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# Parent heparin and daughter LMW heparin correlation analysis using LC-MS and NMR

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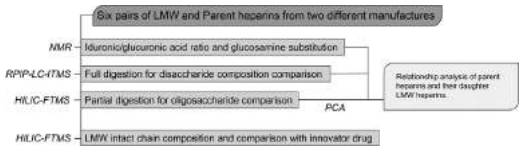
## HIGHLIGHTS

- Low molecular weight heparins prepared from different heparin parents were analyzed.
- An integrated analytical approach relied on LC-MS and NMR analysis.
- Monosaccharide compositional analysis relied on top-down NMR analysis.
- Intact chain, oligosaccharide, and disaccharide analyses relied on LC-MS.
- Differences due to parent heparin were observed using principal component analysis.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Heparin is a structurally complex, polysaccharide anticoagulant derived from livestock, primarily porcine intestinal tissues. Low molecular weight (LMW) heparins are derived through the controlled partial depolymerization of heparin. Increased manufacturing and regulatory concerns have provided the motivation for the development of more sophisticated analytical methods for determining both their structure and pedigree. A strategy, for the comprehensive comparison of parent heparins and their LMW heparin daughters, is described that relies on the analysis of monosaccharide composition, disaccharide composition, and oligosaccharide composition. Liquid chromatography-mass spectrometry is rapid, robust, and amenable to automated processing and interpretation of both top-down and bottom-up analyses. Nuclear magnetic resonance spectroscopy provides complementary top-down information on the chirality of the uronic acid residues and glucosamine substitution. Principal component analysis

*Abbreviations:* LMWH, Low Molecular Weight Heparin; OSCS, Over-sulfated chondroitin sulfate; USP, United States Pharmacopeia; PNNL, Pacific Northwest National Laboratory; API, Active pharmaceutical ingredient; 1D-$H^1$, One-dimensional Proton; 2D-HSQC, Two-dimensional Heteronuclear Single Quantum Coherence Spectroscopy; NMR, Nuclear magnetic resonance; LC-MS, Liquid chromatography-mass spectrometry; ESI–ITMS, Electrospray ion-trap mass spectrometry; RPIP–LC, Reverse-phase ion-pairing liquid chromatography; HILIC, Hydrophilic interaction chromatography; FTMS, Fourier transform mass spectrometry; dp, Degree of Polymerization; PCA, Principal Component Analysis; PC, Principal Component; NRE, Non-reducing End; RE, Reducing End; TrBA, Tributylamine; 0S, ΔUA (1 → 4) GlcNAc; NS, ΔUA (1 → 4) GlcNS; 6S, ΔUA (1 → 4) GlcNAc6S; 2S, ΔUA2S (1 → 4) GlcNAc; NS2S, ΔUA2S (1 → 4) GlcNS; NS6S, ΔNS2SUA (1 → 4) GlcNS6S; 2S6S, ΔUA2S (1 → 4) GlcNAc6S; TriS, ΔUA2S (1 → 4) GlcNS6S; ΔUA, 4-deoxy-β-L-*threo*-hex-4- enopyranosiduronic acid; GlcN, Glucosamine; IdoA, Iduronic acid; GlcA, Glucuronic acid; Ac, Acetyl; S, Sulfate.

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Nuclear magnetic resonance
Correlation

(PCA) was applied to the normalized abundance of oligosaccharides, calculated in the bottom-up analysis, to show parent and daughter correlation in oligosaccharide composition. Using these approaches, six pairs of parent heparins and their daughter generic enoxaparins from two different manufacturers were comprehensively analyzed. Enoxaparin is the most widely used LMW heparin and is prepared through controlled chemical β-eliminative cleavage of porcine intestinal heparin. Lovenox®, the innovator version of enoxaparin marketed in the US, was analyzed as a reference for the daughter LMW heparins. The results, show similarities between LMW heparins from two different manufacturers with Lovenox®, excellent lot-to-lot consistency of products from each manufacturer, and detects a correlation between each parent heparin and daughter LMW heparin.



## 1. Introduction

Heparin is a structurally complex mixture of linear anionic polysaccharides with an average molecular weight of 16 kDa and is widely used as a clinical anticoagulant [1]. Low molecular weight (LMW) heparins (average molecular weight 4 kDa to 6 kDa) are prepared through the controlled chemical or enzymatic depolymerization of heparin [2,3]. Due to their improved bioavailability and pharmacodynamics LMW heparins have replaced heparin for many therapeutic applications [4].

Heparin is a natural product, extracted from food animal tissues rich in mast cells, such as porcine intestine [1]. The complex supply chain for the manufacture of heparin and LMW heparins, involving both the food industry and the pharmaceutical industry, has made the sourcing of safe product quite complex [3,5]. Impurities, such as viruses and prions of this food animal product, as well as shortages of animal sourced materials are of growing concern [6,7]. Moreover, it is difficult to distinguish heparin prepared from approved food animal sources, such as porcine intestine, from heparins prepared from non-approved tissues, such as bovine lung or bovine intestine [7,8]. In 2007-8, there was a heparin crisis that resulted in a number of US patient deaths. Heparin, prepared in China, was adulterated with an inexpensive semi-synthetic polysaccharide, over-sulfated chondroitin sulfate (OSCS) [5,9].

As a result of manufacturing regulatory concerns, there have been intensified efforts to develop improved methods for the analysis of both heparin and LMW heparins [10–16]. Our laboratory has contributed methods for disaccharide analysis [17–20], bottom-up analysis [21,22], top-down analysis [23,24] and integrated approaches for heparin analysis [25,26]. One aspect of heparin analysis that has remained relatively unexamined is the relationship between parent heparin and daughter LMW heparin prepared from different manufacturers using variants of a specific manufacturing process.

