaparin injections were analyzed to demonstrate the application of this method for evaluating the sameness of LMWH products.
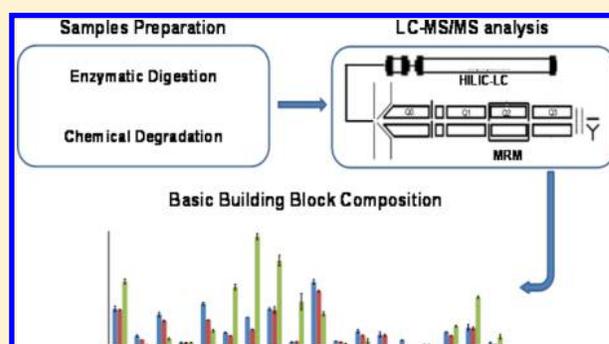
This year, 2016, is the 100th anniversary of the discovery of heparin and the 80th anniversary of the first use of heparin as a clinical anticoagulant drug.[1,2] Over the century, heparin and its derivatives have remained the most important anticoagulant agents, although their structures have not yet to be fully determined. As a family of linear, highly sulfated, negatively charged, and microheterogeneous polysaccharides, the sequencing and quantification every individual chain in heparin and low-molecular weight heparins (LMWHs) are still a major challenge for analytical chemists.[3] The structure of heparin is composed of uronic acid and hexosamine repeating building blocks that are 1,4-glycosidically linked. The uronic acid residue can be either D-glucuronic acid (D-GlcA) or L-iduronic acid (L-IdoA), while the hexosamine residue is D-glucosamine (D-GlcN) with or without N-acetyl (NAc) or N-sulfo (NS) substitution. Heparin can be modified at 2-position of uronic acid residues and 3- and 6-position of hexosamine residues with a O-sulfo group.[4] LMWHs have been produced

by either controlled chemical or enzymatic depolymerization of heparin to reduce the bleeding and thrombocytopenia risk and improve their bioavailability over that of unfractionated heparin.[5] Enoxaparin, manufactured by benzyl esterification and alkaline depolymerization of heparin, has taken the major share of the worldwide LMWH market.[6] Although molecular weight (MW) is decreased in going from heparin to enoxaparin, structural complexity is increased because of the introduction of chemical modifications (Figure 1). For example, the non-reducing end (NRE) of enoxaparin can either retain the saturated uronic acid residue of parent heparin or be modified to an unsaturated Δ4,5-glycuronic acid residue.[7] Similarly, the reducing end (RE) of enoxaparin can contain the linkage region
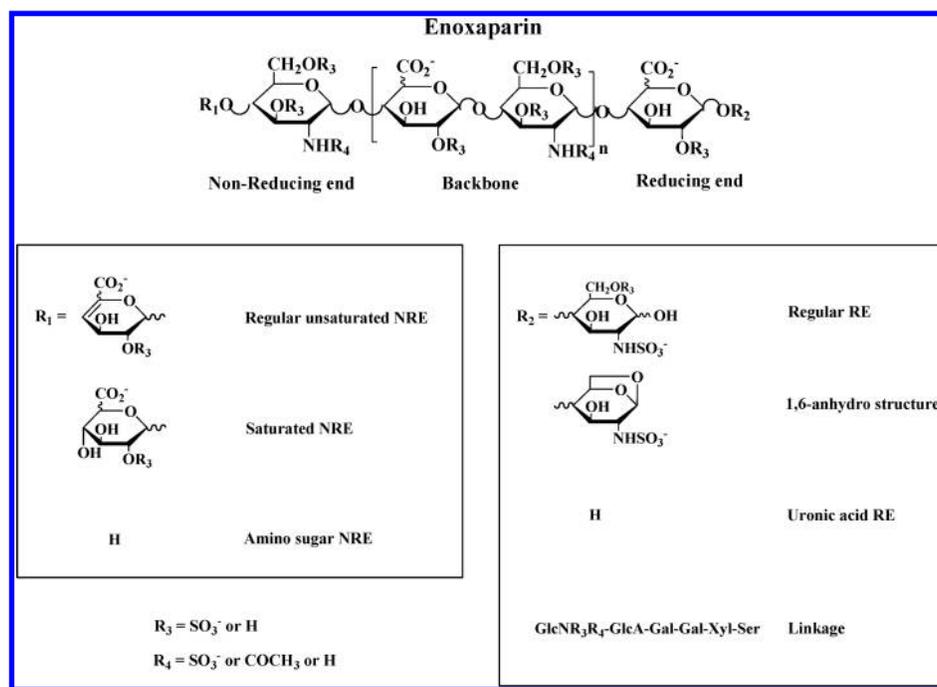
Figure 1. Structure of enoxaparin.

