# Preparation of low molecular weight heparins from bovine and ovine heparins using nitrous acid degradation

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ABSTRACT

Low molecular weight heparins (LMWHs) are important anticoagulant drugs. Nitrous acid degradation is a major approach to produce LMWHs, such as dalteparin. Due to the foreseeable shortage of porcine intestinal mucosa heparin and other potential risks, expansion of other animal tissues for heparin preparation is necessary. Heparins from different tissues differ in structure and bioactivity potency, and these variations may be carried over to the LMWH products. Sophisticated analytical techniques have been applied to compare various versions of dalteparins produced from porcine intestinal, bovine lung and ovine intestinal heparins to elucidate the effects of different animal tissues starting materials and processing conditions on the properties of final dalteparin products. With adjusted depolymerization conditions, versions of dalteparins that qualify under the European Pharmacopeia (EP) specifications were manufactured using non-porcine heparins. Dissimilarities among the three interspecies animal tissue heparin-derived dalteparins regarding fine structures are also disclosed, and their origins are discussed.

## 1. Introduction

Low molecular weight heparins (LMWHs) are a family of anticoagulant drugs prepared from unfractionated heparin. Based on the modes of the depolymerization reactions and characteristic structures of the final products, LMWHs can be categorized into different types, including enoxaparin, dalteparin, nadroparin, tinzaparin, and ardeparin (Linhardt & Gunay, 1999). LMWHs have been dominant in the prevention and treatment of thrombotic diseases, and the continuously growing demands on LMWHs will inevitably lead to a shortage of heparin materials (Melnikova, 2009). Currently, only porcine intestinal mucosa derived-heparin is permitted in most counties, including the U.S. and European Union. Other animal tissues, such as bovine lung, bovine intestine, and ovine intestine, have been actively investigated to produce heparin that is interchangeable with porcine intestinal heparin to expand the sources of heparin (Keire et al., 2015; Watt, Yorke, & Slim, 1997). Since the majority of heparin is consumed by LMWH manufacturing, the similarities and differences of processing conditions and properties between LMWHs prepared from porcine intestinal heparin and other animal tissue-derived heparins represent important areas of study. Although bovine intestine is currently the major

resource to prepare non-porcine heparins, bovine lung heparin and ovine intestinal heparin are more comparable to porcine intestinal heparin (Ange et al., 2016; Fu et al., 2013), and the chance of producing biosimilar LMWHs would be greater by starting with bovine lung and ovine intestinal heparins.

Enoxaparin sodium, the most important type of LMWH, is produced by the esterification of heparin followed by alkaline depolymerization. We have previously compared the enoxaparin prepared from bovine lung heparin and porcine intestinal heparin (Guan et al., 2016). Liu et al. (2017) have also investigated LMWHs prepared from bovine lung and bovine intestinal mucosa and concluded that LMWHs bioequivalent to enoxaparin could be potentially obtained from non-porcine species. However, to the best of our knowledge, an in-depth investigation of preparing dalteparin, another important type of LMWH, using heparins extracted from animal tissues other than porcine intestine has never been reported. The preparation of dalteparin involves nitrous acid depolymerization followed by sodium borohydride reduction (Fig. 1).

The major challenge of developing an LMWH manufacturing process is to understand the relationship between the mode of depolymerization and the properties of the resulting oligosaccharide chains. LMWHs consist of extremely complicated and heterogeneous

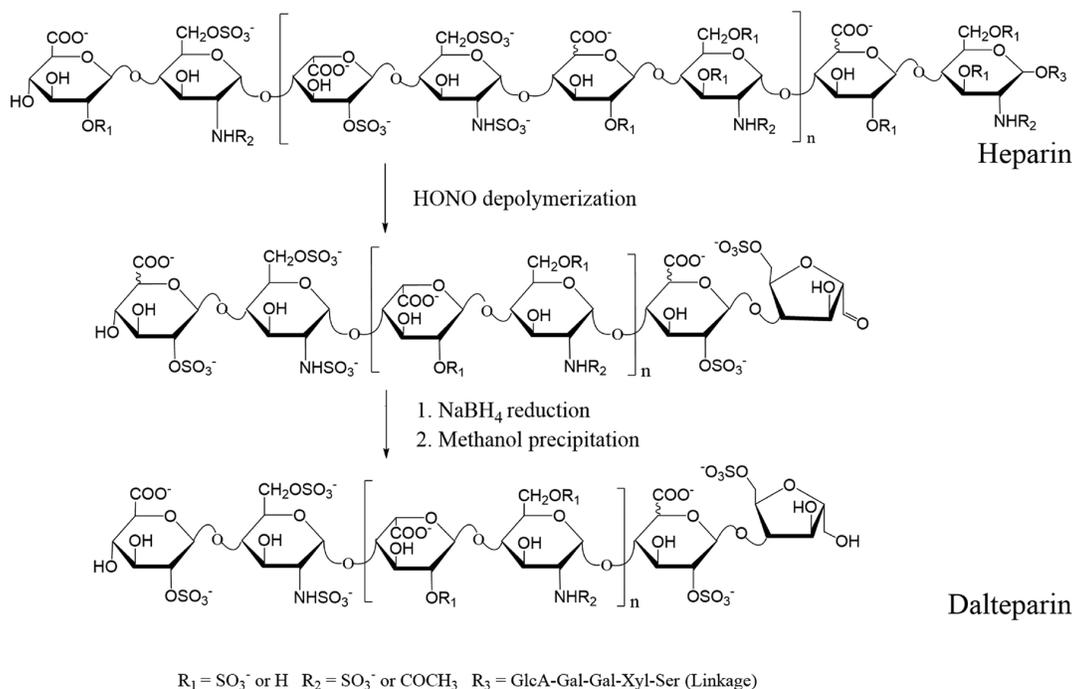R₁ = SO₃⁻ or H   R₂ = SO₃⁻ or COCH₃   R₃ = GlcA-Gal-Gal-Xyl-Ser (Linkage)

