Sequencing the oligosaccharide pool in the low molecular weight heparin dalteparin with offline HPLC and ESI–MS/MS

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ABSTRACT
Low molecular weight heparins (LMWHs) are widely used anticoagulant drugs. The composition and sequence of LMWH oligosaccharides determine their safety and efficacy. The short oligosaccharide pool in LMWHs undergoes more depolymerization reactions than the longer chains and is the most sensitive indicator of the manufacturing process. Electrospray ionization tandem mass spectrometry (ESI–MS/MS) has been demonstrated as a powerful tool to sequence synthetic heparin oligosaccharide but never been applied to analyze complicated mixture like LMWHs. We established an offline strong anion exchange (SAX)-high performance liquid chromatography (HPLC) and ESI–MS/MS approach to sequence the short oligosaccharides of dalteparin sodium.

With the help of in-house developed MS/MS interpretation software, the sequences of 18 representative species ranging from tetrasaccharide to octasaccharide were obtained. Interestingly, we found a novel 2,3-disulfated hexauronic acid structure and recomfirmed it by complementary heparinase digestion and LC–MS/MS analysis. This approach provides straightforward and in-depth insight to the structure of LMWHs and the reaction mechanism of heparin depolymerization.

1. Introduction
Heparin and low molecular weight heparins (LMWHs) have been widely used as anticoagulant drugs for prevention and treatment of thrombotic diseases due to their high binding affinity to antithrombin III (ATIII), which then inactivates several enzymes of the coagulation system (Gray, Mulloy, & Barrowcliffe, 2008). LMWHs, the depolymerized derivatives of heparin, are more frequently used than their parent heparin and dalteparin sodium, the two most important types of LMWHs, are produced by alkaline hydrolysis of heparin benzyl ester and by borohydride reduction after nitric acid depolymerization, respectively. Both of these LMWHs inherit the backbone structures of parent heparin, the repeating disaccharide of iduronic acid (IdoA) or glucuronic acid (GlcA) residue 1→4 linked to glucosamine (GlcN) residue, with the variable sulfation at 2-O-position of hexuronic acid (HexA) residues and the 3-O, 6-O- and/or N-position of the GlcN residues. In addition, the termini of LMWHs are modified by the different depolymerization reactions used in their generation and are distinct from one another. For example, the non-reducing end (NRE) and reducing end (RE) of dalteparin are a 2-O-sulfate-α-L-idopyranosyluronic acid residue and a 6-O-sulfate-2,3-anhydro-o-mannitol residue (Fig. 1a), while the terminal structures of enoxaparin are an unsaturated uronic acid residue at the NRE and a D-GlcN or 1,6-anhydro structure at the RE in a small percentage (ranging from 15% to 25%) of the enoxaparin chains (Guerrini, Guglieri, Naggi, Sastriekharan, & Torri, 2007).

LMWHs are comprised of linear polyanionic oligosaccharides, and the sequence and composition of these oligosaccharides are essential for their clinical efficacy and safety. Some defined sequences are known to be responsible for the anticoagulant activity. For example, a unique pentasaccharide sequence, −GlcNS/Ac6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S−, is indispensable for activating ATIII to inhibit coagulation factor Xa (Bisio et al., 2009). A much longer oligosaccharide sequence, containing at least 18 saccharide units and the same pentasaccharide domain, is necessary to inhibit coagulation factor IIa by forming a ternary complex with ATIII together (Chuang, Swanson, Raja, & Olson, 2001). Heparin-induced thrombocytopenia, a major adverse effect of heparin and LMWHs, is closely related to the structure of these drugs (Ahmed, Majeed, & Powell, 2007). Heparin is recognized as one of the

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most difficult molecules to analyze due to its structural complexity and heterogeneity. The chemical modifications during the depolymerization process make the structural elucidation of LMWHs even more challenging.