In the current study, we examine six parent heparins coming from two different manufacturers, one located in the U.S. and one located in Europe. These heparins were each converted to LMW heparin products, generic versions of the innovator drug, Lovenox®, through controlled chemical β-eliminative depolymerization [2,3]. While the exact processes, used in converting a heparin to an enoxaparin, are trade secrets, they generally involve the steps shown in Fig. 1. Slight process differences or differences in heparin starting material might result in structural differences in the resulting LMW heparin product. Generic LMW heparins, presumed to be structurally identical, were compared with the innovator drug and one another and with their heparin parents using an integrated analytical approach. The goal of this forensic study is to understand whether a daughter LMW heparin could be traced to its parent heparin and whether different variations of a manufacturing processes result in structural signatures in LMW heparin products.

## 2. Experimental section

### 2.1. Materials

Lovenox®, drug product from Sanofi-Aventis (Bridgewater, NJ) was obtained from commercial suppliers. Six pairs of parent heparin and generic enoxaparin were provided as their active pharmaceutical ingredients (APIs), three pairs form a U.S. based and three from a European based manufacturer. All heparin and LMW heparins were analyzed at the same time prior to their expiration dates. Unsaturated heparin disaccharide (dp2) standards (0S: ΔUA (1 → 4) GlcNAc; NS: ΔUA (1 → 4) GlcNS; 6S: ΔUA (1 → 4) GlcNAc6S; 2S: ΔUA2S (1 → 4) GlcNAc; NS2S: ΔUA2S (1 → 4) GlcNS; NS6S: ΔUA (1 → 4) GlcNS6S; 2S6S: ΔUA2S (1 → 4) GlcNAc6S; TriS: ΔUA2S (1 → 4) GlcNS6S, where ΔUA is 4-deoxy-β-L-*threo*-hex-4-enopyranosiduronic acid, GlcN is glucosamine, Ac is acetyl, and S is sulfate) (Fig. S1) were purchased from Iduron (Manchester, UK). Tributylamine (TrBA) was purchased from Sigma Chemical (St. Louis, MO, USA). Ammonium acetate ($NH_4OAc$), calcium chloride ($CaCl_2$), acetic acid (HOAc), water, and acetonitrile are of HPLC grade (Fisher Scientific, Springfield, NJ). Microcon centrifugal filter units YM-10 was obtained from Millipore (Bedford, MA, USA). *Escherichia coli* expression and purification of the recombinant *Flavobacterium heparinum* heparin lyase I, II, III (Enzyme Commission (EC) #s 4.2.2.7, 4.2.2.X, 4.2.2.8) were performed in our laboratory as previously described [27]. LMWHs were desalted by dialysis using 1-kDa molecular weight cut-off (MWCO) dialysis tube (Spectrum Laboratories, CA, USA) and lyophilized before nuclear magnetic resonance (NMR) analysis and re-dissolved in distilled water into stock solution (20 μg/μL) for liquid chromatography (LC)-mass spectrometry (MS) analysis. Digestion buffer (50 mM $NH_4OAc$, 2 mM $CaCl_2$, pH 7.0) was used for heparin lyase treatment.

### 2.2. Disaccharide analysis

Samples (100 μg) were added to 100 μL digestion buffer and mixed with heparin lyase I, II and III (20 mU each in Tris-HCl buffer, pH 7.0). Samples were sufficiently digested in a 37 °C water bath for 12 h. Removing the enzymes using a 10-kDa MWCO spin column terminated the enzymatic digestion. The filtrates were lyophilized and re-dissolved in distilled water at a concentration of 1 μg/μL. Reverse-phase ion-pairing liquid chromatography (RPIP–LC) with on-line electrospray ion-trap mass spectrometry (ESI–ITMS) analysis was performed on an Agilent 1200 LC/MSD instrument (Agilent Technologies, Wilmington, DE, USA) equipped with a 6300 ion-trap and a binary pump [18].

### 2.3. Bottom-up analysis

Samples (100 μg) were added to 100 μL of digestion buffer and mixed with heparin lyase II (20 mU in Tris-HCl buffer, pH 7.0).