**Fig. 1.** Structure and manufacturing process from heparin to dalteparin.

oligosaccharides arising from the parent heparin and depolymerization modifications. Heparin is a linear polysaccharide comprised of repeating disaccharide units of β-1,4-linked hexuronic acid (HexA) and glucosamine (GlcN) residues. The HexA residue can be epimerized to either α-*L*-iduronic acid (IdoA) or β-*D*-glucuronic acid (GlcA), and its C2 position can be substituted by an *O*-sulfo group. The GlcN residues can be substituted by an *N*-sulfo group, *N*-acetyl group, and/or an *O*-sulfo group at the C3/C6 positions (Rabenstein, 2002). The pentasaccharide sequence of −GlcNAc/NS6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S- constitutes the antithrombin III (ATIII) binding site motif and is essential for the anticoagulant activity of heparin (Lindahl et al., 1984; Streusand, Björk, Gettins, Petitous, & Olson, 1995). The heparin polysaccharide chains also contain a linkage tetrasaccharide, -GlcA-Gal-Gal-Xyl, at their reducing ends (REs), through which they are originally attached to the core proteins as proteoglycans in animal tissues (Iacomini et al., 1999). The molecular weight (MW) of heparin generally ranges from 12,000 Da to 25,000 Da, while the average MWs of LMWHs are reduced to less than 8000 Da by depolymerization reactions. In the meantime, the structure of either one or both termini of the newly generated oligosaccharide chains is usually modified. For example, the RE of dalteparin is a characteristic 6-*O*-sulfo-2,5-anhydro-*D*-mannitol residue (Racine, 2001). Additionally, side-reactions may occur during the process and result in certain unusual structures, such as ones containing an odd number of saccharide residues, arising from a peeling reaction and epoxide structures due to 2-*O*-desulfation of IdoA2S. Because these reactions are not related to nitrous acid degradation, the corresponding structures should be originally generated during the heparin process and carried over to dalteparin (Rej & Perlin, 1990; Shriver, Gapila, & Sasisekharan, 2012). The composition and sequence of oligosaccharides determine the drug efficacy and safety of LMWHs, and the minor structures particularly indicate the subtle differences in heparin starting materials and the manufacturing process. Both the U.S. Food and Drug Administration (FDA) and European Medicine Agency (EMA) require comprehensive structural characterizations with sophisticated analytical techniques from multiple aspects to evaluate the bioequivalence or biosimilarity between generic and

innovator LMWHs (García-Arieta & Blázquez, 2012). These guidelines are referred in our study to evaluate the dalteparins prepared from different sources. For example, disaccharide building block analysis is fundamental to reveal the basic pieces that construct the LMWHs (Sun et al., 2016; Sun et al., 2017; Wang et al., 2014; Zhang et al., 2013), fragment mapping offers an alternative way to elucidate the reoccurring sequences (Li, Steppich et al., 2014; Xu et al., 2015), and intact chain mapping provides a high resolution distribution and composition of oligosaccharides (Li, Zhang, Zaia, & Linhardt, 2012; Li, Chi et al., 2014). In addition, the anticoagulant potency of LMWHs can be measured by anti-factor IIa and Xa chromogenic assays (Martinez, Savadogo, Agut, & Anger, 2013; Suzuki, Ishii-Watabe, & Hashii, 2013).

In this study, we developed processes for preparing dalteparin-like LMWHs from bovine lung heparin and ovine intestinal heparin. The bovine and ovine versions of dalteparin were compared to the authentic porcine dalteparin using an integrated analytical approach comprised of liquid chromatography mass spectrometry (LC–MS) and 2D nuclear magnetic resonance (NMR). The relationship between the depolymerization conditions and product properties was investigated, and the potential for expanding non-porcine animal tissues as new resources for producing LMWHs is discussed.

## 2. Materials and methods

### 2.1. Materials

Porcine intestinal heparin, bovine lung heparin and ovine intestinal heparin were gifts from the Innokare Bio-Pharmaceutical Tech Co., Ltd. (Suzhou, China). Dalteparin European Pharmacopoeia (EP) reference standard (EPD-S) and heparin reference standard were purchased from the EP Commission (Strasbourg, France). Heparinase I, II and III, ATIII, factor IIa, factor Xa, and chromogenic substrates S-2238, S-2222 and S-2765 were obtained from Adhoc International Technologies (Beijing, China). Sodium nitrite, hydrochloric acid and sodium borohydride were purchased from the Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All other chemicals and reagents are of the highest purity

available.

## 2.2. Preparation of dalteparin

Three batches of porcine intestinal heparin-derived dalteparin (PID) were produced using the following procedure. One gram of porcine intestinal heparin was dissolved in 10 mL of water. Sodium nitrite (29.5 mg) and 0.59 mL of 2.5 M hydrochloric acid solution were added to the heparin solution to generate nitrous acid, which then depolymerizes the heparin chains at 4 °C for 90 min. Sodium hydroxide was used to neutralize excess nitrous acid, and the aldehyde groups in the newly generated chains were reduced by reacting with $NaBH_4$ solution (at a weight ratio of 1% $NaBH_4$ to heparin starting materials, pH 10.0) overnight. After the reaction, acetic acid was added to remove excess $NaBH_4$ by adjusting the pH to 4.0, which was then neutralized by sodium hydroxide. The final products were precipitated using 67% methanol aqueous solution and lyophilized. The concentration of nitrous acid during the depolymerization step and concentration of methanol in the precipitation step were adjusted for bovine lung heparin-derived dalteparin (BLD) and ovine intestinal heparin-derived dalteparin (OID) based on the MW and MW distribution of the starting heparins.

