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Hydrophilic interaction chromatography-multiple reaction monitoring mass spectrometry method for basic building block analysis of low molecular weight heparins prepared through nitrous acid depolymerization

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ABSTRACT

Low molecular weight heparins (LMWHs) are important anticoagulant drugs that are prepared through depolymerization of unfractionated heparin. Based on the types of processing reactions and the structures of the products, LMWHs can be divided into different classifications. Enoxaparin is prepared by benzyl esterification and alkaline depolymerization, while dalteparin and nadroparin are prepared through nitrous acid depolymerization followed by borohydride reduction. Compositional analysis of their basic building blocks is an effective way to provide structural information on heparin and LMWHs. However, most current compositional analysis methods have been limited to heparin and enoxaparin. A sensitive and comprehensive approach is needed for detailed investigation of the structure of LMWHs prepared through nitrous acid depolymerization, especially their characteristic saturated non-reducing end (NRE) and 2,5-anhydro-D-mannitol reducing end (RE). A maltose modified hydrophilic interaction column offers improved separation of complicated mixtures of acidic disaccharides and oligosaccharides. A total of 36 basic building blocks were unambiguously identified by high-resolution tandem mass spectrometry (MS). Multiple reaction monitoring (MRM) MS/MS quantification was developed and validated in the analysis of dalteparin and nadroparin samples. Each group of building blocks revealed different aspects of the properties of LMWHs, such as functional motifs required for anticoagulant activity, the structure of heparin starting materials, cleavage sites in the depolymerization reaction, and undesired structural modifications resulting from side reactions.

1. Introduction

Heparin and its depolymerized derivatives, low molecular weight heparins (LMWHs), are the most important pharmaceutical polysaccharides, and are widely used clinically as anticoagulant drugs [1]. The major advantages of LMWHs, over their unfractionated heparin parent, include improved bioavailability, more predictable anticoagulant activity, and more controllable side effects [2]. Different chemical or enzymatic depolymerization reactions are used by the pharmaceutical industry to produce various types of LMWHs [3]. Nitrous acid can cleave glycosidic bonds of heparin to afford chains that possess a saturated uronic acid residue at their non-reducing end (NRE) and a hexosamine-derived aldehyde residue at their reducing end (RE). Sodium borohydride is commonly used to reduce the hexosamine-derived aldehyde residue to a 2,5-anhydro-D-mannitol to remove the reactive aldehyde group and prevent further side reactions [4,5]. Two types of LMWHs, dalteparin and nadroparin, are produced through this

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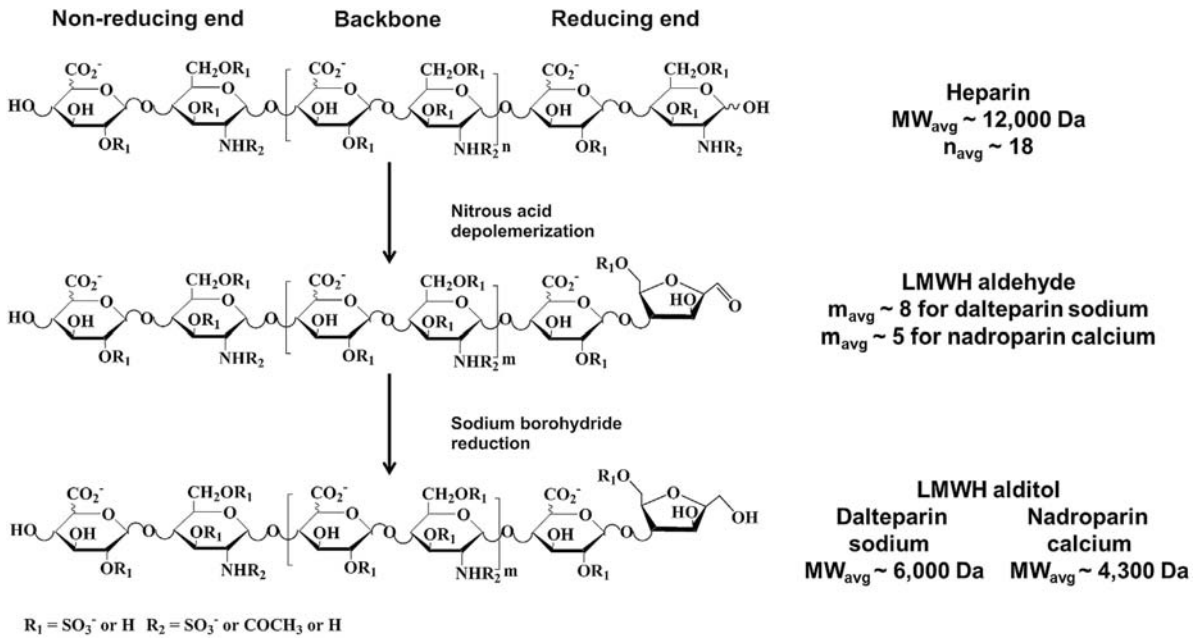

Fig. 1. The structure and process from heparin to LMWHs using nitrous acid depolymerization.

approach [6,7]. Dalteparin is a sodium salt with a molecular weight (MW) of approximately 6000 Da, while nadroparin is a calcium salt with a lower MW of 4300 Da (Fig. 1) [8]. They hold the second and third place in worldwide market share of LMWH drugs.