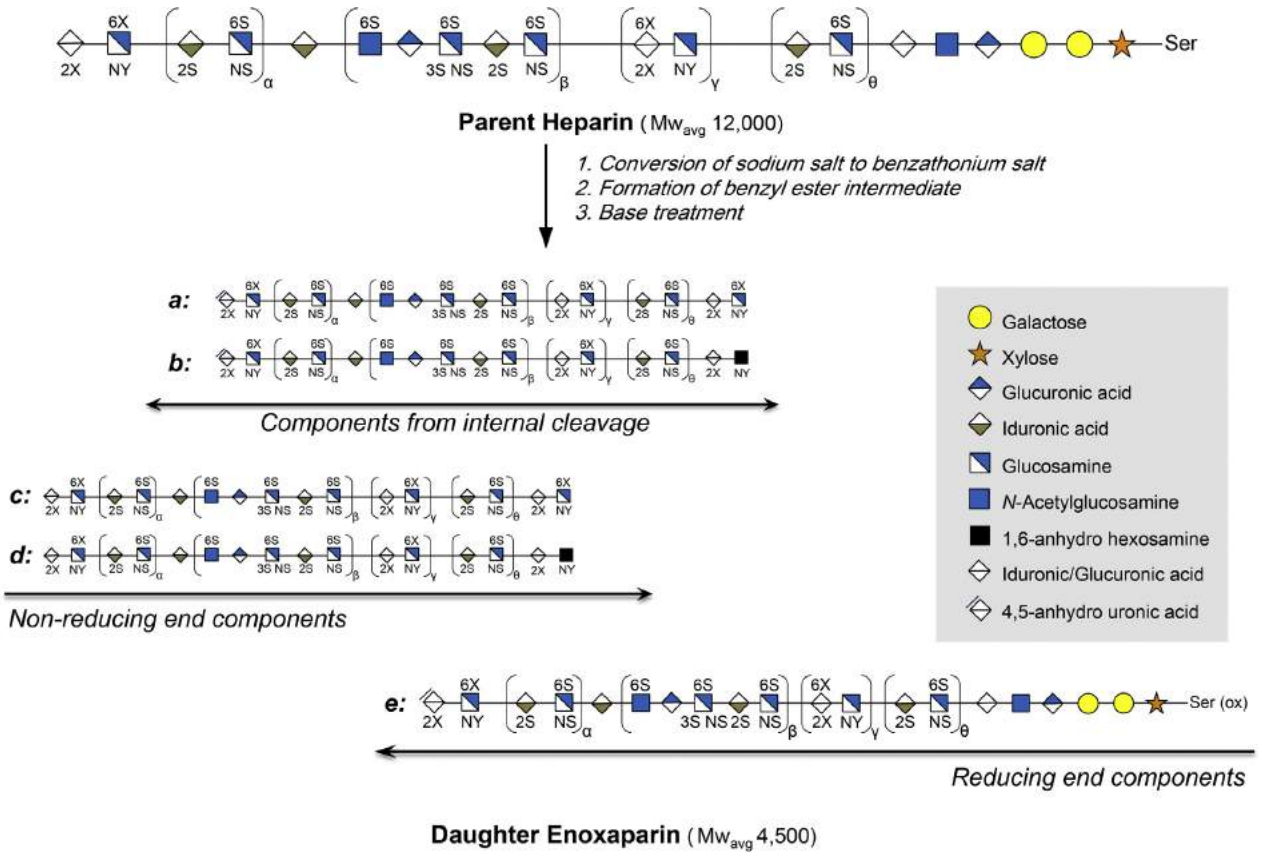


**Fig. 1.** Schematic of the preparation and structures of heparin and its chemical β-elimination-derived enoxaparin product. Structures **a** and **b,** components from internal cleavage carrying unsaturated non-reducing ends (NREs) observed in top-down HPLC-MS analysis. Structures **c** and **d,** components from the non-reducing end carrying saturated NREs from parent heparin observed in the top-down analysis. Structure **e,** components from the reducing end carrying linkage region structures. α, β, γ and θ represent different domains in heparin structure, α and θ represent trisulfated domains, γ represents under-sulfated domains, β represents antithrombin pentasaccharide binding sites. $\alpha + \theta$ ~13, $\gamma = 0$ or 1, β ~5 in heparin. $\alpha + \theta$ ~6, $\gamma = 0$ or 1, β ~2 in enoxaparin.

Samples were digested in 37 °C water bath for 12 h. Enzymes were removed using a 10-kDa MWCO spin column. The filtrates were lyophilized and re-dissolved in distilled water at a concentration of 1 μg/μL. Online hydrophilic interaction chromatography (HILIC) Fourier transform mass spectrometry (FTMS) was applied [21]. The LC column was connected online to a standard ESI source of LTQ-Orbitrap XL FT-MS (Thermo Fisher Scientific, San-Jose, CA). A Luna HILIC column (2.0 × 50 mm$^2$, 200 Å, Phenomenex, Torrance, CA) was used. Mobile phase A was 5 mM $NH_4OAc$ prepared with HPLC grade water. Mobile B was 5 mM $NH_4OAc$ prepared in 98% HPLC grade acetonitrile with 2% of HPLC grade water. The gradient was used from 5% A to 70% A in 7 min then reset to 5% A at a flow rate of 250 μL/min.

### 2.4. Top-down analysis

Online HILIC-FTMS was performed as previously described [23]. Samples were diluted into 1 μg/μL with 10% HILIC mobile phase A and the injection volume is 5 μL. A Luna HILIC column (2.0 × 150 mm$^2$, 200 Å, Phenomenex, Torrance, CA) was used. HPLC binary pump was used to deliver the gradient from 10% A to 35% A over 40 min at a flow rate of 150 μL/min.

### 2.5. Bioinformatics

Charge deconvolution was auto-processed by DeconTools software (web source from Pacific Northwest National Laboratory (PNNL) at http://omics.pnl.gov/). Heparin and enoxaparin oligosaccharide structural assignments were done by automatic processing using GlycReSoft 1.0 software developed at Boston University (http://code.google.com/p/glycresoft/downloads/list) [28]. A heparin oligosaccharide theoretical database was generated by GlycReSoft 1.0 automatically [23] Heparin linkage region oligosaccharide theoretical molecular weights were added into the hypothesis file manually. After matching with the hypothesis generated in GlycReSoft, the compositional data were further compared using GlycCompSoft [29]. The bottom-up comparison results were checked manually to confirm that the presence and absence of the components were accurate. The relative quantitative data were normalized to the total peak volume of the 10 most abundant oligosaccharides (in the format of percentage, %).

### 2.6. Nuclear magnetic resonance analysis

Samples were exchanged by $D_2O$ and resolved into 20 mg/mL in $D_2O$ before analysis. One-dimensional (1D) proton ($H^1$) NMR was operated on a 600 MHz spectrometer (Bruker Bio-Spin, Billerica, Massachusetts). Two-dimensional (2D) heteronuclear single quantum coherence spectroscopy (HSQC)-NMR was performed on an Advance II 800 MHz spectrometer (Bruker Bio-Spin, Billerica, Massachusetts). Processing of integration was done using TopSpin

2.1.6 (Bruker Bio-Spin, Billerica, Massachusetts) on the $H^1$ spectra using an average of triplicate measurements.

Detailed LC-MS and NMR parameters are presented in the supplementary information.

## 3. Results and discussion

Parent heparins and daughter LMW heparins **1**–**3**, obtained from one manufacturer, and parent heparins and daughter LMW heparins **4**–**6**, obtained from a second manufacturer, were used in the parent and daughter correlation study. The daughter generic LMW heparins were prepared from their parent heparins using trade secret, chemical β-eliminative processes, modeled on the process believed to be used for the preparation of the innovator drug, Lovenox®. Cleavages occur randomly during the β-eliminative process, generating five types of oligosaccharides with different terminal structures. The structures and relative amounts of these oligosaccharides might be used to indicate a relationship between a parent heparin and its daughter LMW heparin (Fig. 1). In current study, NMR analysis was applied to provide a comparison of the overall monosaccharide composition (Fig. 2). The major disaccharide repeating units (α, γ, θ) (Fig. 1) were analyzed using the LC-MS compositional disaccharide analysis (Fig. 3, Fig. S1). AT III binding domains (β), as well as the terminal disaccharides, were analyzed by LC-MS bottom-up analysis (Figs. 4–6). Finally, intact oligosaccharide chains, carrying enoxaparin terminal structures, were analyzed by LC-MS top-down analysis (Figs. S2 and S3). Comparing each component separately, we then correlated the bottom-up and NMR analysis dataset for parent heparin and daughter LMW heparin using PCA (Fig. 7).