## 2.3. Gel permeation chromatography (GPC) analysis

Molecular weight profiles of all of the LMWH samples were obtained using a Superdex™ Peptide 10/300 GL column (13 μm, 10.0 × 310 mm, GE Healthcare, Uppsala, Sweden) and Shimadzu LC-20A HPLC system equipped with a RID-10A refractive index detector (Tokyo, Japan). A 0.2 M $NH_4HCO_3$ aqueous solution was used as the mobile phase, and the flow rate was at 0.3 mL/min.

A GPC multi-angle laser light scattering (MALLS) system, consisting of a Waters 515 HPLC (Milford, USA), a Wyatt DAWN HELEOS II MALLS detector and a Wyatt Optilab rEX refractive index detector (Santa Barbara, USA), was used to measure the absolute MWs and MW distributions for the LMWHs. The column was a Shodex OHpak SB-806M HQ GPC column (13 μm, 8.0 × 300 mm, Showa Denko, Tokyo, Japan), and the mobile phase was 0.2 M $NaNO_3$ with 2 g/L $NaN_3$. The flow rate was at 0.5 mL/min for 30 min. All data were processed by the software ASTRA (Wyatt Technology, Santa Barbara, USA).

## 2.4. NMR analysis

Representative lots of PID, BLD, OID as well as EPD-S were analyzed by two-dimensional $^1H$-$^{13}C$ heteronuclear single quantum coherence (2D-HSQC)-NMR. Spectra were acquired on a DD2 600 MHz NMR spectrometer equipped with a SmartOne Probe (Agilent Technologies, Santa Clara, USA). One hundred milligrams of each sample was dissolved in $D_2O$ (99.9%), lyophilized and then re-dissolved with 500 μL of $D_2O$ again for NMR analysis. The qHSQC pulse sequence was used for the spectra acquisition with the $^1J_{CH}$ set as 155 Hz.

## 2.5. Intact chain mapping analysis

Hydrophilic interaction chromatography (HILIC) and electrospray ionization (ESI)-MS was used to delineate the intact chain fingerprint of the dalteparin samples. The system includes a Shimadzu LC-20A HPLC system and a Thermo LTQ-Orbitrap XL mass spectrometer (San Jose, USA). A Luna HILIC column (3.0 μm, 200 Å, 2.0 × 150 mm, Phenomenex, Torrance, USA) was used to separate the LMWH chains, and their high resolution mass spectra were acquired in the negative-ion mode. The LC conditions were as follows: mobile phase A, 5 mM ammonium acetate aqueous solution; mobile phase B, 5 mM ammonium acetate in 98% acetonitrile; step gradient, 80% B for 5 min, 80% B to 60% B from 5 min to 45 min, and 60% B from 45 min to 55 min; and flow rate, 150 μL/min. The MS parameters were as follows: spray voltage, 4.2 kV; capillary voltage, −40 V; tube lens voltage, −50 V;

capillary temperature, 275 °C; sheath flow rate, 20 arbitrary units; and auxiliary gas flow rate, 5 arbitrary units. The mass range was set from 400 to 2000 with a resolution of 30,000. All data were processed by Thermo Xcalibur 2.5 software.

## 2.6. Basic building block composition analysis

Dalteparin samples were completely digested into their basic building blocks by a cocktail of heparinase I, II and III at the sample/enzyme ratio of 1 μg to 0.6 mIU in digestion buffer at a pH of 7.0 containing 0.1 g/L bovine serum albumin, 2 mM calcium acetate and 100 mM sodium acetate. The incubation temperature was 37 °C, and the incubation time was 48 h. An 1100 series LC system (Agilent Technologies, Santa Clara, USA) connected to a Thermo TSQ Quantum Ultra triple quadrupole mass spectrometer was used to perform the basic building block composition analysis in the multiple reaction monitoring (MRM) mode. A Click-Mal HILIC column (5 μm, 2.1 × 150 mm, Acchrom Technologies, Beijing, China) was used. Mobile phase A was 5 mM ammonium acetate in water, and mobile phase B was 5 mM ammonium acetate in 95% acetonitrile. The step gradient was 90% B for 5 min, 90% B to 77% B for 50 min, 77% B to 50% B for 10 min, and 50% B for 5 min. The flow rate was set at 250 μL/min. The MRM-MS/MS scan was performed with the following parameters: ESI mode, negative; 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 were set according to the method that had been previously developed in our laboratory (Sun et al., 2017). All data were processed by Thermo Xcalibur 2.5 software.

## 2.7. Anti-factor IIa and anti-factor Xa activities

The bioactivities of the starting materials and dalteparin samples were determined by the same chromogenic assays as described previously (Guan et al., 2016). Briefly, the heparin reference standard was dissolved in buffer containing 50 mM Tris, 7.5 mM EDTA, 175 mM NaCl and 0.1% polyethylene glycol 6000 (pH = 8.4) to make standard solutions at four different concentrations. ATIII solution was then mixed with the standard solution or test solution and incubated for 4 min at 37 °C. Substrate S-2238 and S-2222 were used separately for the anti-factor IIa assay and the anti-factor Xa assay, respectively. After the reaction was quenched by acetic acid solution, the UV absorbance at 405 nm was measured using a microplate reader. For dalteparins, substrate S-2765 was used to replace S-2222 in the anti-factor Xa assay. The values of the bioactivities were calculated using the parallel-line models.

## 3. Results and discussion

### 3.1. Adjustment of dalteparin processing conditions for different heparins

The manufacturing procedure of dalteparin involves three major steps: cleavage of the glycosidic bonds of heparin by nitrous acid; reduction of the newly formed oligosaccharides with sodium borohydride; and recovery of the final LMWH products using methanol precipitation. The amount of acid and the reaction time in the first step are crucial for controlling the chain length and distribution of the newly generated oligosaccharides, while the concentration of alcohol used in the third step is the key factor for cutting-off the size of oligosaccharides to be recovered or discarded. These two steps jointly determine the MW profiles of dalteparin products. The purpose of the second step is to protect the newly generated oligosaccharides from peeling reactions and other oxidation-reduction side reactions by converting the active aldehydes at the newly formed REs to alditols (Li, Chi et al., 2014), thus, this step does not impact the MW of the final products.