Structural characterization is critical to ensuring the efficacy and safety of LMWHs. It is also essential for the evaluation of sameness between generic LMWHs and the corresponding innovator products [9]. However, due to the structural complexity and microheterogeneity of LMWHs, the full elucidation of the individual chains remains a significant challenge for analysts. Heparin consists of repeating disaccharide of D-glucuronic acid or L-iduronic acid residues that are 1,4-glycosidically linked to hexosamine residue, with variable sulfation at the 2-*O*-position of uronic acid residues, 3-*O*, 6-*O*, and *N*-positions at the hexosamine residues and *N*-acetylation at the hexosamine residues [10,11]. LMWHs inherit the backbone disaccharide building blocks from the parent heparin, while one or both of chain termini are typically modified during the depolymerization process. Compositional analysis of the basic building blocks is generally used to reveal the fundamental structural characteristics of LMWHs. Numerous approaches for building block analysis have been developed in recent decades. In general, heparin or LMWHs are first exhaustively digested into disaccharides or tetrasaccharides by a cocktail of heparinase I, II, and III. Unsaturated uronic acid residues are generated at the enzymatic cleavage sites, which contain a chromophore that absorbs ultraviolet (UV) at 232 nm [12]. Next, the basic building blocks are separated with liquid chromatography (LC) or capillary electrophoresis (CE) methods, and they are then detected by UV detection or mass spectrometry (MS) [13–16]. Pre-column or post-column fluorescence derivatization is sometimes used to improve the separation resolution and/or detection specificity and sensitivity [17,18]. Most of these building block analysis methods have only studied heparin or enoxaparin, a LMWH prepared through alkaline depolymerization, and they are unsuitable for the analysis of LMWHs prepared by nitrous acid depolymerization. This is because the saturated NRE of dalteparin and nadroparin lacks a chromophore and the alditol RE is unreactive to fluorescence derivatization reagents, thus, these methods overlook one or both of these terminal building blocks. Only a pre-column florescence labeling reverse phase (RP) LC method relying on dual detection by UV and fluorescence was able to observe both the NRE and RE building blocks of dalteparin, allowing the identification and quantification of a total of 17 species [19]. Recently, we reported that as many as 31 building blocks from enoxaparin could be detected [20]. Considering their similar level of structural complexity, we assume that there are many undiscovered building blocks of LMWHs prepared though nitrous acid depolymerization.

The first challenge in developing a comprehensive method for basic building block analysis of LMWHs is the chromatographic separation. Due to the structural similarity of the large number of the possible oligosaccharide components, a method having superior resolution is needed to resolve all building blocks in a relatively short analysis time. Hydrophilic interaction chromatography (HILIC) has become a popular approach for separating heparin disaccharides and oligosaccharides [21,22]. In contrast to strong anion exchange and ion pairing (IP) RP methods, HILIC is easily coupled to on-line electrospray (ESI)-MS. There are several types of HILIC columns, such as diols, amides, zwitterions and maltose immobilized onto the silica gel surface of solid phase [23–25]. It is important to understand the interactions between heparin oligosaccharides and the different types of functional groups present on the HILIC columns. A second challenge is to detect the minor components present in very low abundance. These unusual building blocks are often particularly important as they are sensitive indicators of the processing conditions used during the manufacture of a LMWH. Multiple reaction monitoring (MRM)-MS/MS is a powerful tool for quantitative measurement of target molecules, and can potentially detect most of the basic building blocks present in LMWHs prepared through nitrous acid depolymerization.

In the current study, we establish an HILIC-MRM-MS/MS approach for compositional analysis of LMWHs that were prepared by nitrous acid depolymerization. A modified HILIC column shows significant improvements in resolution with a reduction in separation time. Our method represents the most comprehensive analytical approach to date for determining the structures of dalteparin and nadroparin, allowing the detection and quantification of over 30 different basic building blocks.

Table 1
The building blocks identified in the nitrous acid depolymerized LMWHs and their standards for quantification.

