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Top-down and bottom-up analysis of commercial enoxaparins

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ABSTRACT

A strategy for the comprehensive analysis of low molecular weight (LMW) heparins is described that relies on using an integrated top-down and bottom-up approach. Liquid chromatography-mass spectrometry, an essential component of this approach, is rapid, robust, and amenable to automated processing and interpretation. Nuclear magnetic resonance spectroscopy provides complementary top-down information on the chirality of the uronic acid residues comprising a low molecular weight heparin. Using our integrated approach four different low molecular weight heparins prepared from porcine heparin through chemical β-eliminative cleavage were comprehensively analyzed. Lovenox™ and Clexane™, the innovator versions of enoxaparin marketed in the US and Europe, respectively, and two generic enoxaparins, from Sandoz and Teva, were analyzed. The results which were supported by analysis of variation (ANOVA), while showing remarkable similarities between different versions of the product and good lot-to-lot consistency of each product, also detects subtle differences that may result from differences in their manufacturing processes or differences in the source (or parent) porcine heparin from which each product is prepared.

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1. Introduction

This year marks the 100th anniversary of the discovery of heparin by Jay McLean a second year medical student at Johns Hopkins University [1]. By 1935, heparin was produced by Connaught Laboratories at the University of Toronto in sufficient quantities for clinical studies on controlling blood coagulation. The introduction of anticoagulant heparin predated the formation of the US Food and Drug Administration. In contrast, low molecular weight (LMW) heparins (Fig. 1), derived from heparin through its controlled chemical or enzymatic depolymerization (Fig. 2), went through a rigorous approval process beginning in the 1990s and continuing today [2,3]. Thus, there has been intensive research in the analytical chemistry required for the characterization of LMW heparins.

Heparin and the related LMW heparins are structurally complex mixtures of sulfated, linear polysaccharides prepared from porcine intestinal tissues [4]. These chains consist of a 1,4-linked disaccharide repeating-unit comprised of uronic acid and glucosamine residues having a range of chain lengths. Heparin and LMW heparins exert their anticoagulant activities by binding to the serine protease inhibitor antithrombin III (AT) causing it to undergo a conformational change, becoming a potent inhibitor of the coagulation serine proteases, thrombin (factor IIa) and factor Xa [5]. Heparin and LMW heparins are applied to control blood coagulation for different diseases and in extracorporeal therapies, and are critical drugs for the practice of modern medicine [6]. Heparin is administered *intravenously* and plasma levels need to be closely monitored, while LMW heparins can also be administered *subcutaneously*, requiring little or no monitoring, and, thus, these are more widely used in the US, reducing the costs of anticoagulant therapy [4]. Furthermore, there are some differences in the activity, specificity and applications of heparin and LMW heparins [4,7].

The FDA approval of the first LMW heparin in 1993 relied on analytical, biological and pharmacological data limited by the technology available in the early 1990s for characterizing complex mixtures of a polysaccharide natural product, like heparin

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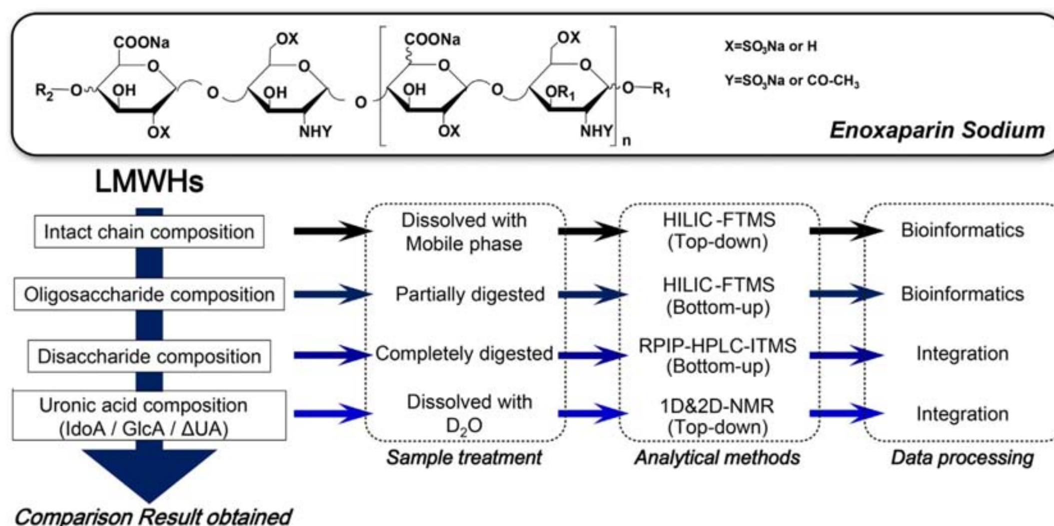