A great number of analytical methods have been established for structural characterization of heparin and LMWHs from different aspects. Gas chromatography (GC) has been used to determine the monosaccharide constituents of sulfated glycosaminoglycans (Zanetta,
Pervin, Al-Hakim, & Linhardt, and Guerrini, Guglieri, Naggi, Mourier & Li, Steppich et al., 2014. Wang, Li, Sun, Bai, Doneanu, LC, gel electrophoresis and NMR, pay more attention to the overall approaches provide in-depth structural elucidation for LMWHs, but Chi, 2015. of heparinase for fragment mapping analysis (partially degraded to oligosaccharide fragments by using only one kind more cleavage reactions than the longer oligosaccharides during the analysis (part (help of bioinformatics tools for individual oligosaccharides with size up are used to separate LMWH chains and obtain their high-resolution Chen, & Gebler, 2009. Charge based separations, such as strong anion exchange (SAX) and cetyltrimethylammonium (CTA)-SAX, resolve LMWH oligosaccharides carrying different negative charge densities in an effective way (Mourier, Agut, Souaifi-Amara, Herman, & Viskov, 2015; Mourier & Viskov, 2004). Reversed phase (RP) HPLC is not suitable for LMWHs, but it can be modified by using ion paring reagents to form an ion paring reversed phase chromatography (RPIP) system or label the oligosaccharides with hydrophilic and fluorescent agents to make them retainable on a RP column (Galeotti & Volpi, 2013; Wang, Li, Sun, Bai, Jin, & Chi, 2014; Kinoshi & Sugahara, 1999). Because dalteparin oligosaccharides lack chromophore at the NRE and do not contain re-active RE for fluorescence labelling, a mass spectrometer is necessary to serve as a detector following LC separation. Directly coupling the LC separation to a mass spectrometer (MS) is usually referred as on-line LC-MS. Alternatively, the LC separation can be collected, selectively combined or desalted, and then injected into a mass spectrometer, which is correspondingly referred as an offline LC-MS method.

In recent years, MS techniques are becoming the mainstay for characterizing oligosaccharides in LMWHs. A top-down and bottom-up strategy, similar to that used in proteomics analysis, has been applied to LMWH analysis. In the top-down approaches, hyphenated techniques, such as SEC (Zhang et al., 2013), RPPIP (Li, Chi et al., 2014; Doneau, Chen, & Gebler, 2009), HILIC (Li, Zhang, Zaia, & Linhardt, 2012) and CE (Sun et al., 2016a,b) coupled with electrospray ionization (ESI)-MS are used to separate LMWH chains and obtain their high-resolution analysis. The tetrasaccharide pool of enoxaparin sodium has been sequenced by orthogonal experiments, including fractionating tetrascaric acids using SEC, purifying individual chains using semi-preparative SAX-HPLC and characterizing each component with MALDI-MS and NMR (Lee et al., 2013). However, the short oligosaccharides in dalteparin sodium cannot be analyzed using the same approach. Unlike enoxaparin, dalteparin chains do not have chromophores that can be easily monitored during the chromatographic separation. Additionally, the MW of dalteparin is larger than enoxaparin, and the major components in the short oligosaccharide pool of dalteparin are heexasaccharides and octasaccharides, rather than the tetrascaric acids found in enoxaparin. The increased size of dalteparin chains make it impractical to obtain individual dalteparin oligosaccharides of satisfactory purity for NMR analysis.

In this study, we developed an offline SAX-HPLC and ESI-MS/MS approach for sequencing the short oligosaccharide pool of dalteparin sodium. The oligosaccharides with sizes equal or smaller than octasaccharides were separated from dalteparin using SEC. These chains were further separated using high resolution SAX-HPLC column. The fractions were desalted and determined by ESI-MS. Finally, the individual chains were sequenced using optimized MS/MS conditions. This work provides a sensitive and indispensable tool to evaluate the fine structures of LMWHs that can be used in conjunction with top-down and bottom-up chain mapping approaches.
2. Materials and methods

2.1. Materials

Dalteparin reference standard was obtained from the European Pharmacopoeia. Heparin disaccharide standard ΔIVA (ΔUA-GlcNAc) was purchased from Iduron (Manchester, UK). Heparin lyases I, II, and III were obtained from Aglyco (Beijing, China). Arixtra™ was obtained from the hospital pharmacy. HPLC grade acetonitrile, methanol and water were from Fisher-Scientific (Springfield, NJ). All other reagents and chemicals were of the purest grade available.

2.2. SEC separation

The short oligosaccharides were separated by SEC with a Superdex TM Peptide 10/300 GL column (13 μm, 10 × 300 mm) from GE Healthcare (Uppsala, Sweden) on an LC-20A HPLC system (Shimadzu, Kyoto, Japan) equipped with a refractive index detector. Isocratic mobile phase of 0.2 M ammonium bicarbonate was used at the flow rate of 0.4 mL/min. The short oligosaccharide pool was collected and the volatile salts were removed by repeated lyophilization.