### 3.1. Comparison of Lovenox® with 6 generic LMW heparins

Various methods have been described to compare generic and innovator products [30,31]. The similarity, of the six daughter enoxaparins and Lovenox®, was first determined to evaluate the quality of the generic enoxaparin drugs. After analysis by the integrated LC-MS approaches, component compositions were calculated by taking the mean of the three batches of enoxaparin, prepared by a single manufacturer, with error bars corresponding to the standard deviation of the components of these products. A single batch of Lovenox® was analyzed in triplicate as a control, with error bars for Lovenox® corresponding to analytical variation. The analytical error was less than half of the batch-to-batch variability observed for the generic enoxaparins. Based on the small standard deviations in these data, we conclude that the three enoxaparins from each manufacturer showed low batch-to-batch variability. The disaccharide composition (Fig. S1) showed that generic enoxaparins **1**–**3** had a higher sulfate level than Lovenox® and generic enoxaparins **4**–**6**. In the top-down analysis (Fig. S2), in which intact LMW heparin chains were analyzed by HILIC-FTMS, similar chain compositions were observed in all samples. Generic enoxaparins **1**–**3** had a higher abundance in long chains (A:[0,4,4,0,12], B: [1,5,6,0,17], where saccharide structure is [ΔHexA, HexA, HexN, Ac, $SO_3^-$]) and lower abundance in short chains (A:[0,2,2,0,6], B: [1,1,2,0,6]) compared to Lovenox® than did generic enoxaparins **4**–**6**. The relative quantification of terminal structures was also obtained by top-down analysis.

The chains within enoxaparin could be grouped into three major types (Fig. 1): 1. components from internal cleavage carrying unsaturated non-reducing ends (NREs); 2. saturated NREs coming from parent heparin, and 3. minor reducing end (RE) components carrying linkage region structure. Both generic enoxaparins **1**–**3** and **4**–**6** had terminal structural compositions very similar to the innovator product, Lovenox® (Fig. S3).

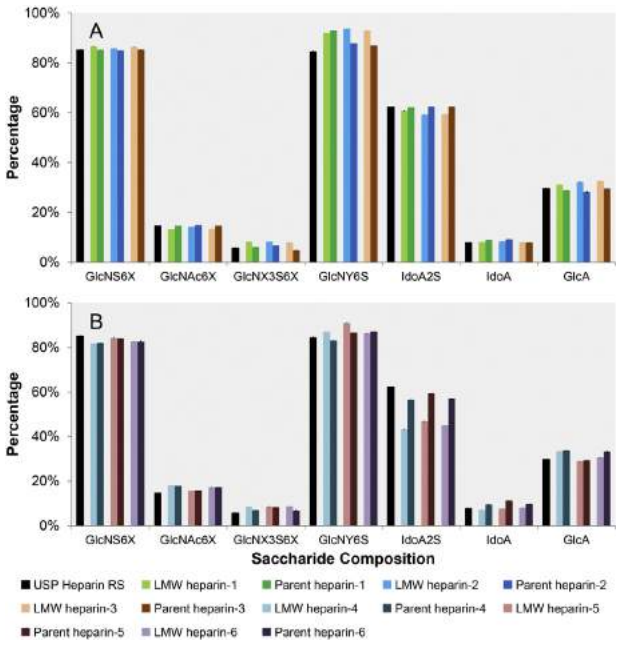


**Fig. 2.** Comparison of the $^1$H NMR analysis of the monosaccharide composition of parent and their daughter LMW heparins. Panel **A**, shows parent **1**–**3** and their daughter LMW heparins **1**–**3**. Panel **B**, shows parent **4**–**6** and their daughter LMW heparins **4**–**6**.

### 3.2. Monosaccharide and disaccharide analysis of heparins and LMW heparins

Monosaccharide analysis relied on 1D $^1$H NMR spectroscopy performed on the intact chains. The United States Pharmacopeia (USP) reference standard and Lovenox® were used for the initial assignments of signals using 2D-HSQC-NMR [32–36] before the six parent heparin and six generic LMW heparin samples were similarly analyzed by 1D-NMR. Spectral assignments of parent heparin **1** and daughter LMW heparin **1** are shown in Fig. S4, as an example. An overlay of the spectra for parent heparins **1**–**3** and daughter LMW heparins **1**–**3** are shown in Fig. S5. The relative quantities of seven types of monosaccharides in each (GlcNS6X, GlcNAc6S, GlcNX3S6X, GlcNY6S, iduronic acid (IdoA), IdoA2S and glucuronic acid (GlcA)) were determined (see supplementary information for parameters used in spectral integration). The monosaccharide compositions of all of the samples were remarkably similar (Fig. 2, Table S3). Of particular importance is the determination of the chirality of the uronic acid residues (IdoA2S, IdoA, and GlcA) as these cannot be conveniently assessed using MS analysis. The results showed the parent heparins and daughter LMW heparins were very similar. The few differences observed include the appearance of the 1, 6-anhydro, the ΔUA and ΔUA2S peaks in the LMW heparins. No appreciable differences in the size, shape or intensities of peaks were apparent. While high-resolution NMR analysis is also widely used provide sequence information on some heparin-derived oligosaccharides [37,38], heparins and LMW heparins pose unique challenges. First, their chain lengths often extend beyond the resolution of even high-field (*e.g.*, 800 MHz) instruments but more importantly, these products are complex polycomponent mixtures that are not currently amenable to structural solution by NMR.