Fig. 1. The structure of target LMWH (enoxaparin sodium) and the strategy of commercial LMWH comparison. ('R_1' and 'R_2' are reducing end structures, non-reducing end structures, respectively, detailed structures are shown in Fig. 2).

[8]. Since the initial approval different types of LMW heparins as well as generic versions of specific types of LMW heparins have been approved [3,9–11], the criteria for the characterization of these drugs have grown increasingly more sophisticated. Our laboratory [12–23] and others [24–30] have developed a number of different methods useful for characterizing LMW heparins. The current study focuses on the application of an integrated top-down, bottom-up approach to characterize and compare the LMW heparin, enoxaparin, prepared by multiple manufacturers that have been approved by the FDA as generic versions and innovator LMW heparins, Lovenox™ (sold by Sanofi-Aventis in the US) and Clexane™ (sold by Sanofi-Aventis in Europe). Enoxaparin sodium is currently the most widely used LMW heparin and represents nearly half of the total anticoagulant drug market valued at over $13B [5]. This integrated approach uses mass spectrometry to look at the composition and distribution of intact LMW heparin chains, and following enzyme treatment examines oligosaccharide composition, disaccharide composition and finally NMR analysis to determine the amounts of different types of uronic acids present in these LMW heparins (Figs. 1 and 2). Moreover, this study examines the structural similarity of generic and innovator products and whether regulatory drift has resulted in structural differences between Lovenox™ and Clexane™ that initially began as identical products but been produced for years under different regulatory environments.

2. Material and methods

2.1. Samples and reagents

Lovenox™, and Clexane™ from Sanofi-Aventis (Bridgewater, NJ), enoxaparin sodium injections (generic versions of Lovenox™) from Teva Parenteral Medicines (Irvine, CA) and Sandoz (Princeton, NJ) were obtained from commercial suppliers (3 current lots of each). Unsaturated heparin disaccharide 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 sulfo) 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 [31]. LMWHs were desalted by dialysis using 1-kDa molecular weight cut-off (MWCO) dialysis tube (Spectrum Laboratories, CA, USA) and lyophilized before NMR analysis and re-dissolved in distilled water into stock solution (20 μg/μL) for LC–MS analysis.

2.2. Enzymatic Digestion of LMWHs

Samples (100 μg in 5 μL of distilled water) were added to 100 μL digestion buffer (50 mM NH_4OAc, 2 mM $CaCl_2$, pH 7.0). Heparin lyase (10 mU each in Tris-HCl buffer, pH 7.0) were added and mixed well (heparin lyase I, II and III mixture for disaccharide analysis, only heparin lyase II for bottom-up analysis). Samples were sufficiently digested in 37 °C water bath for 12 h. Enzymatic digestion was terminated by removing the enzymes using a 10-kDa molecular weight cut-off (MWCO) spin column. The filtrates were lyophilized and re-dissolved in 100 μL of distilled water at a concentration of 1 μg/μL.