2.3. SAX separation

The short oligosaccharide pool was further separated on a ProPac PA1 SAX column (4 × 250 mm) from Thermo Scientific (Barrington, IL). Mobile phase A was 0.2 M NaCl, pH 7.0 and mobile phase B was 2 M NaCl, pH 7.0. A step gradient of 0% mobile phase B in 5 min, from 0 to 30% B in 45 min, from 30 to 85% B in 150 min and from 85 to 100% B in 10 min was used. The flow rate was at 1 mL/min. The eluents were collected using an automatic fraction collector by setting as 1 min/tube and collecting from 0 min to 120 min. The fractions were then desalted using a Sephadex G-10 column (10 × 400 mm) from Dalian Elite (Dalian, China). Equal volume of disaccharide standard ΔIVA (0.08 μg/μL) was added to each fraction before desalting to serve as an internal standard.

2.4. ESI–MS and MS/MS analysis

Each desalted fraction dissolved in 30 μL water was directly introduced to the ESI–MS interface of a Thermo LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The negative ion mode was used with the sheath gas flow rate at 20 arb, aux gas flow rate at 5 arb, spray voltage at 3.5 kV, capillary temp at 275.00 °C, S-lens level, 10%. The MS/MS parameters were the same as above.

2.5. Sequence assignment using python 2.7 software

A program was written in Python to facilitate the interpretation of MS/MS spectra. It included two compartments, database and fitting algorithm. The theoretical fragment ion database was generated for each oligosaccharide according to its composition. A fitting algorithm with 0.02 Da mass acceptance range was used to match the experimental fragment ions by searching against the theoretical fragment ion database.

2.6. Enzymatic digestion and LC–MS/MS analysis

A digestion solution was prepared by mixing 12.5 μL of 0.4 IU/mL each of heparinases I, II and III with 8.75 μL of sodium acetate/calcium acetate buffer (pH 7.0). The oligosaccharide was digested into disaccharide blocks by incubating at 37 °C for 24 h. The digest was then analyzed using an Agilent 1100 capillary HPLC system (Agilent Technologies, Santa Clara, CA) to a Thermo LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The column was a Phenomenex Luna HILIC column (2 × 150 mm, 200 Å, Torrance, CA). Mobile phase A was 5 mM ammonium acetate aqueous solution, while mobile phase B was 5 mM ammonium acetate in 95% acetonitrile. A gradient elution with 95% mobile phase B in 5 min, followed by 95–77% B in 102 min, was used at a flow rate of 0.15 mL/min. The mass spectrometer was operated in the negative ion mode with the following parameters: sheath gas flow rate (arb), 10; auxiliary gas flow rate (arb), 2; spray voltage, 3.5 kV; capillary temperature, 275.00 °C; S-lens level, 10%. The MS/MS parameters were the same as above.