Disaccharide analysis of the heparin and LMW heparin samples were next performed [18] and all the samples showed nearly the

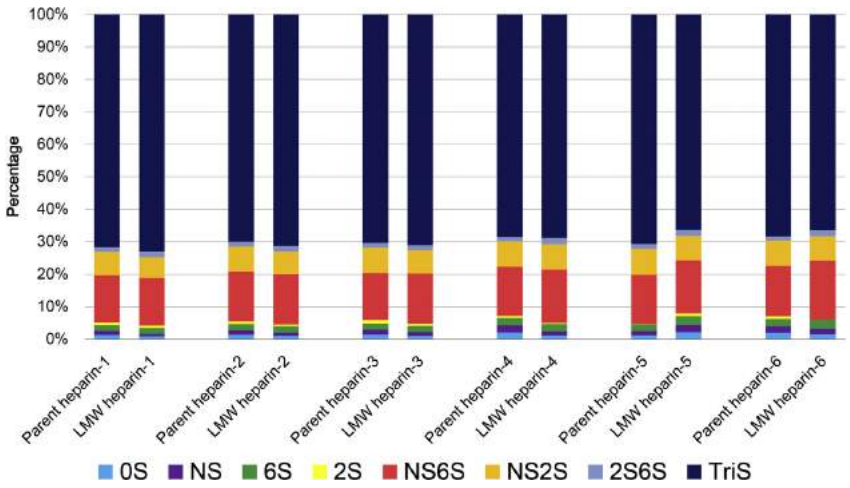


**Fig. 3.** Comparison of disaccharide composition of parent heparins and their daughter LMW heparins (See Table S2 for the raw data and the experimental section for structure of disaccharides).

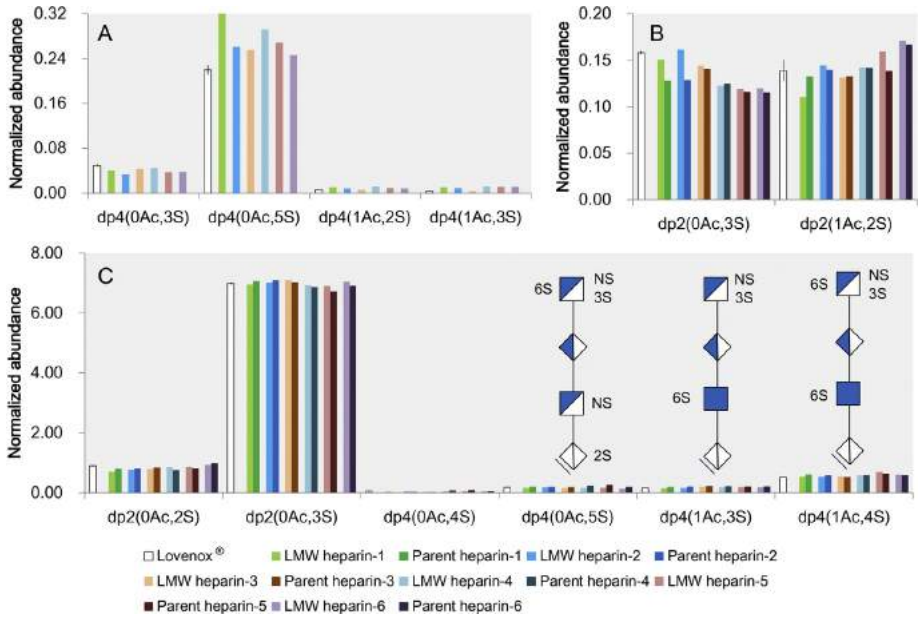


**Fig. 4.** Bottom-up analysis of heparin lyase II treated oligosaccharide by HILIC-FTMS comparison of six parent heparins and their corresponding daughter LMW heparins with Lovenox®. **A**, Tetrasaccharides with both unsaturated NREs and 1,6 anhydro REs. **B**, Disaccharides with saturated NREs. **C**, Disaccharides, tetrasaccharides with unsaturated NREs.

same compositions (Fig. 3). In these analyses, the heparin or LMW heparin sample is exhaustively treated with a cocktail of heparin lyases 1, 2 and 3 to convert each sample to primarily eight unsaturated disaccharide products (Fig. S6). Small amounts of resistant tetrasaccharides (dp4), containing 3-*O*-sulfo groups at their NRE, cannot be converted to disaccharide products [39], but these can be accounted for in the $^{1}$H NMR monosaccharide analysis as GlcNX3S6S (Fig. 2) and in the bottom-up analysis.

### 3.3. Bottom-up analysis for comparison of parent heparins with their daughter LMW heparins

Oligosaccharides generated by heparin lyase II digestion of heparins and enoxaparins are shown in Fig. 4 (see all components including the low abundance oligosaccharides in Table S1). Oligosaccharides with double dehydration, which are 1,6-anhydro in RE and 4,5-anhydro in NRE, were only present in Lovenox® and the daughter LMW heparins **1**–**6** (Fig. 4A), since the 1,6-anhydro structure is generated in the chemical β-elimination reaction used in enoxaparin synthesis (Fig. 1). Saturated NREs come from the NRE of parent heparin since both heparin lyase II-catalyzed cleavage and chemical β-elimination generates a 4,5-dehydration in the ΔUA residue at the NRE. Disaccharides with saturated NRE structure were observed in both parent and their daughter LMW heparins (Fig. 4B). The 3-*O*-sulfo group-containing tetrasaccharides are of particular significance since they arise from the pharmacologically relevant antithrombin pentasaccharide binding site that is resistant to heparin lyase II cleavage [40]. These 3-*O*-sulfo group-