The EP monograph has defined the mass-averaged MW of dalteparin within the range from 5600 Da to 6400 Da, the chains with MW lower

than 3000 Da are not more than 13%, and the chains with MW higher than 8000 Da range between 15% and 25% of the total oligosaccharides. The procedure of preparing qualifying dalteparin was first established using porcine intestinal heparin as the starting material. The key parameters were optimized as follows: nitrous acid concentration, 10 mL of 1 g of porcine intestinal heparin mixed with 2.95% (w:w) sodium nitrite and 5.9% (v:v) of 2.5 M hydrochloric acid; and the methanol percentage for precipitation, 67%. Three lots of PIDs were produced using these conditions, and their MWs were measured and calculated as 6122 Da, with an average portion of 8.3% of oligosaccharides having an MW less than 3000 Da. The same processing conditions were then used to treat bovine lung heparin and ovine intestinal heparin. Both of the products (BLD-1 and OID-1) failed to meet the EP specifications, with MW of 4850 Da and 5008 Da, respectively. Since the MW of bovine lung heparin and ovine intestinal heparin are lower than that from porcine intestine, milder acid degradation conditions are needed to avoid excessive depolymerization. When a more dilute nitrous acid solution prepared by mixing 2.50% sodium nitrite (w:w) and 5.0% hydrochloric acid (v:v) was used, the corresponding BLD-2 and OID-2 were within acceptable MW values of above 5600 Da. However, both of these LMWHs still failed to meet the EP specification with regards to the percentage of chains with MW lower than 3000 Da, as well as the percentage of chains with MW higher than 8000 Da. The concentration of methanol used in the precipitation step was next reduced to 61% for BLD and 65% for OID to remove the excess shorter oligosaccharides. The amounts of shorter oligosaccharides ranging from a degree of depolymerization (dp)6 to dp10 significantly decreased, and the MW profiles of BLD-3 and OID-3 became very similar to those of the porcine version of dalteparin (Fig. 2). Two additional replica lots for bovine (BLD-4 and 5) and ovine materials (OID-4 and 5) were produced for further structural characterization. All MW information on starting heparin materials from different animal tissues and LMWH lots obtained with different processing conditions are summarized in Table 1.

### 3.2. NMR spectroscopy

Previous reports have shown the structural difference among porcine intestinal, bovine lung and ovine intestinal heparins using ¹H NMR spectroscopy (Fu et al., 2013; Watt et al., 1997). Porcine intestinal

heparin contains many more GlcNAc residues than the other two heparins. Another differentiable structural feature among these heparins is the epimerization ratio of HexA residues. Three major forms of HexA are present in heparin, including IdoA2S, IdoA and GlcA. The ratio of GlcA to (IdoA2S + IdoA) for bovine lung heparin was lower than porcine intestinal heparin and ovine intestinal heparin.

2D-HSQC NMR was used to provide detailed structural information on dalteparin derived from different heparins. The nitrous acid depolymerization and sodium borohydride reduction reactions provided a characteristic 6-O-sulfo-2,5-anhydro-D-mannitol structure at the RE of dalteparin, which was reconstructed by the several distinct signals in Fig. 3. The 2-N-acetyl-2-deoxy-D-glucosidic bond to GlcNAc in heparin is insensitive to the nitrous acid cleavage reaction but it appears that a higher level of GlcNAc in porcine intestinal heparin does not impact the depolymerization efficiency. However, the structural dissimilarities, particularly with regards to the content of GlcNAc residues and ratio of HexA epimerization, carry over from the parent heparin to corresponding LMWH product. The level of GlcNAc residues ranked from highest to lowest in PID, OID and BLD, while the content of GlcA in BLD was lower than that in PID and OID.

### 3.3. Profiling of intact oligosaccharide chains by HILIC-ESI–MS

The intact chain mapping profiles of triplicate lots of dalteparin produced from porcine intestinal, bovine lung and ovine intestinal heparins were obtained by HILIC-ESI–MS analyses. Oligosaccharides were eluted based on their sizes and substitutions, and their structural compositions and were precisely assigned based on their high-resolution mass spectra (Fig. 4). Over 60 different oligosaccharide species ranging from dp6 to dp22 were identified and annotated in the form of dp*n*(*x*S, *y*Ac), where *n* is the size of the oligosaccharide, *x* is the number of sulfo groups, and *y* is the number of N-acetyl groups. The ESI–MS analysis further confirmed that all components consisted of a saturated NRE and an anhydro-D-mannitol RE, suggesting that a proper dalteparin structure had been generated under our process conditions.