3. Results and discussion

3.1. Separation of short oligosaccharide pool from dalteparin

Dalteparin is comprised of a broad range of nitrous acid depolymerized heparin oligosaccharides, with a typical mass-average relative molecular mass of 6000 Da. A reference standard sample from the European Pharmacopoeia was analyzed using HILIC-ESI–MS to profile the main chain distribution of dalteparin. A total of 123 chain composition and group substitution pattern or stereoisomers having different HexA chirality, RPIP can separate heparin oligosaccharide isomers to some extent, but the ion pairing reagents are difficult to remove and can interfere in the MS/MS fragmentations (Li, Chi et al., 2014; Doneanu et al., 2009). SAX is the traditional way to separate acidic oligosaccharides. A ProPac PA1 SAX column has been used to separate LMWH oligosaccharides and gives the best resolution (Ozug et al., 2012). The dalteparin oligosaccharides are not detectable by the UV detector due to the lack of a chromophore or by using a refractive index detector because of the salt gradient required for their elution. The high concentration of salts in the mobile phase also precludes the direct infusion of the SAX column eluent into the ESI interface. Thus, we developed an off-line SAX and ESI–MS approach to further separate the short oligosaccharide pool of dalteparin. The eluents were collected every minute by an automatic fraction collector and equal amount of heparin disaccharide ΔIVA was added to each fraction. Following desalting, each fraction was analyzed by ESI–MS to identify the components and determine their relative quantities in comparison with the internal standard. The SAX chromatogram was then plotted and shown in Fig. 2a. All the short oligosaccharides detected in the HILIC-ESI–MS analysis were found again in the SAX analysis. In addition, some minor
The Amster group discovered that the neutral loss of sulfo groups could be minimized by adding sodium hydroxide to deprotonate all acidic groups on the oligosaccharides (Kailemia et al., 2012). They have successfully sequenced a chemically synthesized heparin pentasaccharide and a series of biochemically synthesized heparan sulfate oligosaccharides with sizes up to dp12 (Kailemia et al., 2013). However, the Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer used in their research is not readily accessible in most laboratories. In contrast the readily accessible LTQ-Orbitrap mass spectrometer, operating in the CID mode, failed to sufficiently fragment the oligosaccharide (Kailemia et al., 2012). We optimized the ESI–MS/MS conditions on an LTQ-Orbitrap XL mass spectrometer using a synthetic heparin pentasaccharide, Arixtra™. Arixtra™ has a known sequence of GlcNS6S-GlcA-GlcNS6S3S-IdoA2S-GlcNS6S-OCH$_3$. First, the electrospray parameters were optimized to achieve the highest sensitivity without obvious loss of sulfo groups. At the spray voltage of $-3.5$ kV and capillary temperature of $275^\circ$C, sufficient parent ions with complete deprotonation of all acidic groups were generated by infusing Arixtra™ with 1 mM NaOH, and the three most abundant peaks were [M-10H + 7Na]$^-$, [M-10H + 6Na]$^-$ and [M-10H + 5Na]$^-$.

According to the literature, combining the fragment ions from these parent ions with different charge states allows the determination of the full sequence of Arixtra™. Next, the dissociation parameters were investigated. At the normalized collision energy of 55%, enough glycosidic and cross-ring fragmentations were formed with minimal loss of sulfo groups. The lowest amount of sample required for obtaining the full sequence of Arixtra™ was 1 µg using the regular ESI interface and 100 ng using the nanoESI (see Supplementary Fig. S1).

### 3.4. Development of computer-assisted MS/MS spectra interpretation tool

Heparin oligosaccharides can generate hundreds of fragment ions using CID, and it normally takes quite a few hours for an experienced analyst to assign the spectra to deduce a sequence. A program was developed using Python 2.7 to facilitate the interpretation of MS/MS spectra. When the composition of an oligosaccharide is obtained by high-resolution ESI–MS, all theoretical sequences with different possible sulfation and N-acetylation patterns were automatically generated. A database containing all likely fragmentations as well as various sodium adductions, corresponding to these sequences, was established. The workflow of using this tool to interpret the MS/MS results for Arixtra™ is shown in Supplementary Fig. S2. It took only a few minutes to accurately assign the spectra and export the correct sequence.

### 3.5. Sequencing oligosaccharides in dalteparin

Eighteen representative oligosaccharides were selected for ESI–MS/MS and sequence analysis (Table 2). The optimized experimental conditions, obtained by infusing Arixtra™, were used for analyzing dalteparin short oligosaccharides. The concentration of NaOH in the electrospray solution was adjusted according to the chain length and sulfation degree of each oligosaccharide to ensure the parent ions were fully deprotonated.

Octasaccharides are the largest oligosaccharides to be analyzed. According to the definition of dalteparin (Fig. 1a), an octasaccharide can have up to 11 sulfo groups. Oligosaccharide 1 with the composition dp8 (11S, 0Ac) is the fully sulfated form and its sequence was successfully determined. Three precursor ions with all 15 acidic protons replaced by Na$^+$, m/z $= 403.79$, $-6$ charged, m/z $= 489.14$, $-5$ charged, and m/z $= 617.17$, $-4$ charged, were subjected to CID analysis to yield complementary fragment ions. Glycosidic bond cleavage fragment ions were used to assign sulfo and N-acetyl groups to each monosaccharide residue, and the cross-ring cleavage fragment ions were helpful to locate the substitution position of sulfo groups within each monosaccharide residue. For example, Y$_6$ and Y$_5$ ions demonstrated that one sulfo group was located at residue f, and the O$_5$X$_3$ ion
Table 2  Representative oligosaccharide species for sequencing analysis.