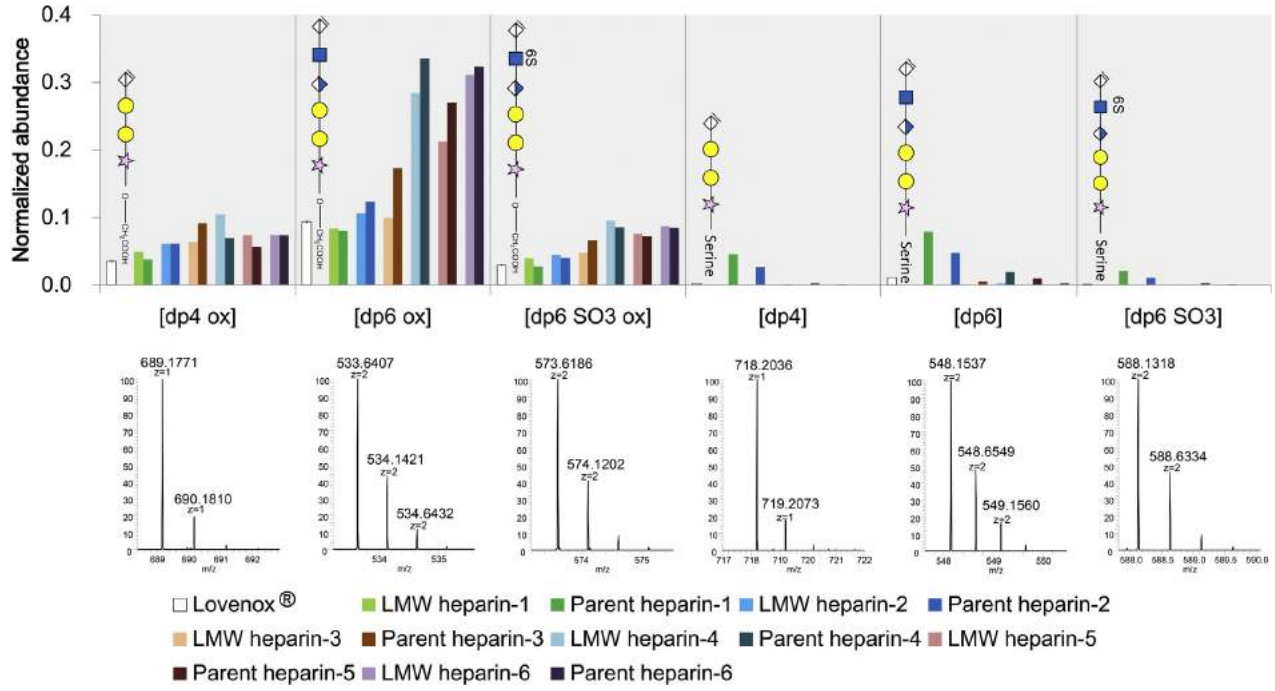


**Fig. 5.** Comparison of linkage region tetrasaccharides and hexasaccharides prepared for parent and LMW heparins by heparin lyase II treatment in the bottom-up analysis. The schematic structures are shown beside the bars and the mass spectra are shown below.

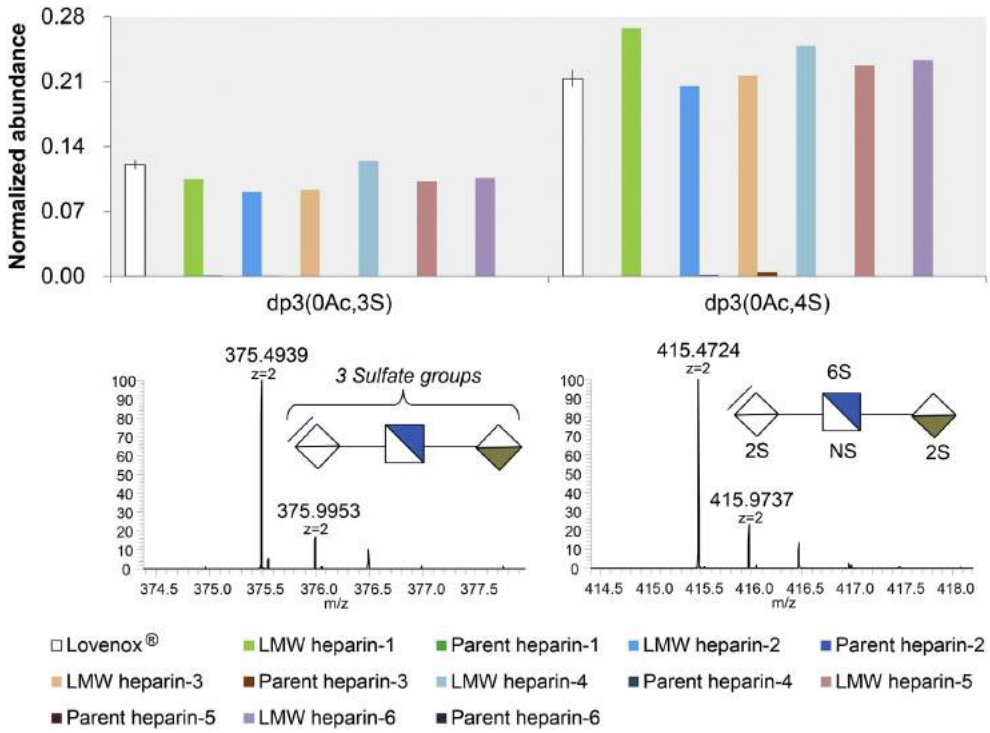


**Fig. 6.** Comparison of trisaccharides in heparin lyase II treated samples observed in the bottom-up analysis. The schematic structures and their mass spectra are shown below.

containing tetrasaccharides were previously reported [21] and are detected as dp4(0Ac,5S), dp4(1Ac,3S) and dp4(1Ac,4S) (Fig. 4C). The major repeating unit of heparin, comprising 60–90% of its structure, is [→4) IdoA2S (1 → 4) GlcNS6S (1→]$_n$ [1]. When heparin (or