While 2D-HSQC NMR analysis provided the overall structural comparison for dalteparins derived from different animal tissues, the HILIC-ESI–MS analysis emphasized the similarity or dissimilarity of individual oligosaccharide chains among various samples. In previous
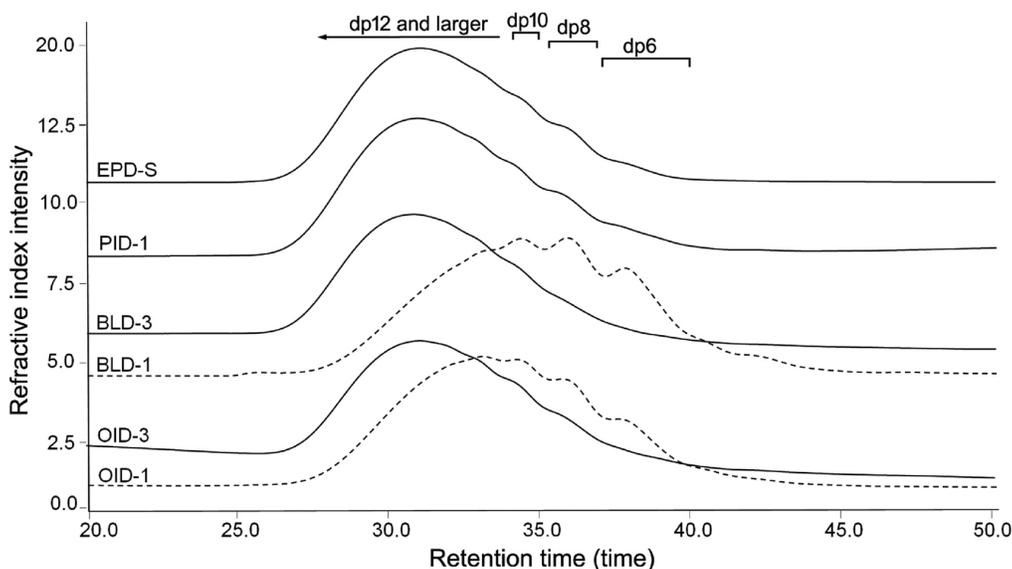


**Fig. 2.** GPC chromatograms of various lots of dalteparins prepared from starting heparins derived from different animal tissues or using different processing conditions. The column was a Superdex™ Peptide 10/300 GL column, and the detector was a refractive index detector. The dashed lines are BLD and OID prepared using the same processing conditions as PID, while the solid lines were dalteparins prepared by finalized processing conditions.

**Table 1**
MWs MW distributions, anti-factor IIa activities and anti-factor Xa activities of dalteparins and their starting materials.

| | Sample | MW (Da)[a] | Polydispersity | < 3000 Da (%) | > 8000 Da (%) | Anti-IIa (IU/mg) | Anti-Xa (IU/mg) |
|---|---|---|---|---|---|---|---|
| **Heparin** | Porcine intestinal heparin | 17,620 | 1.139 | – | – | 198 | 185 |
| | Bovine lung heparin | 15,240 | 1.265 | – | – | 131 | 121 |
| | Ovine intestinal heparin | 14,640 | 1.090 | – | – | 214 | 198 |
| **Dalteparin** | Dalteparin EP requirement | 5600–6400 | – | < 13 | 15–25 | 35–100 | 110–210 |
| | PID-1 | 6044 | 1.142 | 8.9 | 21.2 | 56 | 138 |
| | PID-2 | 6094 | 1.148 | 8.4 | 21. | 60 | 146 |
| | PID-3 | 6227 | 1.157 | 7.7 | 23.4 | 69 | 164 |
| | PID$_{avg}$[b] | 6122 | 1.149 | 8.3 | 22.2 | 62 | 149 |
| | BLD-1 | 4850 | 1.273 | 25.0 | 9.4 | – | – |
| | BLD-2 | 5603 | 1.192 | 16.9 | 19.8 | – | – |
| | BLD-3 | 6023 | 1.177 | 9.5 | 22.4 | 42 | 119 |
| | BLD-4 | 6005 | 1.166 | 10.7 | 21.5 | 36 | 101 |
| | BLD-5 | 5885 | 1.161 | 11.1 | 20.4 | 40 | 114 |
| | BLD$_{avg}$ | 5971 | 1.168 | 10.4 | 21.4 | 39 | 111 |
| | OID-1 | 5008 | 1.210 | 22.0 | 11.9 | – | – |
| | OID-2 | 5743 | 1.183 | 12.9 | 19.1 | – | – |
| | OID-3 | 6092 | 1.177 | 10.6 | 21.8 | 69 | 169 |
| | OID-4 | 6216 | 1.197 | 9.9 | 23.4 | 75 | 184 |
| | OID-5 | 6101 | 1.179 | 10.4 | 22.7 | 66 | 163 |
| | OID$_{avg}$ | 6136 | 1.184 | 10.3 | 22.6 | 70 | 172 |

[a] The MW values in this table were measured using the MALLS method.

[b] The average of 3 batches of samples with MW and MW distribution qualifying the United States Pharmacopeia (USP) and EP dalteparin monograph.
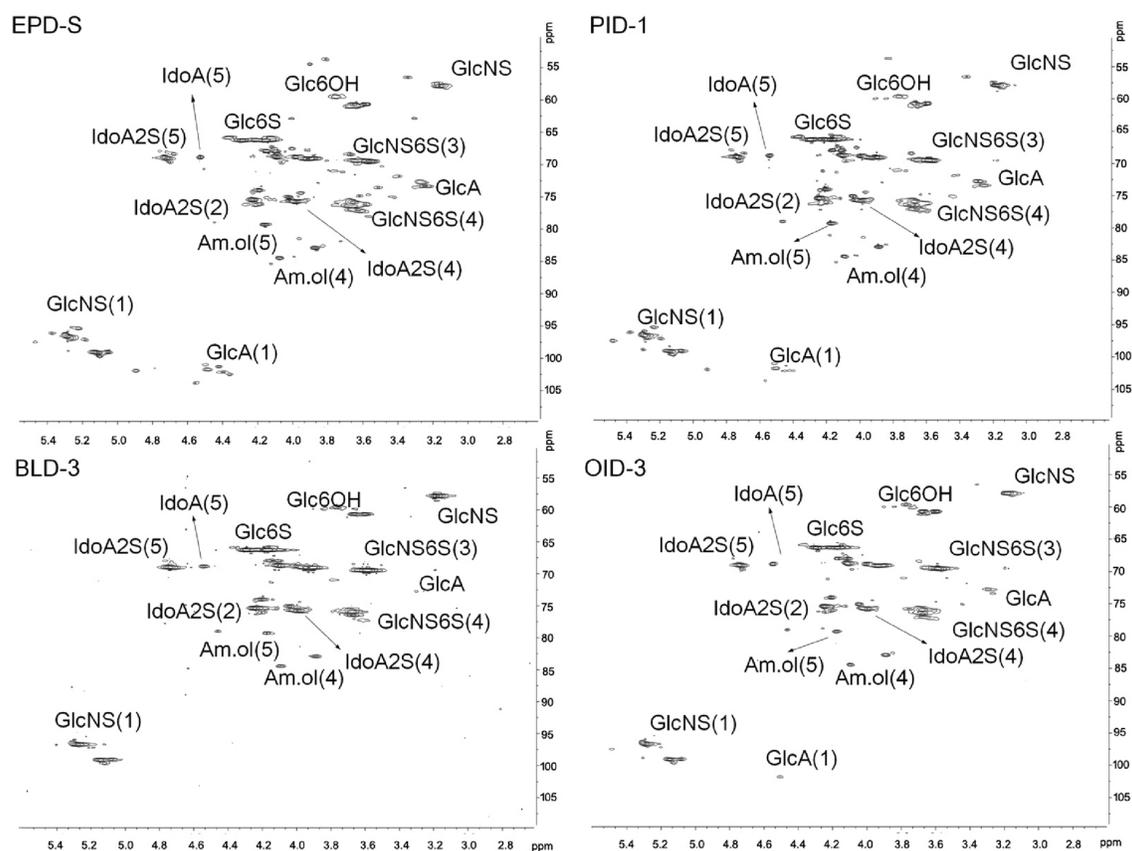