that had attached the original heparin chain to a serine residue of its core protein, a reducing glucosamine aldehyde resulting from the chemical cleavage used in its preparation or a 1,6-anhydro structure as a byproduct from the alkaline hydrolysis.[8] Peeling reactions can also occur, resulting in a hexuronic acid residue at the RE.[9]

Advanced analytical approaches are being developed to deal with the structural complexity of LMWHs. Two dimensional nuclear magnetic resonance (2D-NMR) is an essential tool to determine the substitutions and monosaccharide composition of LMWHs, but the relatively low sensitivity inherent to NMR may result in overlooking some minor components.[10−13] Disaccharide compositions can be obtained using anomeric signals in 2D-NMR.[14] Mass spectrometry (MS) methods, including top-down strategy and bottom-up strategy, provide the high-resolution fingerprinting of LMWH chains. In top-down analysis, intact LMWH chains are separated using size exclusion chromatography, ion pairing reversed phase (IPRP) chromatography, hydrophilic interaction chromatography (HILIC), or combination of two chromatographic methods, then electrospray ionized (ESI) and analyzed by a high-resolution mass analyzer, such as, a time-of-flight (TOF) or a Fourier transform (FT) mass analyzer.[15−20] The composition of hundreds of oligosaccharides, including the number of hexuronic residues and glucosamine residues, the number of O-sulfo, N-sulfo, and/or N-acetyl substitutions, and the type of NRE and RE, can be determined.

In bottom-up approaches, LMWHs are usually exhaustively depolymerized to disaccharides and small oligosaccharides by chemical or enzymatic treatments. The basic building blocks are then analyzed using capillary electrophoresis (CE), liquid chromatography (LC), or LC−MS. Bottom-up analysis is still the primary fingerprinting method in heparin and LMWH characterization and provides valuable complementary information to the top-down analysis. The traditional basic building blocks analysis was restricted to the eight common heparin disaccharides.[21−23] The strong anion exchange (SAX) LC

method for enoxaparin analysis was developed by the United States Pharmacopeia (USP) Convention, emphasizing three characteristic 1,6-anhydro derivatives.[24] This SAX approach can offer a high separation resolution that is an obligatory analysis for all enoxaparin products, although the high salt concentrations in the mobile phase make SAX incompatible with MS analysis.[25,26] Some hyphenated techniques were developed to recover as many building blocks as possible for enoxaparin, including 3-O-sulfated tetrasaccharides, saturated NRE structures, odd number oligosaccharides, and linkage region structures. For example, an IPRP LC−MS method was reported to identify eight species,[21] a CE−MS approach was able to detect 16 components,[16] a precolumn derivatization reversed phase LC−ultraviolet (UV)-MS method was able to detect and quantify 16 components,[7] and a LC−MS/MS method using a Hypercarb column was able to separate and quantify 12 disaccharides.[27] HILIC-LC−MS methods were reported to analyze the disaccharides or oligosaccharide fragments prepared by complete digestion with heparinases cocktail, partial digestion with heparin lyase II, or chemical degradation with nitrous acid.[28,29] However, none of these methods was capable of covering all the reported enoxaparin basic building block structures. Furthermore, several special structures are known to be generated during the heparin or enoxaparin manufacturing process, including galacturonic acid (GalA) residues,[17] epoxide structure on uronic acid residues,[30] and sulfo group directly connected to the carbon of sugar rings (C−S).[31] Some novel structures may even yet be discovered and elucidated. Thus, a sensitive, robust, and comprehensive method that could characterize all possible basic building blocks for enoxaparin is in great demand. Most LC−MS methods quantify the identified species based on their MS intensities and may afford unsatisfactory quantitation performance due to the lack of standards for all known building blocks. The multiple reaction monitoring (MRM) function of the triple quadrupole mass spectrometer is widely recognized for its superior quantitation capability, and MRM based LC−MS/MS methods