2.3. Disaccharide analysis

Reverse-phase ion-pairing liquid chromatography (RPIP–LC) with on-line electrospray ion-trap mass spectrometry (ESI–ITMS) analysis were performed on an Agilent 1200 LC/MSD instrument (Agilent Technologies, Wilmington, DE, USA) equipped with a 6300 ion-trap and a binary pump [14]. A Poroshell 120, EC-C18 column (2.7 μm, 2.1 × 100 mm, Agilent Technologies, Wilmington, DE, USA) was used for separation. Eluent A was water/acetonitrile (85:15, v/v), and eluent B was water/acetonitrile (35:65, v/v). Both eluents contained 12 mM TrBA and 38 mM NH_4OAc and their pH values

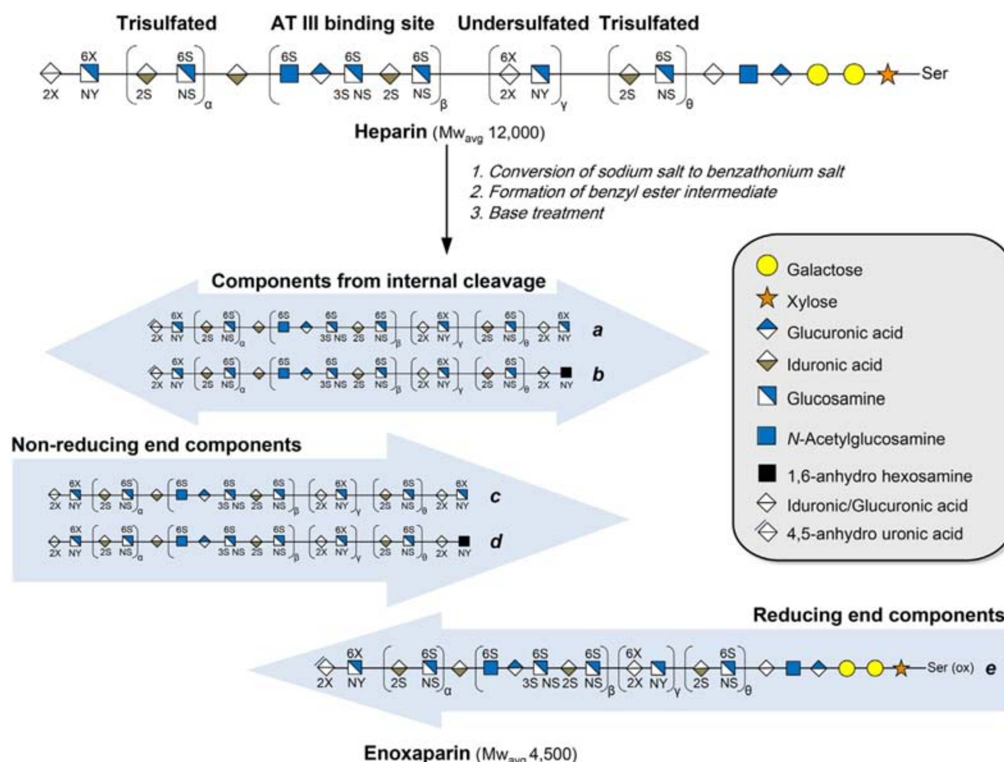


Fig. 2. Synthesis and schematic structures of enoxaparin. Chains generated from the internal structure of parent heparin chains are the most abundant components. Oligosaccharides carrying heparin non-reducing end (NRE) and heparin reducing end (RE) structures are minor components. (α, β, γ and θ represent different domains in heparin structure. $\alpha+\theta\sim13$, $\gamma=0$ or 1, $\beta\sim5$ for heparin. $\alpha+\theta\sim6$, $\gamma=0$ or 1, $\beta\sim2$ for enoxaparin.).

were adjusted to 6.5 with HOAc. Detailed LC–MS parameters are shown in supporting information.

2.4. Bottom-up and top-down analysis

Online hydrophilic interaction chromatography (HILIC) Fourier transform mass spectrometry (FTMS) was applied to analyze the intact chains and the oligosaccharide fragments generated by heparin lyase II digestion [17,18]. 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 LC column was directly connected online to the standard ESI source of LTQ-Orbitrap XL FT-MS (Thermo Fisher Scientific, San-Jose, CA). Detailed LC–MS parameters are presented in supporting information.