**Fig. 3.** The HSQC NMR spectra of dalteparin reference standard EPD-S and representative PID, BLD and OID samples. Am.ol represents the 6-O-sulfo-2,5-anhydro-D-mannitol residue, which is the characteristic structure for dalteparin.

NMR analysis, the most obvious difference between bovine lung heparin starting material along with its dalteparin product and those from two other animal tissues was the content of GlcNAc residues. The distribution of this characteristic into the individual oligosaccharide chains was revealed by HILIC-ESI–MS. For example, in the subgroup of decasaccharides, all BLD oligosaccharide species denoted with one N-acetyl group, including dp10(13S,1Ac), dp10(12S,1Ac), dp10(11S,1Ac), dp10(10S,1Ac) and dp10(9S,1Ac), were significantly
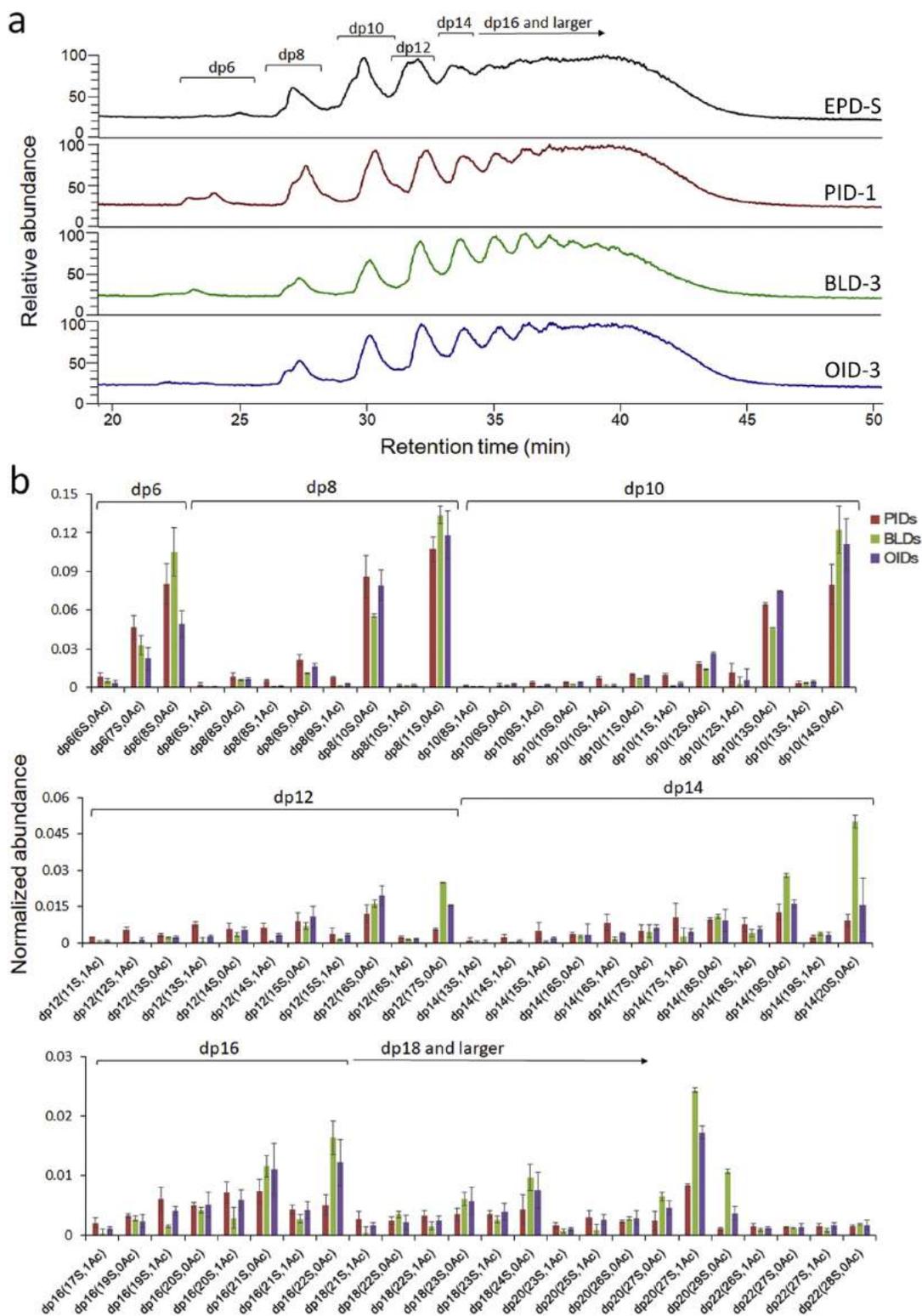
**Fig. 4.** HILIC-ESI–MS intact chain analysis of dalteparin samples. (**a**) Total ion chromatograms (TICs) of EPD-S, PID-1, BLD-1 and OID-1. (**b**) Comparison of individual oligosaccharides identified in PIDs, BLDs and OIDs. The oligosaccharides were represented as dp*n*(*x*S, *y*Ac), where *n* is the chain length of the oligosaccharide and *x* and *y* are the number of sulfo groups and *N*-acetyl groups, respectively. All oligosaccharides have a 2,5-anhydro-*D*-mannitol residue at their RE.