Table 1. Building Blocks Identified in Enoxaparin

no.	name	relative composition	no.	name	relative composition
		Enzymatic Digested Building Blocks			
1	ΔIS	70.1% ± 11.3%	16	ΔIIA-IIS$_{glu}$	0.8% ± 0.6%
2	ΔIIS	6.5% ± 0.7%	17	ΔIIS-IIS$_{glu}$	0.3% ± 0.2%
3	ΔIIIS	5.4% ± 2.1%	18	NRE dp2 (2S)	0.3% ± 0.03%
4	ΔIVS	3.3% ± 1.7%	19	NRE dp2 (3S)	0.8% ± 0.3%
5	ΔIA	0.003% ± 0.003%	20	linkage	0.0005% ± 0.0001%
6	ΔIIA	1.1% ± 0.6%	21	linkage$_{ox}$	0.0001% ± 0.00001%
7	ΔIIIA	0.6% ± 0.3%	22	Δdp3 (2S)	0.02% ± 0.02%
8	ΔIVA	0.6% ± 0.4%	23	Δdp3 (2S, 1Ac)	0.02% ± 0.02%
9	1,6-anhydro ΔIIS	0.001% ± 0.0001%	24	Δdp3 (3S)	0.07% ± 0.05%
	1,6-anhydro ΔIIS$_{epi}$		25	Δdp3 (4S)	0.8% ± 0.5%
10	1,6-anhydro ΔIS	0.2% ± 0.1%	26	ΔIVS$_{gal}$	1.0% ± 0.50%
11	1,6-anhydro ΔIS-IS$_{epi}$	3.0% ± 1.9%	27	ΔIIS$_{gal}$	4.2% ± 1.3%
12	ΔI-H	0.6% ± 0.4%	28	Δdp2 (C-S)	0.002% ± 0.001%
13	ΔII-H	0.09% ± 0.07%	29	epoxide	0.06% ± 0.05%
14	ΔIII-H	0.08% ± 0.06%	30	NRE dp3	0.02% ± 0.02%
15	ΔIV-H	0.001% ± 0.0004%	31	Δdp4 (HexA2S3S)	0.1% ± 0.07%
	NRE building blocks			RE building blocks	
1	NRE ΔIS	7.7% ± 1.5%	1	RE dp2 (3S)	54.4% ± 3.2%
2	NRE ΔIIS	3.5% ± 0.3%	2	RE dp2 (2S)	1.2% ± 0.4%
3	NRE ΔIIIS	2.4% ± 1.0%	3	RE dp3 (5S)	16.1% ± 1.1%
4	NRE dp3 (4S)	2.8% ± 0.6%	4	RE dp3 (4S)	25.8% ± 3.7%
5	NRE dp4 (6S)	17.7% ± 2.6%	5	RE dp3(4S, HexA-ol)	1.0% ± 0.4%
6	NRE dp4 (5S)	66.1% ± 3.6%	6	RE dp3 (3S, 1Ac)	1.5% ± 0.5%

(Note: "Chemical Degraded Building Blocks" spans the bottom section, with "NRE building blocks" on the left and "RE building blocks" on the right.)

are usually considered as the gold standard for quantifying pharmaceutical agents.

Here, we describe an LC−MS/MS method aimed at sensitively, accurately, and comprehensively reflecting the detailed structural characters for enoxaparin samples. The LC separation was optimized based on previously reported HILIC method to resolve the complicated basic building blocks generated by enzymatic digestion or oxidative degradation. To our knowledge, this method is capable of detecting the most number of LMWH basic building blocks, and their relative compositions can be determined by MS/MS using the MRM mode.

■ EXPERIMENTAL SECTION

Materials. Enoxaparin reference standards were purchased from the USP convention. Heparin lyase I, II, and III were obtained from Aglyco (Beijing, China). Heparin disaccharide standards, including eight nature disaccharides (ΔIA to ΔIVA and ΔIS to ΔIVS), four N-unsubstituted disaccharides (ΔIH to ΔIVH), and one synthetic disaccharide, ΔUA2S-GlcNCOEt6S (ΔIP), were purchased from Iduron (Manchester, U.K.). Two GalA residue-containing disaccharides, ΔGalA-GlcNS6S (ΔIIS$_{gal}$) and ΔGalA-GlcNS (ΔIVS$_{gal}$), were prepared in our lab. The preparation procedures and structure characterizations are presented in the Supporting Information (Figure S-1). Water, acetonitrile, and ammonium acetate were HPLC grade and purchased from Fisher Scientific (Springfield, NJ). Sodium borohydride, hydrogen peroxide, and all other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO). Lovenox and Clexane Injections are obtained from the hospital. In-house enoxaparin was prepared in our lab.