<table>
<thead>
<tr>
<th>Oligosaccharide No.</th>
<th>Composition</th>
<th>Retention Time (min)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>dp8 (11S, 0Ac)</td>
<td>76</td>
<td>HexA2S-GlcNS3S/6S-HexA2S-GlcNS3S/6S-HexA2S-Mnt6S-2,5-anhydro</td>
</tr>
<tr>
<td>2</td>
<td>dp8 (11S, 0Ac)</td>
<td>69</td>
<td>HexA2S-GlcNS6S-HexA2S-GlcNS3S/6S-HexA2S-GlcNS6S-HexA2S-Mnt6S-2,5-anhydro</td>
</tr>
<tr>
<td>3</td>
<td>dp8 (10S, 0Ac)</td>
<td>62</td>
<td>HexA2S-GlcNS6S-HexA2S-GlcNS3S/6S-HexA2S-GlcNS6S-HexA2S-Mnt6S-2,5-anhydro</td>
</tr>
<tr>
<td>4</td>
<td>dp8 (10S, 0Ac)</td>
<td>59</td>
<td>HexA2S-GlcNS6S-HexA2S-GlcNS3S/6S-HexA2S-GlcNS6S-HexA2S-Mnt6S-2,5-anhydro</td>
</tr>
<tr>
<td>5</td>
<td>dp8 (9S, 0Ac)</td>
<td>50</td>
<td>HexA2S-GlcNS6S-HexA2S-GlcNS3S/6S-HexA2S-GlcNS6S-HexA2S-Mnt6S-2,5-anhydro</td>
</tr>
<tr>
<td>6</td>
<td>dp8 (9S, 1Ac)</td>
<td>74</td>
<td>HexA2S-GlcNS6S-HexA2S-GlcNS6S-HexA2S-Mnt6S-2,5-anhydro</td>
</tr>
<tr>
<td>7</td>
<td>dp6 (8S, 0Ac)</td>
<td>54</td>
<td>HexA2S-GlcNS6S-HexA2S-GlcNS6S-HexA2S-Mnt6S-2,5-anhydro</td>
</tr>
<tr>
<td>8</td>
<td>dp6 (8S, 0Ac)</td>
<td>62</td>
<td>HexA2S-GlcNS6S-HexA2S-GlcNS6S-HexA2S-Mnt6S-2,5-anhydro</td>
</tr>
<tr>
<td>9</td>
<td>dp6 (9S, 0Ac)</td>
<td>73</td>
<td>HexA2S-GlcNS6S-HexA2S-GlcNS6S-HexA2S-Mnt6S-2,5-anhydro</td>
</tr>
<tr>
<td>10</td>
<td>dp6 (7S, 0Ac)</td>
<td>34</td>
<td>HexA2S-GlcNS6S-HexA2S-GlcNS6S-HexA2S-Mnt6S-2,5-anhydro</td>
</tr>
<tr>
<td>11</td>
<td>dp6 (7S, 0Ac)</td>
<td>43</td>
<td>HexA2S-GlcNS6S-HexA2S-GlcNS6S-HexA2S-Mnt6S-2,5-anhydro</td>
</tr>
<tr>
<td>12</td>
<td>dp6 (7S, 0Ac)</td>
<td>46</td>
<td>HexA2S-GlcNS6S-HexA2S-GlcNS6S-HexA2S-Mnt6S-2,5-anhydro</td>
</tr>
<tr>
<td>13</td>
<td>dp4 (5S, 0Ac)</td>
<td>33</td>
<td>HexA2S-GlcNS6S-HexA2S-GlcNS6S-HexA2S-Mnt6S-2,5-anhydro</td>
</tr>
<tr>
<td>14</td>
<td>dp4 (4S, 0Ac)</td>
<td>15</td>
<td>HexA2S-GlcNS6S-HexA2S-GlcNS6S-HexA2S-Mnt6S-2,5-anhydro</td>
</tr>
<tr>
<td>15</td>
<td>dp7 (11S, 0Ac)</td>
<td>94</td>
<td>GlcNS3S/6S-HexA2S-GlcNS6S-HexA2S-GlcNS6S-HexA2S-Mnt6S-2,5-anhydro</td>
</tr>
<tr>
<td>16</td>
<td>dp7 (10S, 0Ac)</td>
<td>69</td>
<td>GlcNS3S/6S-HexA2S-GlcNS6S-HexA2S-GlcNS6S-HexA2S-Mnt6S-2,5-anhydro</td>
</tr>
<tr>
<td>17</td>
<td>dp7 (9S, 0Ac)</td>
<td>61</td>
<td>GlcNS3S/6S-HexA2S-GlcNS6S-HexA2S-GlcNS6S-HexA2S-Mnt6S-2,5-anhydro</td>
</tr>
<tr>
<td>18</td>
<td>dp5 (8S, 0Ac)</td>
<td>82</td>
<td>GlcNS3S/6S-HexA2S-GlcNS6S-HexA2S-Mnt6S-2,5-anhydro</td>
</tr>
</tbody>
</table>