2.5. Bioinformatics for bottom-up and top-down analysis

Charge deconvolution was auto-processed by DeconTools software (web source from PNNL at http://omics.pnl.gov/). LMWH structural assignment was done by automatic processing using GlycReSoft 1.0 software developed at Boston University (http://code.google.com/p/glycresoft/downloads/list) [32]. GlycReSoft 1.0 parameters were set as Minimum Abundance, 1.0; Minimum Number of Scans, 1; Molecular Weight Lower Boundary, 200 Da; Molecular Weight Upper Boundary, 6000 Da; Mass Shift, ammonium; Match Error (E_M), 5.0 ppm; Grouping Error (E_G), 80 ppm; Adduct Tolerance (E_A), 5.0 ppm. A theoretical database was generated by GlycReSoft 1.0. All of the relative quantitative data were normalized (as percentages, %) to the total identified oligosaccharide peak area.

2.6. Nuclear magnetic resonance (NMR) analysis

Samples were exchanged by D_2O and re-dissolved into 20 mg/mL in D_2O before analysis. One-dimensional (1D) ^1H NMR was performed on a 600 MHz spectrometer (Bruker Bio-Spin, Billerica, Massachusetts). The 1D-^1H NMR experiments were performed using a 30° flip angle for 12 scans with a 5 s relaxation delay. Two-dimensional (2D) HSQC-NMR spectra were obtained on an Advance II 800 MHz spectrometer (Bruker Bio-Spin, Billerica, Massachusetts). The 2D-HSQC had a J coupling value of 145 Hz, with 24 scans in the direct dimension (^1H), 210 experiments for the indirect dimension (^{13}C) with a transmitter offset of 4.703 ppm (^1H) and 80.0 ppm (^{13}C). Processing of integration was performed using Topspin 2.1.6 (Bruker Bio-Spin, Billerica, Massachusetts) on the ^1H spectra using an average of triplicate measurements.

3. Results and discussion

The analysis of LMW heparins has been a work in progress since the initial approval of Lovenox^{TM} (enoxaparin sodium) in 1993. The initial approval of this first LMW heparin was based on a relatively simple characterization of its molecular weight by gel permeation chromatography and its activity by the clotting assays that had been used for heparin at the time [8]. Subsequently, more sophisticated analysis including disaccharide compositional analysis [14,20] and oligosaccharide analysis [18,21] were introduced as bottom-up approaches to better understand the building blocks that made up LMW heparins. One building block are oligosaccharides with 1,6-anhydro sugar residues at their reducing ends (REs), introduced into a fraction of the polysaccharide chains of enoxaparin during its manufacture [33,34]. The 1,6-anhydro sugar