Enzymatic Digestion of LMWH. Enoxaparin was enzymatically digested by a cocktail of equal amount of heparin lyase I, II, and III with an enzyme to sample ratio of 0.6 mIU to 1 μg in the digestion buffer (100 mM sodium acetate/2 mM calcium acetate, pH 7.0, containing 0.1 g/L bovine serum albumin) at 25 °C for 72 h to achieve the exhaustive digestion. The digests were ultracentrifuged using a 30 kDa MW cutoff spin column to remove enzymes. The pass-through was vacufuge dried and then redissolved with water to a series of concentrations from 1 μg/μL to 20 μg/μL. Enoxaparin injections were dialyzed against 1 kDa dialysis membrane and lyophilized prior to enzymatic digestion.

Reduction and Oxidative Degradation of LMWH. Enoxaparin samples were first reduced by sodium borohydride to modify the reducing end to sorbitol amine. Briefly, samples were dissolved in freshly prepared 20 μg/μL sodium borohydride solution with a concentration of 10 μg/μL. The reaction was quenched by adding acetate acid to adjust the pH to 6−7 after overnight incubation at room temperature. Then the samples were lyophilized and redissolved in 0.1 M sodium acetate−acetic acid buffer (pH 7.0) containing 0.2 mM copper(II) acetate with a concentration of 1 μg/μL. The oxidative degradation was carried out by adding 2% (v/v) of 30% hydrogen peroxide to the reduced LMWH samples and incubated for 5 h at 45 °C. Excess hydrogen peroxide was removed by lyophilization. Sample solutions with a series of concentration from 1 μg/μL to 20 μg/μL were prepared.

LC−MS/MS MRM Analysis. LC separation of basic building blocks of LMWH samples was performed on an Agilent 1100 series LC system (Agilent Technologies, Santa Clara, CA) using a Luna HILIC column (2.0 mm × 150 mm, 200 Å, Phenomenex, Torrance, CA). Mobile phase A was 5 mM ammonium acetate aqueous solution, while mobile phase B was 5 mM ammonium acetate in 95% acetonitrile. The composition of mobile phase B was started at 95% and lasted for 5 min, decreased to 77% in 102 min, then decreased to 50% in 5 min and kept for 23 min to elute all compounds.
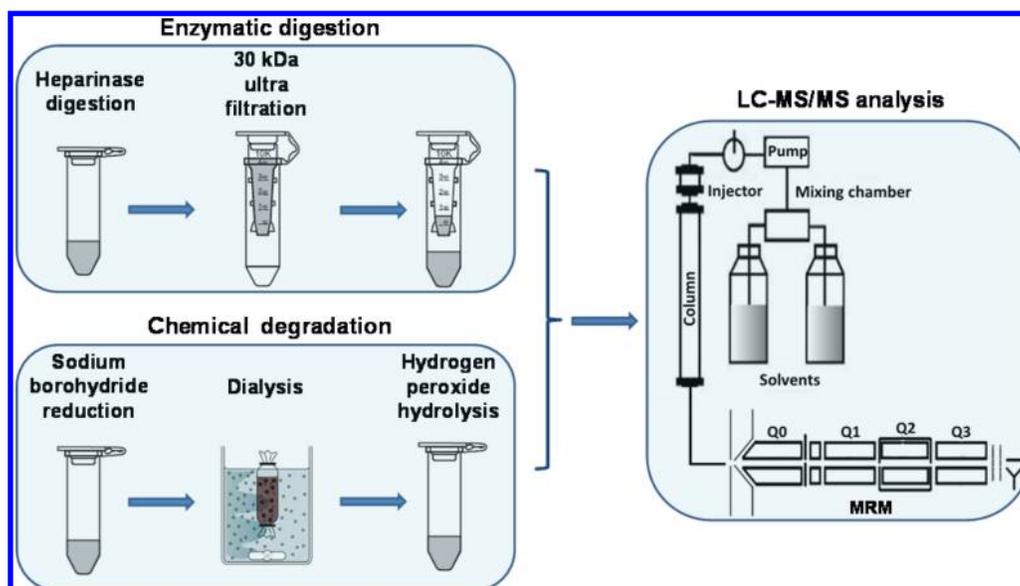
Figure 2. Analytical workflow for basic building block compositional analysis of enoxaparin.