provided further evidence to identify it as a 2-0-sulfation. The mass difference between Y3 and Y4 ions deduced that residue e contained an O-sulfo and an N-sulfo group, and the O-sulfo group can be further identified as a 6-0-sulfation by the presence of 2,4-A4ion. The sequence of oligosaccharide 1 was deduced as HexA2S-GlcNS5S/6S-HexA2S-GlcNS6S-HexA2S-GlcNS6S-HexA2S-GlcNS6S-HexA2S-Mnt6S-2,5-anhydro (Fig. 3a). Oligosaccharide 2 has the same composition as oligosaccharide 1, and the ESI-MS/MS result revealed its sequence as HexA2S-GlcNS6S-HexA2S-GlcNS3S/6S-HexA2S-Mnt6S-2,5-anhydro (Fig. 3a). 

The subgroup of hexasaccharides was analyzed using the same approach. Oligosaccharide 1 and 2 isomers with different sulfation patterns. Four isomers were eluted at different retention times from the SAX column (Table 1), and three of them were abundant enough for ESI-MS/MS analysis. Compared to the sequence of fully sulfated oligosaccharide 7, the one fewer sulfate group in oligosaccharide 10, 11 and 12 could be determined by corresponding characteristic fragment ions. As shown in Fig. 4d and Supplementary Fig. S4d, the Y3 + 2Na2− ions indicated that the undersulfation of oligosaccharide 10 was on residue c. The B2 and A2 ions disclosed that the 6-O-position was unsubstituted instead of the N-position. Similarly, oligosaccharide 11 and 12 were unambiguously identified to be the 2-O-desulfated on residue d and 6-O-desulfated on residue e by ions [Y3 + Na+]− and [Y3 + 4Na+]− ions [Zn2+ + 2Na+]−, Y3, Zn and Zn2+ ions, respectively. (Fig. 4e, f, Supplementary Fig. S4e and S4f). 

Tetrasaccharides were the shortest chains found in dalteparin. Two species with different degrees of sulfation were sequenced and their annotated MS/MS spectra were shown in Supplementary Fig. S5. Besides these even numbered oligosaccharides, components with odd number of sugar residues, including three heptasaccharides and one pentasaccharide, were also present in the short oligosaccharide pool of dalteparin. Odd numbered oligosaccharides were reported in enoxaparin as byproducts from peeling reaction (Liverani, Mascellini, & Spelta, 2009). However, the borohydride reduction step in the process of dalteparin has already protected the RE of oligosaccharide chains from peeling reaction. All four odd numbered oligosaccharides have an additional GlcN residue at their NRE. Interestingly, MS/MS results suggested that all the terminal GlcN residues were substituted or likely substituted by 3-O-sulfo group. 3-O-sulfation of GlcN residue is a unique structure in heparin and responsible for many important bioactivities including the anticoagulant activity. While the nitrous acid prefer to cleave the 1→4 linkages between GlcN and HexA residues and forms even numbered oligosaccharides, the 3-O-sulfation of GlcN residue may cause the glycosidic bond between HexA and GlcNS residues more cleavable and result in odd numbered oligosaccharides. The annotated MS/MS spectra of odd numbered oligosaccharides are presented in Supplementary Fig. S6. This provides a novel insight in understanding.
Fig. 3. The annotated sequences of dalteparin octasaccharides obtained from CID-MS/MS analysis. (a) Oligosaccharide 1. (b) Oligosaccharide 2. (c) Oligosaccharide 3. (d) Oligosaccharide 4. (e) Oligosaccharide 5 and (f) Oligosaccharide 6. The CID-MS/MS spectra of fragmenting fully deprotonated precursor ions with different charge states are presented in Supplementary Fig. S3.
Fig. 4. The annotated sequences of dalteparin hexasaccharides obtained from CID-MS/MS analysis. (a) Oligosaccharide 7. (b) Oligosaccharide 8. (c) Oligosaccharide 9. (d) Oligosaccharide 10. (e) Oligosaccharide 11 and (f) Oligosaccharide 12. The CID-MS/MS spectra of fragmenting fully deprotonated precursor ions with different charge states are presented in Supplementary Fig. S4.
the reaction mechanism of dalteparin process that is distinct from other types of LMWHs.