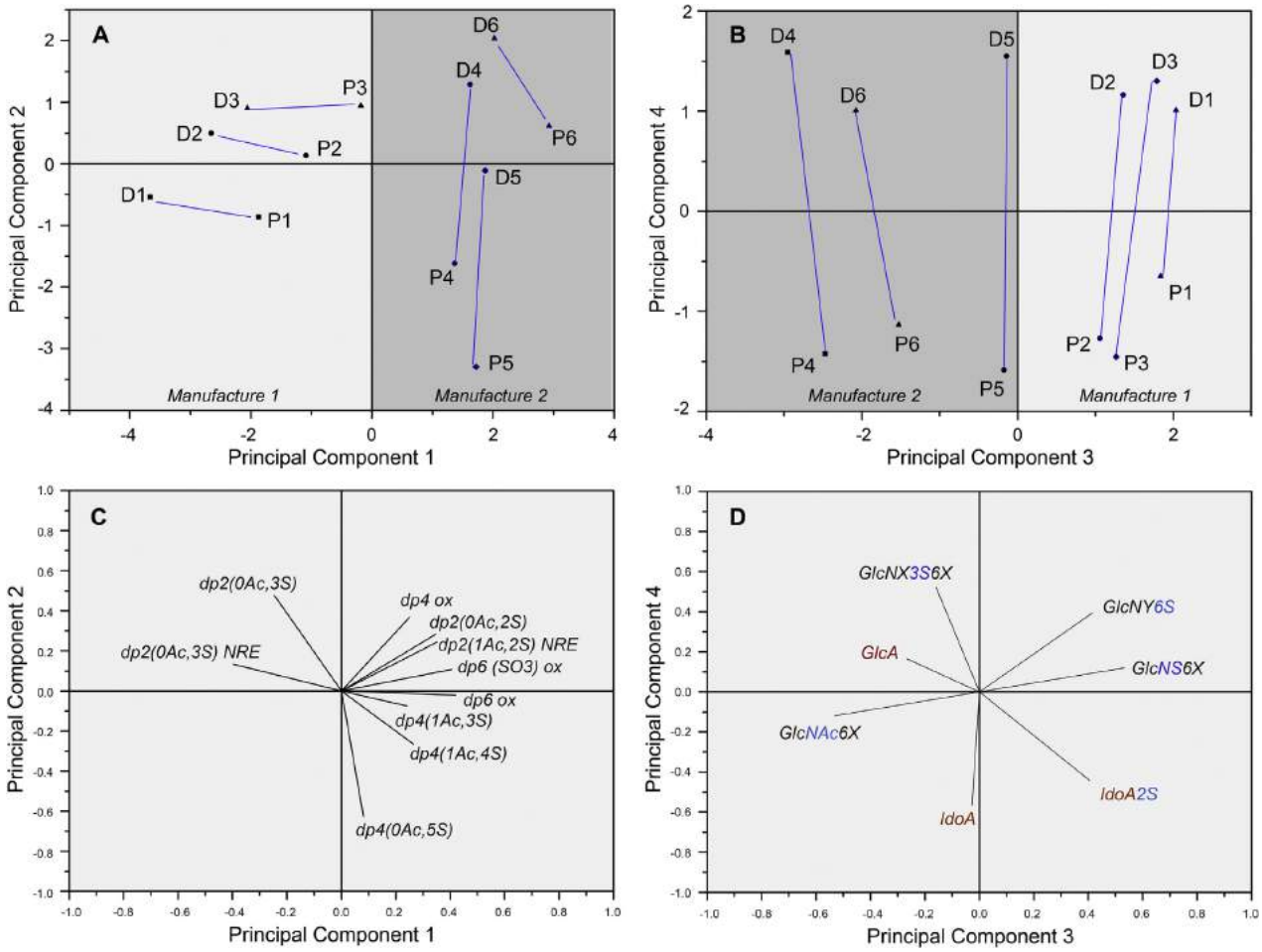


**Fig. 7.** Principal component analysis of parent heparins and their daughter LMW heparins based on normalized abundance calculated in the bottom-up analysis and saccharide composition calculated in the NMR analysis. (Parents were labeled "P", and daughters were labeled "D".) **A,** PCA scores for parent heparins and their daughter LMW heparins based on bottom-up analysis. **B,** PCA scores for parent heparins and their daughter LMW heparins based on NMR analysis. **C,** PCA loadings for the normalized abundance of oligosaccharides calculated in the bottom-up analysis. **D,** PCA loadings for the saccharide composition calculated in the NMR analysis.

LMW heparin) is exhaustively treated with heparin lyases, a trisulfated disaccharide (TriS), ΔUA2S (1 → 4) GlcNS6S is released (Fig. S1). When the insufficient enzyme is applied, an intermediate, resistant tetrasaccharide with the structure ΔUA2S (1 → 4) GlcNS6S (1 → 4) IdoA2S (1 → 4) GlcNS6S is observed [41,42]. This heparin lyase resistant tetrasaccharide can be minimized using a 12 h of incubation and doubling the amount of enzyme.

### 3.4. Comparison of minor components

A comparison of linkage region tetrasaccharides and hexasaccharides (dp6) from the bottom-up analysis, of both heparins and LMW heparins, is shown in Fig. 5. Oxidized serine containing oligosaccharides have been previously sequenced [21]. In the current study, the theoretical molecular weights were manually added to the GlycoReSoft matching hypothesis so that the relative quantification of these components was possible. Oxidized serine carrying oligosaccharides were present in both parent heparins and their daughter LMW heparins. The normalized abundance of oxidized serine containing oligosaccharides is consistent between each pair of daughters and their respective parent. Unoxidized, serine-carrying oligosaccharides were observed in parent heparins but these are present only in very small amounts in their daughter enoxaparins. This suggests the sensitivity of chains containing this linkage region to conditions used in manufacturing enoxaparins. A comparison of trisaccharides (dp3), in the bottom-up analyses, is shown in Fig. 6. These two most abundant trisaccharides [19] are only present in daughter enoxaparin. Their absence in the parent heparin confirms that oligosaccharides with an odd number of saccharide residues are generated during enoxaparin synthesis.