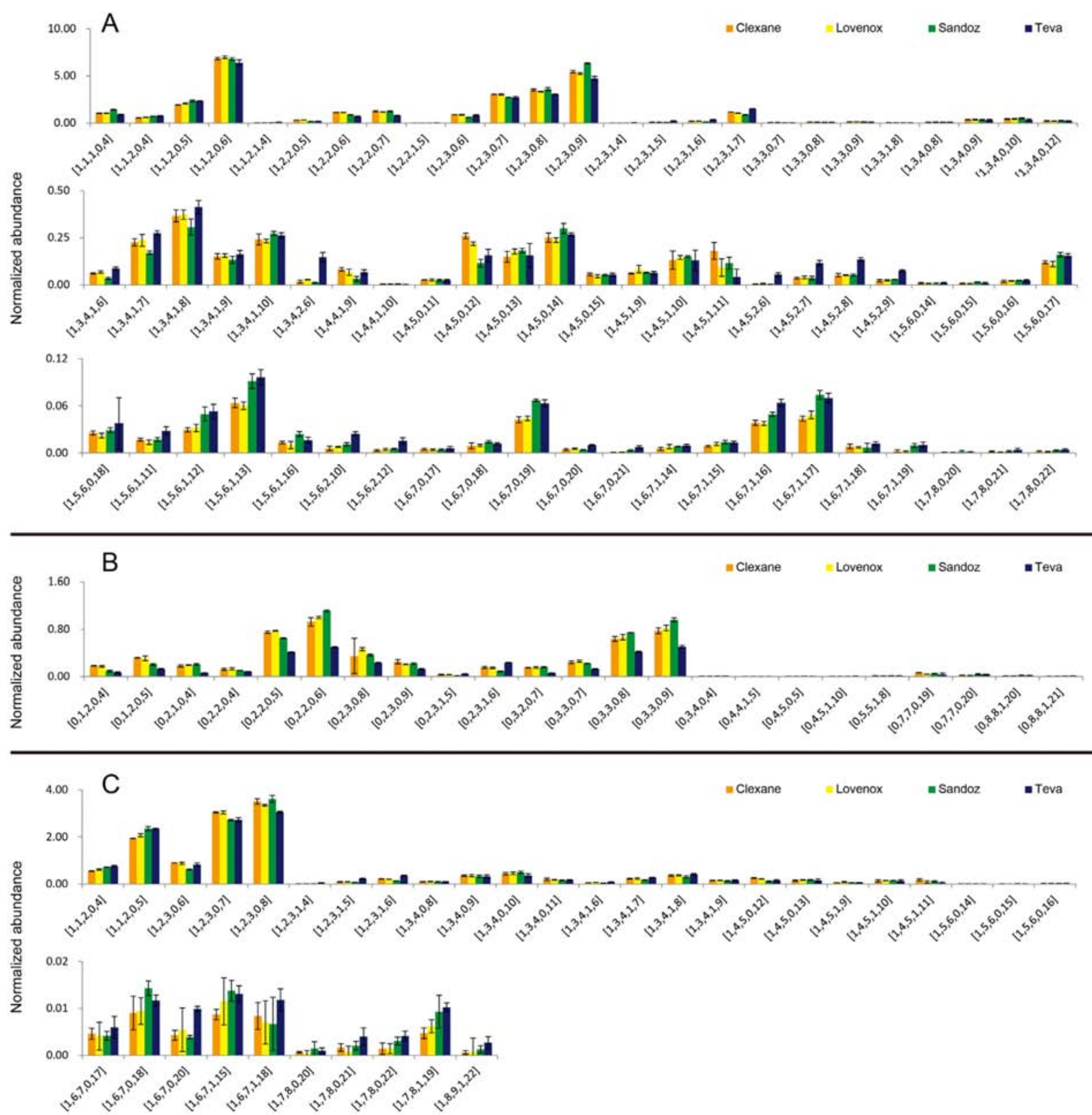


Fig. 3. Intact chain composition comparison by HILIC-FTMS (Top-down analysis results). A. Chains with unsaturated NREs and regular REs. B. Chains with saturated NREs and regular REs. C. Chains with unsaturated NREs and 1,6-anhydro REs. (Saccharide structure is listed as [ΔHexA, HexA, HexN, Ac, SO_3^-]. The error bars indicate the standard deviation of 3 lots of LMW heparins).

content of a LMW heparin now represents a required characteristic for all generic versions of enoxaparin [33,34]. Recently, more sophisticated bottom-up analysis relying on liquid chromatography (LC)- and capillary electrophoresis (CE)- mass spectrometry (MS) have resulted in the characterization of many more components of enoxaparin, including ones having a variety of additional modifications [35,36]. Moreover, as the result of advances in LC–MS and the availability of bioinformatics software [32], top-down analysis using hyphenated-MS analysis of the intact chains of enoxaparin can now also be routinely performed [17]. In addition, one-dimensional and multi-dimensional nuclear magnetic resonance (NMR) spectrometry has been used in the top-down analysis of intact enoxaparin chains [37–39] and can provide detailed information on the stereochemistry of the uronic acid residues that are very difficult [40,41] or impossible to determine using MS analysis. The current study has carefully selected and combined top-down and bottom-up methods, providing a strategy for the more complete characterization of enoxaparin coming from multiple manufacturers.