No.	Name	Structure	Quantification standard
1	ΔIA	ΔUA2S-GlcNAc6S	ΔIA
2	ΔIIA	ΔUA-GlcNAc6S	ΔIIA
3	ΔIIIA	ΔUA2S-GlcNAc	ΔIIIA
4	ΔIVA	ΔUA-GlcNAc	ΔIVA
5	ΔIS	ΔUA2S-GlcNS6S	ΔIS
6	ΔIIS	ΔUA-GlcNS6S	ΔIIS
7	ΔIIIS	ΔUA2S-GlcNS	ΔIIIS
8	ΔIIS_{gal}	ΔGalA-GlcNS6S	ΔIIS
9	ΔIVS	ΔUA-GlcNS	ΔIVS
10	ΔIVS_{gal}	ΔGalA-GlcNS	ΔIVS
11	ΔI-H	ΔUA2S-GlcN6S	ΔI-H
12	ΔII-H	ΔUA2S-GlcN	ΔII-H
13	ΔIII-H	ΔUA-GlcN6S	ΔIII-H
14	$\Delta IIA\text{-}IIS_{glu}$	ΔUA-GlcNAc6S-GlcA-GlcNS3S6S	Fondaparinux
15	$\Delta IIS\text{-}IIS_{glu}$	ΔUA-GlcNS6S-GlcA-GlcNS3S6S	Fondaparinux
16	3-*O*-S Δdp2	1, 0, 1, 0, 4[a]	n.q.[b]
17	3-*O*-S Δdp4 (3S, 1Ac)	1, 1, 2, 1, 3	n.q.
18	3-*O*-S Δdp4 (4S, 0Ac)	1, 1, 2, 0, 4	n.q.
19	3-*O*-S Δdp4 (5S, 1Ac)	1, 1, 2, 1, 5	n.q.
20	3-*O*-S Δdp4 (6S, 0Ac)	1, 1, 2, 0, 6	n.q.
21	NRE dp2 (2S)	IdoA2S-GlcNS	ΔIIIS
22	NRE dp2 (3S)	IdoA2S-GlcNS6S	ΔIS
23	NRE dp3	GlcNS6S-HexA2S-GlcNS6S	ΔIS
24	Δdp2 (1S) RE	ΔUA-Mnt6S	ΔIIA
25	Δdp2 (2S) RE	ΔUA2S-Mnt6S	ΔIA
26	Δdp4 (3S) RE	ΔUA2S-GlcNS-UA-Mnt6S	Fondaparinux
27	Δdp4 (4S) RE	ΔUA2S-GlcNS-UA2S-Mnt6S	Fondaparinux
28	Δdp4 (5S) RE	ΔUA2S-GlcNS6S-UA2S-Mnt6S	Fondaparinux
29	$Linkage_{ox}$	$\Delta UA\text{-}Gal\text{-}Gal\text{-}Xyl\text{-}O\text{-}Ser_{ox}$	Fondaparinux
30	Linkage	ΔUA-Gal-Gal-Xyl-O-Ser	Fondaparinux
31	Δdp3 (2S)	ΔUA-GlcNS6S-HexA	ΔIS
32	Δdp3 (3S)	ΔUA2S-GlcNS6S-HexA	ΔIS
33	Δdp3 (4S)	ΔUA2S-GlcNS6S-HexA2S	ΔIS
34	Δdp2 (C-S)	ΔUA2CS-GlcNS6S	ΔIS
35	Epoxide	ΔUA2S-GlcNS6S-GlcA-2,3-anhydro-GlcNS	Fondaparinux
36	Δdp4 (HexA2S3S)	ΔUA-GlcNS-HexA2S3S-GlcNS	Fondaparinux

[a] The figures represent the number of ΔHexA residues, HexA residues, GlcN residues, acetyl groups and sulfo groups, respectively.
[b] n.q. represents not quantified.

2. Materials and methods

2.1. Materials

Dalteparin reference standard was purchased from the European Pharmacopoeia (EP). Eight natural heparin disaccharide (ΔIS to ΔIVS and ΔIA to ΔIVA), four *N*-unsubstituted disaccharide (ΔIH to ΔIVH) and one synthetic disaccharide (ΔUA2S-GlcNCOEt6S, ΔIP) standards were purchased from Iduron (Manchester, UK). Two galacturonic acid residue-containing disaccharides, ΔGalA-GlcNS6S (ΔIIS_{gal}) and ΔGalA-GlcNS (ΔIVS_{gal}), were prepared in our lab [20]. Heparinase I, II, and III were obtained from Aglyco (Beijing, China). HPLC grade water, acetonitrile and ammonium acetate were purchased from Fisher Scientific (Springfield, NJ, USA). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fragmin, Fraxiparine and Fondaparinux injections were obtained from the hospital formulary. HILIC columns examined include a XAmide column (5 μm, 2.1 × 150 mm), a Click XIon-G column (5 μm, 2.1 × 150 mm) and a Click Mal column (5 μm, 2.1 × 150 mm) from Acchrom Technologies (Beijing, China) as well as a Luna Diol column (3 μm, 2.0 mm × 150 mm) from Phenomenex (Torrance, CA).