lower in percentage than their corresponding components in PIDs and OIDs. Furthermore, BLDs exhibited a higher degree of sulfation, as the fully sulfated (containing three sulfo groups per disaccharide unit, except the terminal disaccharide at the RE has two sulfo groups) decasaccharide, dp10(14S,0Ac), was notably more abundant in BLDs than in PIDs and OIDs. Correspondingly, those undersulfated species, such as dp10(13S,0Ac), dp10(12S,0Ac), dp10(11S,0Ac), dp10(10S,0Ac) and dp10(9S,0Ac), were less abundant in BLDs compared to PIDs and OIDs. The same pattern was observed for other groups of oligosaccharides, and this pattern was independent of chain size. High resolution HILIC-ESI–MS fingerprinting showed that OIDs resembled PIDs in oligosaccharide structure, composition and distribution, whereas BLDs differed in many ways.

### 3.4. Composition of basic building blocks

The cocktail of heparinase I, II and III can break dalteparin into its basic building blocks, most of which are disaccharides. Several larger oligosaccharides, such as trisaccharides and tetrasaccharides, can also be present in the digest if they contain unusual or unnatural structures that are resistant to these enzymes. Compositional analysis of basic building blocks provides essential structural information on LMWHs in a bottom-up manner, which is complementary to intact chain mapping analysis. A total of 28 enzymatic digested species were identified and quantified for PIDs, BLDs and OIDs using LC-MRM-MS/MS analysis (Fig. 5). These building blocks can be sorted into four categories, the backbone disaccharides, the terminal structures, the active motif-derived tetrasaccharides and other minor structures. The subgroup of backbone structures includes eight common heparin disaccharides, ΔIA to ΔIVA and ΔIS to ΔIVS, which reflect the structural features of the

starting materials. Consistent with NMR analysis and intact chain mapping analysis, the BLDs contain more highly sulfated disaccharides ΔIS (53.0%) than PIDs (38.3%) and OIDs (45.5%). Meanwhile, the sums of disaccharides ΔIA to ΔIVA were 1.4% for BLDs, 4.6% for PIDs and 2.6% for OIDs, respectively, indicating that the degree of acetylation of BLDs was lower than those in PIDs and OIDs.

The termini of dalteparin were designated modified structures and further sorted into NRE structures and RE structures. The regular NRE of dalteparin was determined to be two saturated disaccharides, Hex2S-GlcNS and HexA2S-GlcNS6S. The regular RE of dalteparin included two tetrasaccharides of ΔUA-GlcN-HexA-Mnt (Mnt represents a 2,5-anhydro-D-mannitol residue) with three or four sulfate groups and two disaccharides of ΔUA-Mnt with one or two sulfate groups.

Two tetrasaccharides, ΔIIA-IIS$_{glu}$ and ΔIIS-IIS$_{glu}$, were identified as the surrogates of the ATIII binding structural motifs of LMWHs, and their relative abundances were significantly lower in BLDs than in PIDs and OIDs. The backbone structures and active motifs of LMWHs are inherited from the parent heparins, which cannot be adjusted by the manufacturing process. The final subgroup of basic building blocks of dalteparin was created by various mechanisms. The saturated trisaccharide GlcNS6S-HexA2S-GlcNS6S and the linkage structure ΔUA-Gal-Gal-Xyl-Ser were derived from the original termini of the parent heparin. O-desulfation of the IdoA2S residue followed by rearrangement reactions generated two galacturonic acid (GalA) residues containing disaccharides ΔIIS$_{gal}$ and ΔIVS$_{gal}$ as well as one epoxide tetrasaccharide with a 2,3-anhydro at the uronic acid residue. N-desulfation also occurred during the heparin and dalteparin processes and resulted in disaccharides ΔIH, ΔIIH and ΔIIIH. Three different trisaccharides of ΔUA-GlcN-HexA with two, three and four sulfate groups were discovered as the side products of peeling reactions that occurred at the
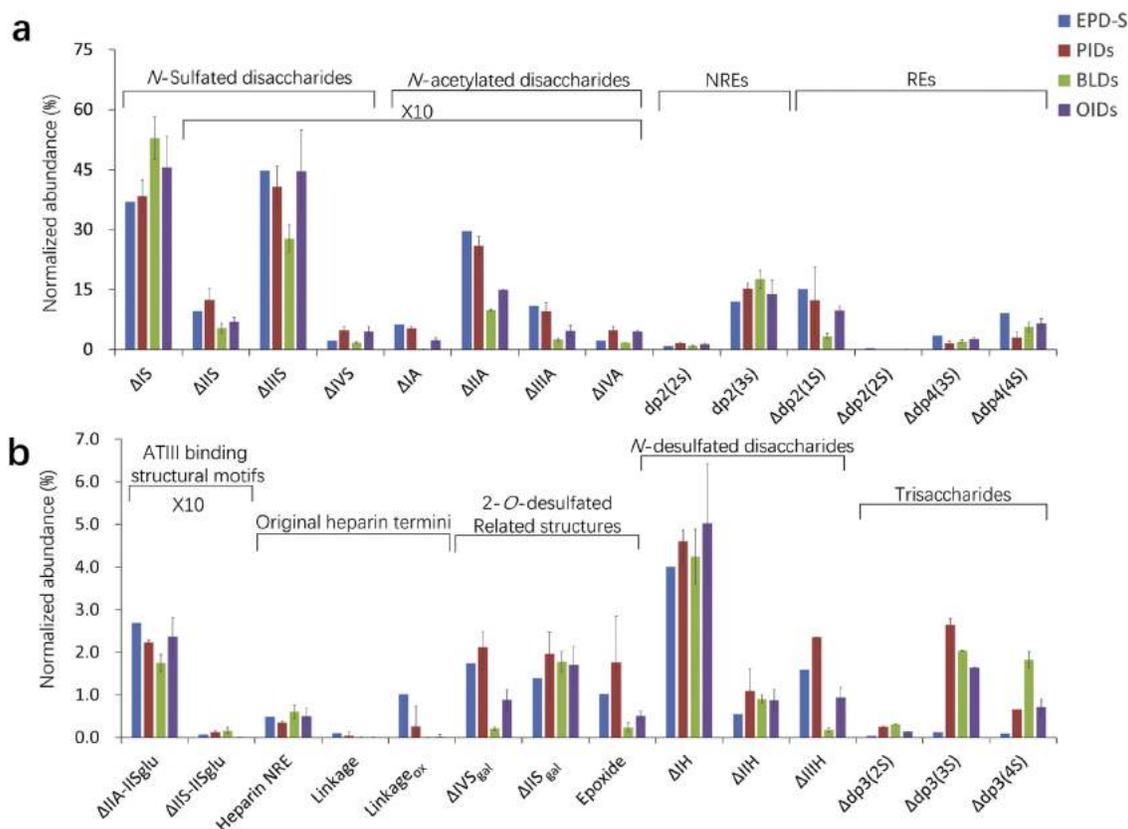