3.1. Top-down analysis

Intact enoxaparin chains from four different enoxaparin products, Clexane™, Lovenox™ (enoxaparin innovator drugs), enoxaparin-Sandoz and enoxaparin-Teva, with up to 18 saccharide units (degree of polymerization (dp)18) were first examined

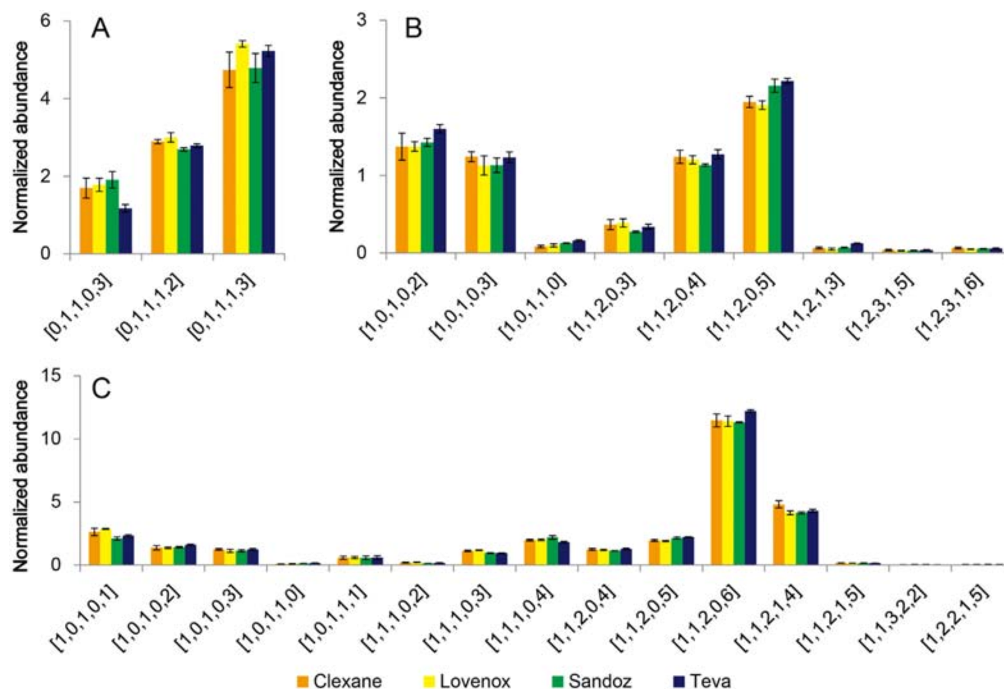


Fig. 4. Oligosaccharide composition comparison by HILIC-FTMS (Bottom-up analysis results). A. Disaccharides with saturated NREs and regular REs. B. Disaccharides, tetrasaccharides, and hexasaccharides with unsaturated NREs and 1,6 anhydro REs. C. Disaccharides, trisaccharides, tetrasaccharides, pentasaccharides with unsaturated NREs and regular RE structure. (Saccharide structure is listed as [ΔHexA, HexA, HexN, Ac, SO_3^-], i.e., the major trisulfated disaccharide product of heparin lyase treated heparin, ΔUA2S (1 → 4) GlcNS6S, is [1,0,1,0,3]. The error bars indicate the standard deviation of 3 lots of LMW heparins).