A Thermo TSQ Quantum Ultra mass spectrometer equipped with an ESI source (Thermo Fisher Scientific, San Jose, CA) was connected for online MS/MS detection in the MRM mode. The instrument settings and collision parameters are as follows, tube lens, −75 V; collision energy, 35. All the building block MRM transitions are listed in Tables S-1 and S-2 in the Supporting Information.

Data processing was performed using the Thermo Xcalibur software. Relative amounts of all components were calculated by comparing the peak area of each component with that of internal standard ΔIP. The compositions of all basic building blocks were then calculated based on their relative quantities and summarized in Table 1.

RESULTS AND DISCUSSION

Depolymerization of LMWHs to Basic Building Blocks. The cocktail of heparinase I, II, and III cleave 1,4-glycosidic bonds of enoxaparin under random endolytic mode and leave an unsaturated Δ4,5-glycuronic acid residue at the NRE of digested disaccharides and oligosaccharides. Some unusual structures, such as 3-O-sulfo groups on the GlcN residues and 1,6-anhydro derivatives, may hinder the enzyme activities and result in unsaturated tetrasaccharides instead of disaccharides. Most of the structural characteristics of enoxaparin can be deduced from the enzymatic digests except for the original NRE and RE of enoxaparin chains. The Δ4,5-glycuronidase has been used to remove the first monosaccharide residue from the NRE of enoxaparin and differentiate it from other hexuronic acid residues in the backbone.[32] Oxidative degradation is an alternative way to keep the original NRE of enoxaparin distinguishable, as chemical cleavage of glycosidic bonds does not generate double bonds on the hexuronic acid residues. For the original RE of enoxaparin chains, reduction with sodium borohydride can modify it to an alditol and add a mass tag of 2 Da. The subset of basic building blocks generated from reduction and oxidative degradation of LMWH was also analyzed by LC−MS/MS MRM to provide complementary information to the enzymatic digestion approach (Figure 2).

LC−MS/MS Analysis of Enoxaparin Building Blocks. HILIC LC separation method was established based on a previously reported approach.[28] A longer column, 150 mm running under an optimized gradient, was used to resolve all enoxaparin's basic building blocks from one another. Except for the eight common heparin disaccharides and four N-unsubstituted heparin disaccharides, all other building blocks are not commercially available. Therefore, the heparin lyases digested USP enoxaparin reference standard was analyzed first using Thermo TSQ mass spectrometer under full scan mode. All ions observed that might correspond to possible building blocks of enoxaparin were then further analyzed using MS/MS under product mode and unique fragments were selected for all components. A total of 31 species were identified in the enoxaparin samples treated by heparin lyases, including 29 known and two novel species (Table 1 and Table S-1 in the Supporting Information). Structures already reported include eight natural unsaturated disaccharides,[22] three 1,6-anhydro structures,[24] four trisaccharides, two 3-O-sulfo group containing tetrasaccharides, two saturated NRE structures, two linkage region oligosaccharides,[28] two unsaturated GalA disaccharides,[17] four N-unsubstituted disaccharides,[26] and two unusual structures containing a C-sulfo group and epoxide.[30,31] These structures are summarized from ones presented in 10 publications, as no single publication provided complete coverage of every building block group. Additionally, we also identified a saturated trisaccharide has an aminosaccharide residue at both RE and NRE, as well as a tetrasaccharide with a 3-O-sulfo group on its uronic acid residue. According to the retention time of all the components, the whole MRM program was divided to three segments and 26 channels in total. The extracted ion chromatograms (EICs) are shown in Figure S-2.

The natural heparin disaccharides are major components of enoxaparin. The eight unsaturated disaccharides corresponded to 87.6% of the sample, with the most abundant ΔIS accounting for 70.1%, as calculated from the MRM analysis. These results are comparable to the composition reported by traditional disaccharide analysis methods[5,22,28] (Table S-3 in the Supporting Information). The N-unsubstituted disaccharides are desulfation products of N-sulfo group containing disaccharide units that are formed during the heparin and LMWH manufacturing process steps, including β-elimination