2.2. Enzymatic digestion of LMWHs

All dalteparin and nadroparin samples for injection were dialyzed against water using a MW cut-off 1000 Da dialysis membrane and then lyophilized. The EP dalteparin standard and LMWH samples were incubated with a cocktail of heparinase I, II and III (0.6 mIU total enzymes per μg of sample). The digestion buffer was 100 mM sodium acetate/2 mM calcium acetate, which contained 0.1 g/L bovine serum albumin (pH 7.0). The LMWHs were exhaustively digested to their basic building blocks at 37 °C overnight. The digests were recovered by an ultra-centrifugation step using a 30 kDa MW cut-off spin column. The flow-through was vacufuge dried and reconstituted in water.

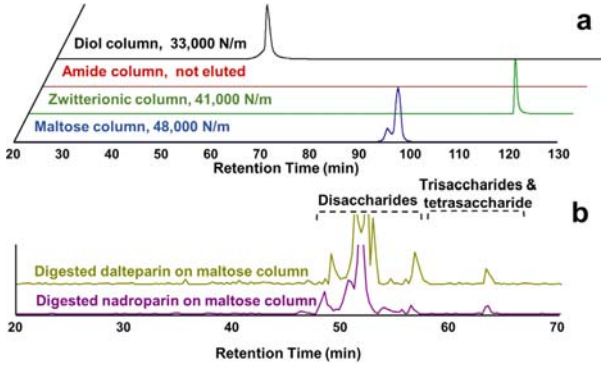

Fig. 2. The separation on HILIC columns. (a) Comparison of different types of HILIC columns using disaccharide standard ΔIS; and (b) Elution of dalteparin and nadroparin building blocks on the maltose column.

2.3. LC separation of building blocks

The LC separation was performed on a 1100 series LC system (Agilent Technologies, Santa Clara, CA). Mobile phases A and B were 5 mM ammonium acetate in aqueous solution and 95% acetonitrile, respectively. A step gradient consisting of 95% mobile phase B for