by top-down LC–MS (Fig. 3). These chains had no more than two *N*-acetyl groups most contained unsaturated non-reducing ends (NREs), resulting from the chemical β-elimination process (Fig. 2) used to prepare enoxaparin [2,42]. The major components of enoxaparin are shown in Fig. 3A. The innovator drugs, Clexane™ and Lovenox™, are believed to be prepared through the same manufacturing process and their major components are almost identical in top-down analysis. The generic enoxaparin-Sandoz shows some subtle differences when compared to the innovator drugs and somewhat greater differences are seen in the generic enoxaparin-Teva. The Teva product was more highly *N*-acetyl substituted chains than other LMWHs, this can be clearly seen in dp10 ([1,4,5,2,7]-[1,4,5,2,9], where the number of each [ΔHexA, HexA, HexN, Ac, SO_3^-] is shown) and dp12 ([1,5,6,1,11]-[1,5,6,2,12]). The enoxaparin-Sandoz shows a similar *N*-acetyl trend as enoxaparin-Teva in dp12 ([1,5,6,1,11]-[1,5,6,2,12]) and dp14. The presence of chains with saturated NREs ([0,. . .], derived from the original NRE of the parent heparin, were also detected (Fig. 3B). Enoxaparin-Teva was lower in these chains suggesting it was derived from a heparin precursor of high molecular weight. Finally, oligosaccharides with 1,6-anhydro RE sugars, coming from a side reaction in the process chemistry, were also detected (Fig. 3). A higher *N*-acetyl in enoxaparin-Teva was also observed in these chains (dp6 [1–3,1,4]-[1,2,3,1,6]).

3.2. Bottom-up analysis

Next bottom-up analysis of the same four heparin products was undertaken following heparin lyase II treatment using LC–MS. Oligosaccharide chains ranging from disaccharide (dp2) to hexasaccharide (dp6) were observed (Fig. 4). The major repeating unit of heparin, comprising 60–90% of its structure is [→ 4) IdoA2S (1 → 4) GlcNS6S (1 → $]_n$ [2,42]. When heparin (or LMW heparin) is treated with heparin lyase, a tetrasaccharde with the structure ΔUA2S (1 → 4) GlcNS6S (1 → 4) IdoA2S (1 → 4) GlcNS6S is formed, which is resistant to heparin lyase breakdown unless excessive amounts of enzyme is used [13,43,44]. Tetrasaccharide [1,1,2,0,6], corresponding to this heparin lyase II-resistant tetrasaccharide is a dimer of the ΔUA2S(1 → 4) GlcNS6S disaccharide [1,0,1,0,3] and this explains the relatively low abundance of this trisulfated disaccharide in this bottom-up analysis (Fig. 4C). Tetrasaccharides [1,1,2,0,5] and [1,1,2,1,4], 3-*O*-sulfo group containing tetrasaccharides, are believed to have the structures, ΔUA2S (1 → 4) GlcNS (1 → 4) GlcA (1 → 4) GlcNS6S3S and ΔUA (1 → 4) GlcNAc6S (1 → 4) GlcA (1 → 4) GlcNS6S3S, based on their retention times and mass [13,16]. These heparin lyase II-resistant tetrasaccharide correspond to a portion of the pentasaccharide AT-binding site and thus provide supporting information on the similar anticoagulant activities of the enoxaparin products.

3.3. Major component composition

The five types of LMW heparin chains were next analyzed (Fig. 2). The percentages of *a*, *b*, *c*, and *d* were generated from LC–MS top-down analysis and shown in Fig. 5A. Different dehydration was used to generate different hypothetical databases in software (GlycReSoft). Chain type *a* have an unsaturated NRE arising from internal cleavage and have a *N*-substituted glucosamine residue at its RE. This is the most abundant chain type in enoxaparin. Chain type *b* corresponds to chains with REs having 1,6-anhydro residues. Chain type *c* corresponds to chains with parent heparin's saturated uronic acid residue and a *N*-substituted glucosamine residue at it's RE. Chain type *d* corresponds to chains with the parent heparin's saturated uronic acid residue at its NRE but with a 1,6-anhydro residue at their REs. In our hypothetical database chain type *d* shows the same dehydration (loss of 18 amu) with type *a.* As the 1,6-anhydro RE structure generation is critical whether the NRE structure is saturated or not, we can calculate these from the equa-