and autoclaving.[33] The loss of anticoagulant potency of heparin products is reported to be associated with these N-desulfation reactions. We were able to detect four disaccharides with free amino groups from the enoxaparin reference standard, and their total relative content was 0.7%. The functional domain in heparin and LMWH is the antithrombin binding pentasaccharide containing a 3-O-sulfo group at the GlcN residue. When this pentasaccharide occurs in a chain, an unsaturated 3-O-sulfo group containing tetrasaccharide or disaccharide is generated by heparinase digestion. Up to 11 different 3-O-sulfated tetrasaccharides have been observed previously by using only heparinase II to treat the sample.[28] In our analysis, six different 3-O-sulfo tetrasaccharides and one tetrasulfated disaccharide were detected by MS (Table S-4 in the Supporting Information). Because of their low abundance and the poor fragmentation capability inherent to the 3-O-sulfation,[34] only two of these were quantified using the MRM analysis. Their combined relative content was 1.1%. If a more complete 3-O-sulfo group containing building block composition analysis is desired, an enrichment step with antithrombin affinity chromatography might be required.

The NRE of enoxaparin either inherits the original NRE of parent heparin or is generated by β-elimination during the LMWH manufacturing process. Two saturated disaccharides with different degrees of O-sulfo group substitution were detected. They are the evidence of the parent heparin and account for 1.1%. A trisaccharide with sequence GlcNS6S-HexA2S-GlcNS6S was also detected with extremely low abundance (Figure 3a). This novel saturated NRE oligosaccharide reveals that the parent heparin chain may start with not only the HexA residue but also with a GlcN residue. A series of unsaturated disaccharides and oligosaccharides, derived from the regular NRE of enoxaparin, were identified from the oxidative degraded sample (Table 1 and Table S-2 in the Supporting Information). It turns out that approximately 95% of unsaturated uronic residues at the NRE are 2-O-sulfated, which is consistent to the 2D-HSQC-NMR analysis of the intact material (Figure S-3 in the Supporting Information).

Similarly, the RE of enoxaparin can either be from the site of cleavage or can contain the original RE of parent heparin. In addition, the 1,6-anhydro derivatives can form as a result of alkaline dehydration. Two linkage oligosaccharides were identified from the enzymatic digests. One is ΔUA-Gal-Gal-Xyl-Ser, through which heparin is linked to its core protein in the biosynthesis pathway, and the other is the oxidized form, ΔUA-Gal-Gal-Xyl-Ser$_{ox}$. The level of linkage region oligosaccharides is much lower than the results afforded through 2D-NMR analysis, which is about 1% based on the signal of xylose residue (Figure S-3 in the Supporting Information). This is due to the poor ionization efficiency and low intensity of product ions of linkage structures in the negative ion mode used for the MS/MS analysis. The 1,6-anhydro structure is characteristic for enoxaparin, and the USP monograph requires their total molar percentage in the range of 15−25%. The relative content of 1,6-anhydro structures was 3% in all basic building blocks, summed up by three 1,6-anhydro derivatives detected in the enzymatic digests. It can be then calculated that approximately 20.3% of the LMWH chains end with 1,6-anhydro structure in the enoxaparin reference standard. Peeling reactions are often observed during the processing of heparin and LMWH.[35] As a result, four unsaturated trisaccharides with different sulfation patterns, were detected and their total relative content was 2.0%.
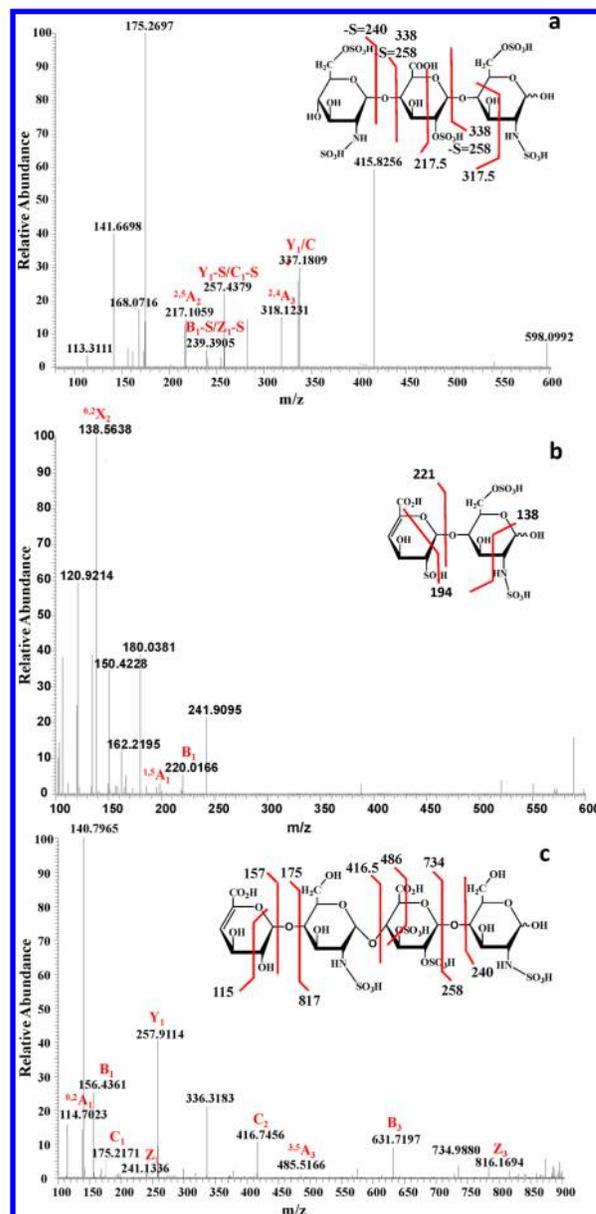