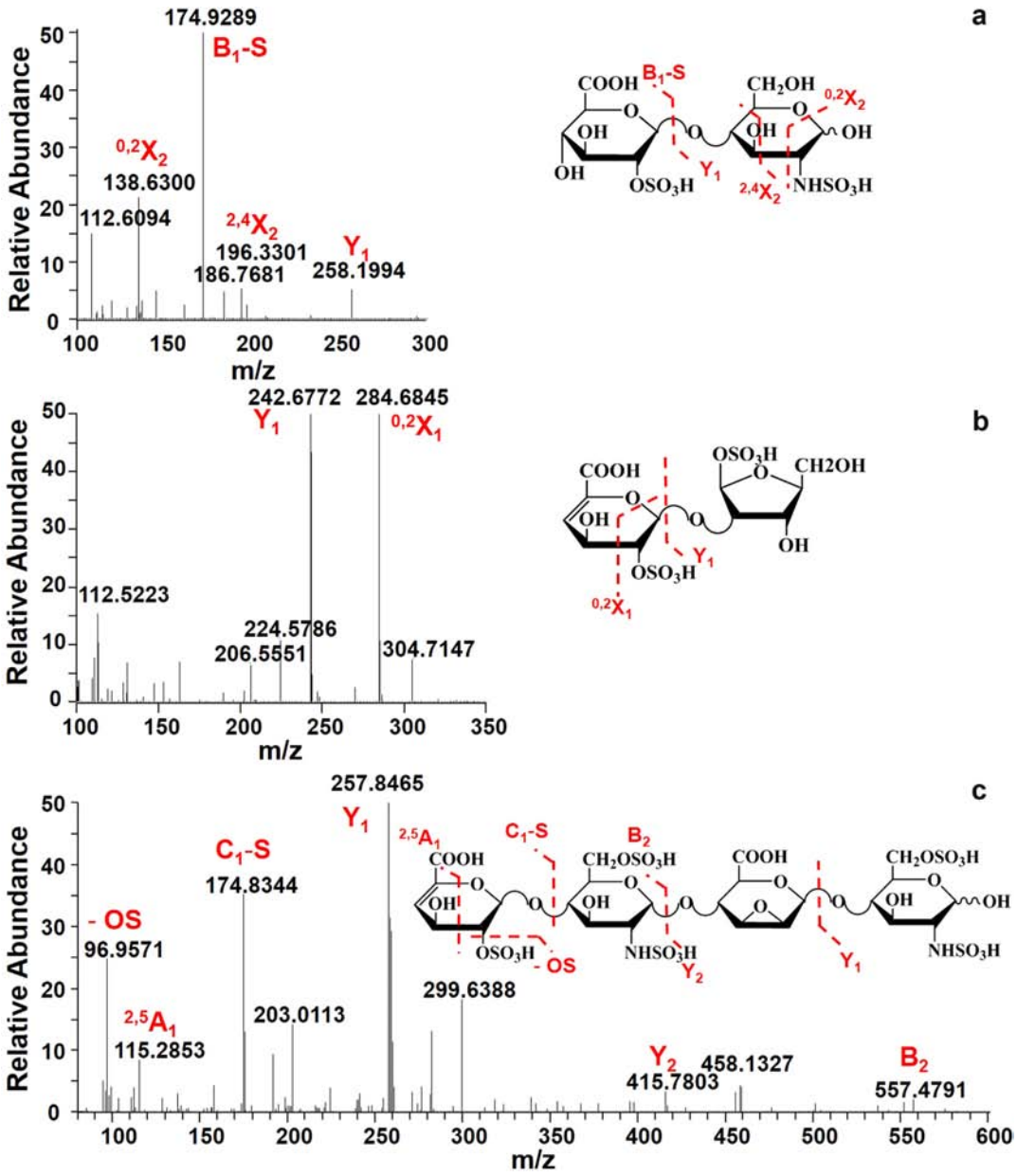

Fig. 3. The MS/MS analysis of terminal and modified building block structures (a) NRE dp2 (3S); (b) Δdp2 (2S) RE; and (c) epoxide tetrasaccharide.

5 min, 95% to 77% mobile phase B from 6 min to 107 min, 77% to 50% from 107 to 112 min, and 50% from 112 min to 135 min was used. Disaccharide standard ΔIS was tested on four different types of HILIC columns, and the numbers of theoretical plates were calculated for each column.

The EP dalteparin reference standard digest was separated using the following conditions. The stationary phase was the Acchrom Click Mal column (5 μm, 2.1 × 150 mm). The gradient of mobile phases was started at 90% mobile phase B and lasted for 5 min, which decreased to 77% in 50 min and then further decreased to 50% in 10 min and then remained steady for 5 min to elute all components. The flow rate was 250 μL/min.

2.4. Identification of building blocks

The digest derived from the EP dalteparin reference standard was analyzed by LC–MS/MS using a Thermo Fisher LTQ-Orbitrap XL mass spectrometer in the negative-ion mode. For the components with standards, their identities were confirmed by the retention times and high resolution MS. For the species without a standard, collision-induced dissociation (CID)-MS/MS was performed to elucidate their structures. The MS and MS/MS parameters were set as follows: spray voltage: 4.2 kV, capillary voltage: −40 V, sheath gas: 15 arb, aux gas: 5 arb, capillary temperature: 275 °C, tube lens: −50 V and collision energy: 50.

2.5. LC-MRM-MS/MS analysis

The MRM-MS/MS analysis was performed on a Thermo Fisher Scientific TSQ Quantum Ultra triple quadrupole mass spectrometer that was equipped with an ESI ion source. The instrument settings are as follows, spray voltage: 3.7 kV, sheath gas: 20 arb, aux gas: 0 arb, capillary temperature: 275 °C, tube lens: −75 V, and collision energy: 35. The MRM transitions used to monitor the building blocks are listed in Fig. 4.

A Thermo Xcalibur workstation was used for data processing. All runs were normalized by the response of internal standard ΔIP to eliminate systematic errors. Additionally, the relative content and compositions of all basic building blocks were calculated based on comparing the peak areas of each component with that of appropriate standards, as listed in Table 1.

3. Results and discussion

3.1. Comparison of HILIC columns

There are a variety of HILIC columns available for separating polar molecules. Only the diol layer coated HILIC column has been previously applied to analyze heparin oligosaccharides [20–22]. Three additional HILIC columns, including amide, zwitterion and maltose modified columns, were compared with the diol column. A heparin disaccharide standard, ΔIS, was used and the numbers of theoretical plates were calculated to demonstrate their capabilities of retaining acidic oligosaccharides. The diol column gave a satisfactory resolution with the number of theoretical plates of 33,000 N/m. The amide column is traditionally used in the separation of complex carbohydrates. However, the heparin disaccharide ΔIS could not be eluted from the amide column, possibly due to the electrostatic interaction between the surface positive charge of the column and the negative charge of ΔIS. The zwitterionic column has amino groups and carboxyl groups on the stationary phase that can be used to separate both positively and negatively charged molecules. It showed better separation resolution compared to the diol column, and the number of theoretical plates was 41,000 N/m. The maltose column is a unique type of HILIC column that separates carbohydrates through controllable hydrogen-bonding force [25]. It offered the highest number of theoretical plate of 48,000 N/m. With the superior resolution of the maltose modified stationary phase, the elution time of all LMWH building blocks was reduced to 70 min, which is nearly half that of the previous method using a diol column (Fig. 2) [20].

3.2. Identification and quantitation of basic building blocks

Many different building blocks were generated from the EP dalteparin reference standard by exhaustive digestion with heparinase I, II and III. For those species with corresponding commercially available standards, including *N*-sulfated disaccharides from ΔIS to ΔIVS, *N*-acetylated disaccharides from ΔIA to ΔIVA, and *N*-unsubstituted disaccharides from ΔIH to ΔIVH, their identities were confirmed by retention times and mass spectra. In-house prepared standards were used to locate two disaccharides containing galacturonic acid residue, ΔIIS_{gal} and ΔIVS_{gal}. For the remaining uncommon building blocks, which lacked standards, their structures were elucidated by tandem MS, performed on an LTQ-Orbitrap mass spectrometer. As in the examples in Fig. 3, the minor components from the NRE, RE and backbone of the dalteparin chains were assigned based on their glycosidic bonds and cross-ring fragmentations in the CID-MS/MS analysis [26]. MRM transitions were set accordingly, and the chromatograms of all building blocks and standards are shown in Fig. 4.