### 3.5. Relationship between parent heparin and daughter LMW heparin

Correlations have been previously reported between two parent bovine heparins with significantly different properties and their daughter LMW heparins [43]. However, based on the monosaccharide, disaccharide and oligosaccharide compositional data we obtained from all these analytical approaches (Figs. 2–6), all six enoxaparin samples and Lovenox® showed very similar structural properties despite the presence of minor structural differences that occur in no clearly identifiable pattern. Principal component analysis (PCA) was applied as a tool that utilizes all the variances obtained from bottom-up LC-MS (Fig. 7A and C) and NMR analysis

(Fig. 7B and D) to identify structural features that could be used to pair parent heparin with daughter LMW heparin and to distinguish manufacturer. PCA is a multivariate analytical method that uses multivariate data to differentiate between observations by reconstructing new vectors and maximizing the variances [44]. The new vectors, which are the principal components (PCs), are linear combinations of all the input variables. The dimensionality of the data can be reduced by selecting two PCs having the largest variance. PCA has been used to characterize complicated processes including ones with as many as 68 components [45]. In our research, some minor differences were apparent in the RE structures (*i.e.*, containing linkage region) and NRE structures (*i.e.*, with saturated uronic acid residues) and were highly correlated between parent and daughter (Figs. 4B and 5). Thus, the normalized abundances of two types of disaccharide generated from saturated NRE structures (Fig. 4B), the three types of the oligosaccharides generated from the linkage region RE structures, (*i.e.*, $dp4_{ox}$, $dp6_{ox}$ and $dp6\,(SO3)_{ox}$ in Fig. 5), the three types of 3-*O*-S tetrasaccharides, and the two types of the most abundant disaccharide (Fig. 4C) were selected as the variables for PCA (Fig. 7A and C). The percent composition of the various glucosamine substitutions and uronic acid compositions (except for ΔUA and ΔUA2S not present in parent heparins) obtained from NMR analysis (Fig. 2) were selected as variables of the second set of PCA (Fig. 7B and D).

The score value plots (Fig. 7A and B) give the amount of the loading data in each of the different samples. All the variables put in the loading plots (Fig. 7C and D) reveal which of the variables are influential. PC 1 and PC2 are the variable combinations of the normalized abundance of 10 fragments (Fig. 7C, Table S5) chosen from the bottom-up analysis dataset. PC3 and PC4 are variable combinations of the percentage composition from the NMR analysis dataset (Fig. 7D, Table S4). For example, Parent 1 (P1), located at [PC1, -1.8), (PC2, -0.8)] (Fig. 7A, Table S5), suggests dominant variables with negative PC1 scores (Fig. 7C, Table S4), such as (dp2(0Ac,3S) NRE and dp2(0Ac,3S)), and dominant variables with negative PC2 scores, such as (dp4(0Ac,5S), dp4(1Ac,4S), dp4(1Ac,3S), and linkage region dp6 ox), are at larger amounts in the fragment composition. P1 also located at [(PC3, +1.8), (PC4, -0.7)] (Fig. 7B), suggests dominant variables with positive PC3 scores (Fig. 7D, Table S4), such as (GlcNAc6X, GlcNY6S, and IdoA2S), and dominant variables with negative PC4 scores, such as (GlcNAc6X, IdoA2S, and IdoA), are in large amounts in the saccharide composition. Parent heparin and daughter LMW heparin have their PCA scores related to their different compositional information.

In the initial steps of the correlation investigation, both parents (P**1**-P**6**) and daughters (D**1**-D**6**) were analyzed without sample information. After the scores were output as 12 unlabeled points on the score value plots (Fig. 7A and B), blue lines were then drawn manually between parent and daughter pairs based on similarities in one of their PC scores. After paring, the individual sample names were labeled to confirm the paring results (Fig. 7A and B, Table S5).

We discovered that in the score value plots, each parent and daughter pair from manufacturer 1 showed close PC2 scores (Fig. 7A) and PC3 scores (Fig. 7B), and each parent and daughter pair from manufacturer 2 showed similarly close PC1 scores (Fig. 7A) and PC3 scores (Fig. 7B, Table S5). D5 and D6 have close PC1 scores making their paring confusing but we could solve their pairing by comparing PCA scores output in Fig. 7B. Thus, these properties can definitively correlate between parent heparins with their daughter LMW heparins.

Interestingly, an obvious division between the PC1 scores and PC3 scores for enoxaparins prepared by manufacturer 1 and manufacturer 2 could be observed in the score value plots (Fig. 7A and B), suggesting the use of different reagents or processes in enoxaparin synthesis. Moreover, all the parent heparins had negative PC4 scores, but all LMW heparin daughters had positive PC4 scores (Fig. 7B). According to the loadings shown in Fig. 7D, we can conclude that the parent heparins have more IdoA while the LMW heparin daughters have more GlcA. This is consistent with the saccharide compositional analysis (Fig. 2) and with the known selectivity of chemical β-elimination, proceeding primarily at the IdoA and IdoA2S residues.

## 4. Conclusion

The top-down, bottom-up, disaccharide compositional analysis, performed by LC-MS, and the monosaccharide compositional analysis, performed by NMR, provide detailed structural information on LMW heparins and their parent heparins. In the case of simple, homogenous drug products (*i.e.*, aspirin), generic forms are structurally identical. In contrast, LMW heparins are structurally complex, polycomponent drugs and, thus, their generic versions can only be structurally similar. Studies by our group [8,28,29,39,43] and others [15,19,30,31,34] have pointed out the structural similarities of various enoxaparins. In the current study we focused on examining subtle differences in enoxaparins and were unable to arrive at definitive analytical correlations based on traditional data plots (Figs. 2–6), which are typically used in generic drug quality evaluation. However, the compositional information from parent and daughter correlation analysis further evaluated using PCA, confirm the parent-daughter relationship of two generic LMW products. Different manufacturers can produce generic LMW heparin products that differ due to both differences in their manufacturing processes and differences in the parent heparins used as starting materials. The results of the current study demonstrate that PCA can detect minor differences and similarities between pairs of generic LMW heparins from different manufacturers and also can be applied to paring unknown parent heparins and daughter LMW heparins.

## Notes

The authors XL, KS, XW, LL, FZ, LC, and RJL declare no competing financial interest.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.aca.2017.01.042.

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