Figure 3. High-resolution LTQ-Orbitrap MS/MS analysis of unusual building block structures: (a) NRE dp3, (b) Δdp2 (C-S), and (c) Δdp4 (HexA2S3S).

Both termini as well as some central residues within enoxaparin can undergo chemical modification during the depolymerization process. Uronic acid dehydration is one of such modification. Desulfation and conversion to GalA residue may take place at the IdoA2S residue, the major uronic acid form in heparin. This reaction affords two GalA residue containing disaccharides, ΔIIS$_{gal}$ and ΔIVS$_{gal}$. They were detected by a CE method and their identities were assumed based on theoretical electrophoretic mobilities.[17] We confirmed these two structures by preparing ΔIIS$_{gal}$ and ΔIVS$_{gal}$ using desulfation and hydrolysis of heparin by heating under weak alkaline conditions (Figure S-1). An unusual structure, with an oxidized sulfur directly linked to a carbon at the C2 position of uronic acid residue, was found likely to be the product of sequential oxidative−reductive treatment.[31] The disaccharide formed through enzymatic digestion has a unique mass of 560.9764 Da. We confirmed the presence of this disaccharide

using a high-resolution LTQ Orbitrap mass spectrometer (Figure 3b) and monitored it with MRM at a very low abundance of 0.002%. An epoxide with a 2,3-anhydro at the uronic acid residue is another unusual structure that has been reported in heparin.[30] It is resistant to heparin lyases and a 2,3-anhydro tetrasaccharide with four sulfo groups was detected from the enzymatic digest. The relative content of the epoxide is 0.06%. Finally, a tetrasaccharide with two O-sulfo groups within the same uronic acid ring was discovered for the first time (Figure 3c). Whether this is a natural heparin structure or modified structure from a chemical reaction is worthy of further investigation.

Method Validation. Since the standards for most enoxaparin basic building blocks are not available, the synthetic heparin disaccharide ΔIP, having a similar structure to natural heparin disaccharide, was added to enzymatically or chemically degraded samples as an internal standard. The relative amount and composition of each component was then normalized in corresponding to the internal standard ΔIP. The USP enoxaparin reference standard was treated by enzymatic digestion and chemical degradation separately. The samples were then prepared at various concentrations, from 1 μg/μL to 20 μg/μL, and analyzed by this newly established LC–MS/MS MRM method. All concentration levels were analyzed in triplicate, and 5 μL was injected for each run. In Figure S-4, curves were drawn of relative amount of each component as a function of injection amount. R^2 values are all greater than 0.97. Furthermore, all relative standard deviations shown in Figure S-4 are low, confirming that this method is reliable and reproducible.