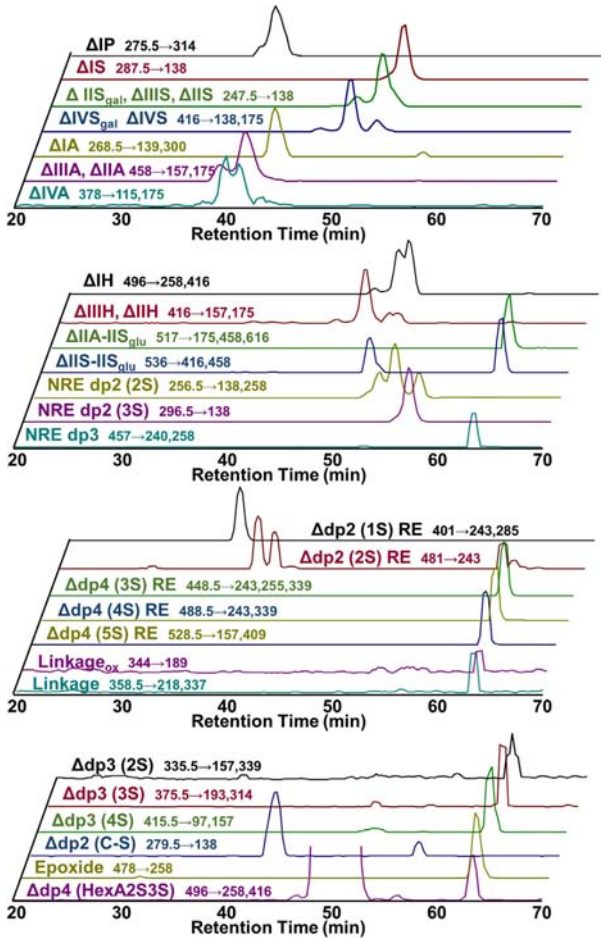

Fig. 4. The EICs of LC–MS/MS-MRM analysis of basic building blocks derived from the EP dalteparin reference standard.

Because of the various chain lengths and sulfation patterns of building blocks, their efficiencies in ionization and fragmentation during MRM analysis are significantly different. A mixed external standard containing equal levels of twelve commercial heparin disaccharides and one synthetic heparin pentasaccharide, Fondaparinux, was used to quantify different categories of building blocks to make the quantitative results closely approximate the real compositions of these building blocks in LMWHs. As summarized in Table 1, the disaccharide and trisaccharide levels were calculated according to disaccharide standards, while the tetrasaccharides and linkage structures were quantified using the pentasaccharide standard. An internal standard, the synthetic heparin disaccharide ΔIP, was also used. After LC-MRM-MS/MS analysis of each sample, all integrated peak areas were first normalized by comparing the intensity of ΔIP in samples to that in external standards. Then, the relative content of each component was calculated based on the comparison of normalized peak areas between samples and external standards.

The digested EP dalteparin reference standard was injected from levels of 5–100 μg in triplicate to validate the LC-MRM-MS/MS quantitation method. The normalized MRM peak area of all the previously identified building blocks exhibited good linearity against the injected amount of reference standard. All R^2 values are higher than 0.98, indicating this method is capable to quantitively determine the building block composition of nitrous acid depolymerized LMWHs.