**Fig. 5.** Comparison of basic building block compositions for PIDs, BLDs and OIDs. The basic building blocks were sorted into (**a**) backbone disaccharides and terminal structures, (**b**) active structural motif-derived tetrasaccharides and other minor components.

RE. The impact of these minor structures on the safety and efficacy of LMWHs are still unknown (Mourier, Herman, Sizun, & Viskov, 2016), but they can serve as sensitive indicators of the subtle changes of starting materials and processing conditions.

## 3.5. Bioactivity assays

The anti-factor II and anti-factor Xa activities are two important values for evaluating the anticoagulant potency of LMWHs. According to the EP monograph, the anti-factor II activity of dalteparin should be in the range from 35 to 100 IU/mg, while the anti-factor Xa activity should be between 110 and 210 IU/mg. In addition, the ratio of anti-factor Xa activity to anti-factor IIa activity has to be between 1.9 and 3.2. The heparin starting materials used in this study possess different bioactivities. As shown in Table 1, anti-factor IIa and anti-factor Xa activities of porcine intestinal heparin were measured as 198 IU/mg and 185 IU/mg, respectively. Ovine intestinal heparin has slightly higher potency than porcine intestinal heparin. Bovine lung heparin exhibited significantly lower activities of 131 IU/mg for anti-factor IIa and 121 IU/mg for anti-factor Xa, which is consistent to previously literature (Ange et al., 2016). The discrepancy on activities were carried over to corresponding dalteparin products. All LMWH samples except one lot of BLD, whose anti-factor Xa value was lower than 110 IU/mg, were able to meet the three EP dalteparin monograph requirements for bioactivity. The values of the bioactivity assays of PIDs and OIDs were closer to those of EPD-S, while the averaged anti-factor II and anti-factor Xa activities of BLDs were at the lower margins of EP monograph specifications. The bioactivity measurement results are consistent to previous basic building block analysis results that BLDs have lower content of active binding sites. Since the bovine lung heparin has a relatively lower potency compared to porcine intestinal and ovine intestinal heparins, a higher dosage of its dalteparin derivatives might need to be administered if they were used in clinics.

## 4. Conclusion

LMWHs are very unique drugs of heterogeneous and complicated carbohydrate chains, which make their manufacturing and characterization highly difficult. However, LMWHs still cannot be completely replaced by small molecule oral anticoagulant drugs, such as warfarin. Conversely, the demand for LMWHs is increasing rapidly, and the inevitable shortage of heparin materials is foreseeable. Dalteparin is the second most widely used LMWH that is depolymerized by a completely different process from enoxaparin. More importantly, dalteparin and enoxaparin are not clinically interchangeable. While the various versions of enoxaparins prepared from different animal tissue-derived heparins have been compared, the feasibility of producing dalteparin using new sources of heparins other than porcine intestinal mucosa has not been initiated until the present work. Two easily accessible materials, bovine lung heparin and ovine intestinal heparin, were investigated using advanced analytical techniques to evaluate their potential as supplementary resources to porcine intestinal heparin for producing dalteparin. Ovine intestinal heparin is more similar to its porcine counterpart with regards to its structure as well as bioactivities. Accordingly, the OIDs were equivalent to PIDs in 2D-NMR analysis, intact chain mapping analysis, basic building block compositions and anti-factor IIa, anti-factor Xa activities. Bovine lung heparin possesses a higher level of sulfation and a lower potency of bioactivities compared to porcine and ovine intestinal heparins. Although BLDs passed the requirements of the EP dalteparin monograph with adjusted depolymerization and purification process conditions, the structural discrepancy was retained, and the anti-factor IIa and anti-factor Xa bioactivities were only marginally within specifications. These results suggest that bioequivalent dalteparins may be produced from non-porcine heparins, while the structural similarity of starting materials determines the level of difficulty of process development.

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