Compositional Comparison of Basic Building Blocks for LMWH Samples. Three LMWH samples, including one lot of Lovenox injection, one lot of Clexane injection, and one lot of in-house prepared enoxaparin, were compared using HILIC-LC–MS/MS after exhaustive enzymatic digestion. Lovenox and Clexane are the innovator's enoxaparin products manufactured by Sanofi Aventis. The in-house enoxaparin was prepared by esterization of unfractionated heparin benzethonium salt with benzyl chloride at 40 °C for 12 h and alkaline depolymerization at 55 °C for 2 h. It was tested for MW, 1,6-anhydro derivatives, NMR, and anti-Factor IIa/Xa according to the USP enoxaparin monograph, and no obvious difference between in-house enoxaparin and reference standard were observed in these tests.[36] Basic building block analysis using MRM provides a much more detailed comparison of these samples. As shown in Figure 4a, the major building block components, including eight natural disaccharides, two GalA residue containing disaccharides, and three 1,6-anhydro derivatives, are similar among three samples, although the in-house enoxaparin showed relatively lower content of 1,6-anhydro structures. Lovenox and Clexane are still highly similar to each other with respect to their minor building block components (Figure 4b). In contrast, some significant differences between innovator's enoxaparin samples and the in-house sample were observed. For example, the total saturated NRE structures of in-house sample are 4.7%, about 3- to 4- fold higher than that of Lovenox (1.4%) and Clexane (1.2%). In-house enoxaparin also contained much less linkage region structures. These results suggest that the parent heparins used to prepare these LMWHs are different. The contents of trisaccharides, epoxides, and C–S containing structures for in-house sample are also dissimilar to the innovator's LMWHs.
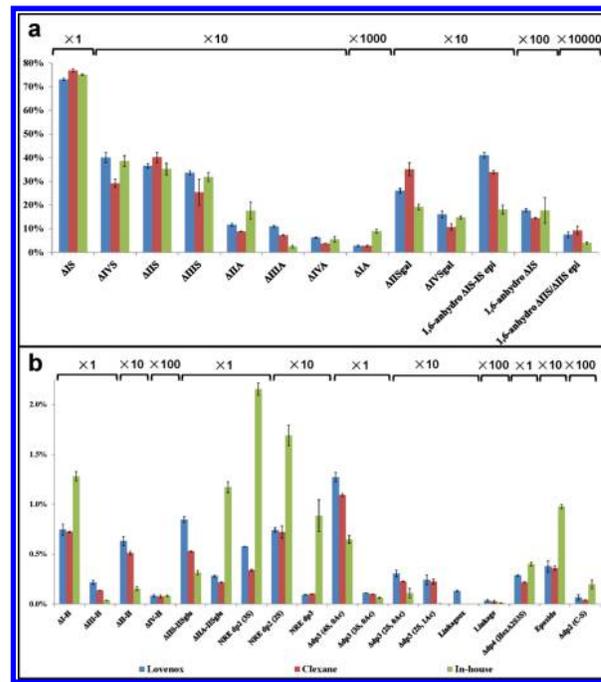

Figure 4. Compositional comparison of three enoxaparin samples: (a) major building block components and (b) minor building block components. Note: ×1, ×10, ×100, ×1000, and ×10 000 are coefficients multiplied to the original values to clearly reveal all building blocks.

Both differences in heparin starting materials and processing conditions are the likely cause of these differences.

■ CONCLUSIONS

Many disaccharide building blocks and compositional analysis methods for heparin and LMWHs have been developed. However, the chromatographic or CE-UV methods cannot detect the building blocks that lack a chromophore. The robustness of LC–MS methods depend on the instrument or operation conditions when quantifying components based on MS peak intensities. In this study, we take advantage of the superior separation resolution of HILIC-LC and excellent quantitation performance of a triple quadrupole mass spectrometer in the MRM mode to establish a sensitive approach to detect most of the basic building block structures in LMWHs. Over 40 different enzymatically digested or chemically degraded components were observed, covering the NRE terminus, the RE terminus, and the backbone region of LMWHs. Some subtle modifications, for example, the 1,6-anhydro derivatives, the epoxide tetrasaccharide, and the disaccharide containing a C–S bond, are also detected. These minor components are useful markers for sensitively detecting the batch-to-batch variability of LMWH products and demonstrating the structural sameness between generic and innovator LMWHs. This analytical method also helps to understand the structural changes from parent heparin to the LMWH products. A couple of new structures were identified, encouraging the continuous efforts on structural study of heparin and LMWH.

ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.anal-chem.6b01709.

Information on all building blocks derived from enzymatic digestion and chemical degradation, comparison of eight natural disaccharides between this method and literature methods, 3-O-sulfo group containing disaccharide and tetrasaccharides detected but not quantified, preparation and structural characterization of GalA residue containing disaccharides, EICs of LC−MS/MS MRM basic building block analysis of USP enoxaparin reference standard, 2D-HSQC-NMR analysis of enoxaparin NRE, reproducibility and repeatability of LC−MS/MS MRM analysis, and MRM mass spectra of all basic building blocks (PDF)

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Notes

The authors declare no competing financial interest.

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