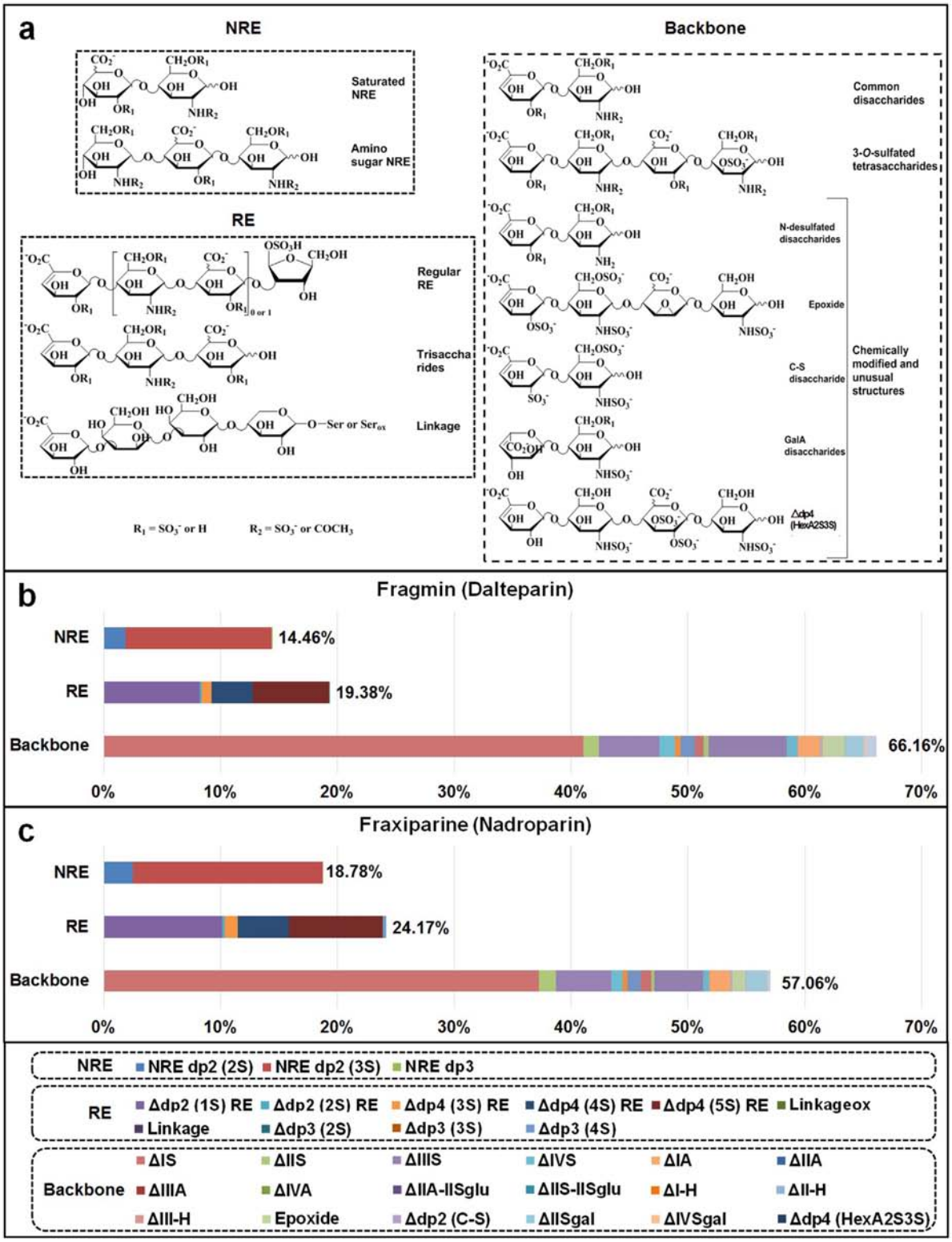

Fig. 5. Compositional analysis of dalteparin and nadroparin samples. (a) Sorting of basic building blocks; (b) building block composition of dalteparin samples; and (c) building block composition of nadroparin samples.

3.3. Compositional analysis of dalteparin building blocks

The building blocks of nitrous acid depolymerized LMWHs can be sorted into three groups, the ones derived from the NRE, the ones from the RE, and the ones from the backbones of LMWH chains (Fig. 5a). The terminal building blocks from the NRE and RE are characteristic for each type of LMWH, and their compositions provide useful information on the chemical cleavage during the LMWH manufacturing process. Two saturated disaccharides with different sulfation degrees were detected. They represented the original NRE of dalteparin chains because building blocks from other locations would contain an unsaturated uronic acid residue at their NRE as a result of heparinase cleavage. The trisulfated disaccharide was dominant, indicating that the nitrous acid preferred to hydrolyze the glycosidic bonds of heparin at the highly sulfated region. Interestingly, a saturated trisaccharide with a glucosamine residue at its NRE was observed at very low abundance, suggesting that the nitrous acid induced hydrolysis mainly occurs at the 1 → 4 glycosidic bonds between glucosamine and uronic acid residues instead of the 1 → 4 glycosidic bonds between uronic acid and glucosamine residues.

The RE of dalteparin is modified by the borohydride reduction to afford a 2,5-anhydro-D-mannitol structure. Five building blocks containing the mannitol structure were observed. Because the unnatural heparin structure was somewhat resistant to the heparinases, three of these were obtained as tetrasaccharides. The ΔUA2S-GlcNS6S-UA2S-Mnt6S and ΔUA2S-GlcNS6S-UA2S-Mnt6S tetrasaccharides were the two most abundant RE building blocks, providing complementary evidence that the highly sulfated region of the parent heparin was more vulnerable to nitrous acid cleavage. Two linkage tetrasaccharides that conserve the original RE of heparin were also observed. In nature, heparin is linked to the serine residues of its core proteins through a defined tetrasaccharide sequence. This unique structure was retained during the depolymerization process from heparin to LMWH, and the ΔUA-Gal-Gal-Xyl-Ser linkage building block and its oxidized form, ΔUA-Gal-Gal-Xyl-Ser_{ox}, were detected from the enzymatic digest of dalteparin. The third subset of RE building blocks involved three trisaccharides that ended with uronic acid residues at their REs. These odd numbered oligosaccharides arose from a side reaction, called the peeling reaction, by which the monosaccharide residues of the LMWH were peeled from the RE.