3.6. Confirmation of unusual 3-O-sulfated HexA structure using enzymatic digestion and LC-MS/MS

In the ESI–MS/MS sequencing analysis, oligosaccharide 9, a hexaascaride containing unusual 2,3-O-disulfated HexA residue, was discovered. However, 3-O-sulfation was known to only occur on the GlcN residue by 3-O-sulfotransferases during the biosynthesis of heparin (Wang et al., 2017). Oligosaccharide 9 was digested with a cocktail of heparinase I, II, III and analyzed using LC–MS/MS to provide more evidence for the presence of this unusual structure. Three enzymatically-digested disaccharides were detected which enable re- construction of the sequence of the parent hexasaccharide: the disaccharide ΔHexA2S-Mnt6S-2,5-anhydro from the RE, the disaccharide Δdp2 (4S, 0Ac) from the middle and the disaccharide HexA2S-GlcNS6S from the NRE (Fig. 5a). The disaccharide Δdp2 (4S, 0Ac) was subjected to ESI–MS/MS analysis. As presented in Fig. 5b, CID fragmentation of the fully deprotonated precursor ion [M-5H + 3Na]⁻ generated two signature fragment ions 85*90 (m/z = 138.97) and [Z; + Na]⁻ (m/z = 341.95), which indicated that there were two sulfo groups on the residue at the RE and the rest two sulfo groups should be on the ΔHex residue. The direct sequencing and enzymatic digestion analysis results provided sufficient evidence that oligosaccharide 9 contains a 3-O- sulfated HexA structure, which has never been reported in dalteparin. Previous basic building block analysis of dalteparin also disclosed a 3-O-sulfated HexA residue containing tetrasaccharide, ΔUA-GlcNS-HexA2S3-GlcNS (Sun et al., 2017). Multiple tests of dalteparin from different aspects have consistently identified and quantified the 3-O- sulfated HexA structure, suggesting that it does exist in dalteparin.

Interestingly, our group has previously discovered the ΔUA-GlcNS-HexA2S3-GlcNS tetrasaccharide in the bottom-up analysis of enoxaparin (Sun et al., 2016a,b). Since the two types of LMWHs are produced through different chemical reactions, the 3-O-sulfation is very possibly a natural modification in heparin. The biosynthesis pathway and function of this novel sulfation pattern of heparin are worth further investigation.

4. Conclusions

Although comprehensive characterization of LMWHs has been widely performed by using integrated analytical methods such as HPLC, CE, LC–MS and NMR, the structural information provided by these approaches is not sufficient to monitor the efficacy and safety of LMWHs drugs. Direct sequencing of oligosaccharides provides straightforward assessment of the structural microheterogeneity for LMWH drugs, and the short oligosaccharide pool is the most sensitive indicator to the depolymerization reaction conditions. While obtaining pure oligosaccharide chains from such complex mixture as LMWHs is still not practical, ESI–MS/MS offers a timely solution with low sample consumption, low requirement of sample purity but rich structural information. In addition, ESI–MS unbiasedly evaluates the oligosaccharides in LMWHs in no matter they contain a chromophore or not. Herein, we developed an offline SAX-ESI–MS/MS approach and demonstrated the sequencing of representative short oligosaccharides with relatively high abundance in dalteparin. Sequencing the minor components is also feasible by accumulating enough samples from multiple runs, developing high throughput desalting method and using the more sensitive nanoESI interface.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2017.11.039.

References