The building blocks from the central portion of the dalteparin chains can be sorted into three subgroups, the common disaccharides, 3-*O*-sulfated oligosaccharides and chemically modified minor structures. The eight common disaccharides, ΔIS to ΔIVS and ΔIA to ΔIVA, construct the majority of heparin. The compositions of these disaccharides are inherent to the nature of heparin starting materials. The 3-*O*-sulfated oligosaccharides were derived from the pentasaccharide sequence, the anticoagulant activity motifs of heparin and LMWHs. The 3-*O*-sulfo group at the glucosamine residue hinder the action of heparinases, leaving tetrasaccharides as major digested products instead of disaccharides. Finally, a variety of building blocks generated by different side reactions during the heparin and LMWH preparation processes were observed. The *N*-desulfation of glucosamine residues is a commonly occurring side reaction in the processing of heparin. Three *N*-desulfated disaccharides, ΔIH to ΔIIIH, were observed in the analysis of dalteparin building blocks. *O*-desulfation is also frequently observed and can lead to complicated rearrangements of sugar rings. For example, the epoxide building block that contains a 2,3-anhydro structure was the result of the 2-*O*-desulfation at the uronic acid residue. It can go through further reactions under oxidation-reduction conditions and form C-S bond containing structures [27]. The corresponding building block Δdp2 (C-S) was detected. The 2-*O*-desulfation reaction of the iduronic acid residue can also change it to a galacturonic acid residue, and two disaccharides, ΔIIS_{gal} and ΔIVS_{gal}, were separately eluted from their isomers, ΔIIS/ΔIIIS and ΔIVS. In addition, a tetrasaccharide containing a 2,3-disulfated uronic acid residue was discovered. This rare heparin structure was also found in enoxaparin, but its possible origins have not been clarified.

The averaged relative composition of each building block from three lots of Fragmin, the brand-named dalteparin manufactured by Pfizer, is presented in Fig. 5b. The summed weight compositions of NRE, backbone and RE building blocks were 14.46%, 66.16% and 19.38%, respectively. The disaccharide ΔIS was the most abundant component, accounting for 41.06% of the total weight of dalteparin. The compositions of eight natural disaccharides within themselves were consistent with the heparin disaccharide compositions reported previously, suggesting that the nitrous acid hydrolysis and borohydride reduction reactions during the dalteparin processing did not significantly affect the structure of the heparin backbone [14,15,17]. A minority of building blocks (5.87%) resulted from side reactions, including the peeling reaction, *N*-desulfation and the *O*-desulfation, which were followed by rearrangements. It is very important to monitor these unusual structures because they may either decrease the efficacy or increase the allergic or toxic risks of the LMWH drug. However, they are very likely to be neglected when analytical methods are not sufficiently sensitive or comprehensive.

3.4. Compositional analysis of nadroparin building blocks

Three lots of Fraxiparine, the brand-named nadroparin from GlaxoSmithKline, were analyzed by the HILIC-MRM-MS/MS method, and their building block compositions are presented in Fig. 5c. The same set of building blocks as the dalteparin was detected for nadroparin. These two LMWHs are prepared using the same depolymerization and reduction reactions, except that nadroparin requires further depolymerization to smaller MW and an ion exchange step to replace the sodium cation with a calcium cation. Compared to dalteparin, nadroparin contains more NRE and RE building blocks and fewer backbone building blocks. This is explained by the shorter chain size of nadroparin compared to dalteparin.

4. Conclusions

A comprehensive and sensitive HILIC-MRM-MS/MS method for the compositional analysis of nitrous acid depolymerized LMWHs was developed. The maltose modified HILIC column showed superior resolution for separating complicated basic building blocks of dalteparin and nadroparin that were derived by heparinase I, II and III digestion. Compared to previous methods, the current method does not require a derivatization step and is capable of discovering the greatest number of building blocks. Over 30 different components were identified and quantified, including the natural structures of heparin as well as the characteristic terminal structures that were modified by nitrous acid hydrolysis and borohydride reduction. A variety of minor components that were induced by different side reactions during heparin and LMWH processing were also determined, including *N*-unsubstituted disaccharides from *N*-desulfation, galacturonic acid residue-containing disaccharides, epoxide tetrasaccharide and C-S bond containing disaccharide from the *O*-desulfation and rearrangement, and odd-number oligosaccharides from the peeling reaction. The compositional analysis of three lots of dalteparin and three lots of nadroparin samples was demonstrated, and the results provided in-depth evaluation of both the starting heparin materials and the manufacturing parameters.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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