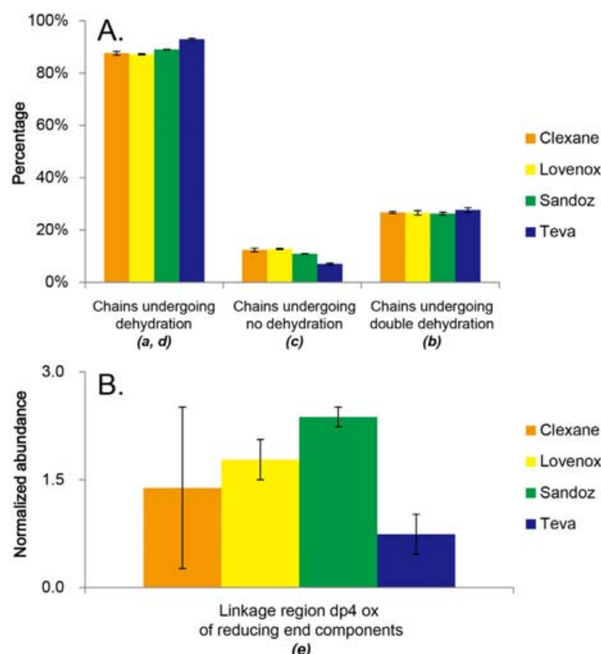


Fig. 5. Relatively quantitative comparison of 5 kinds of oligosaccharide chains (Structures are shown in Fig. 2). A. Percentage of 4 major components calculated from LC–MS top-down analysis. B. The normalized abundance of heparin linkage region tetrasaccharide (Obtained from LC–MS bottom-up analysis).

tion $(a+b)/b=(c+d)/d$ (*a, b, c, d* here correspond to the percentage of *a, b, c, d* type chains.). In Lovenox^{TM}, the total percentage of *a* and *d* type is 87.2%, the percentage of *c* type is 12.8%, and the percentage of *b* type is 26.5%. Using the equation is $(87.2\%-d)/26.5\%=(12.8\%+d)/d$, *d* could be calculated as 3.90%. The results are 3.77% for Clexane^{TM}, 3.26% for enoxaparin-Sandoz, and 2.15% for enoxaparin-Teva.

It is noteworthy that enoxaparin-Teva shows lower abundance in *d* type chains, carrying the parent heparin's NRE (Fig. 5A), which suggests that Teva's parent heparin had a longer chain length. The content of tetrasaccharide arising from the parent heparin's RE is shown in Fig. 5B. The Teva product shows lower normalized abundance as well, consistent with the NRE results.

3.4. Disaccharide and uronic acid composition

The composition of the 8 heparin disaccharides determined by LC–MS is shown in Fig. 6. Standard curves are provided in Supporting Materials Fig. S3. Disaccharide compositional analysis provides information on the sulfation pattern of each glucosamine and uronic acid residue. It is important to note that no glucosamine residues having 3-*O*-sulfo groups are observed in disaccharide analysis, as these sites are resistant to the heparin lyases, and, thus, are found in the tetrasaccharide components observed in the bottom-up analysis. In addition, disaccharide analysis provides no information on the chirality of the uronic acid residues since the C5 proton at the uronic acid is lost in the β-elimination reaction catalyzed by heparin lyases. Sandoz and Teva are both relatively higher in 0S and lower in TriS. This could be the result of desulfation in the manufacturing process or differences in the sulfation content of the porcine heparin precursor.

Top-down NMR analysis also provides information on composition but at the monosaccharide level. Quantitative analysis using 1D NMR [39] was used to determine the content of GlcA, IdoA, IdoA2S, ΔUA and ΔUA2S of each enoxaparin product. The GlcNS6 x peak was integrated from 3.26 ppm to 2.99 ppm, the GlcNAc6X peak was integrated from 1.82 ppm to 2.07 ppm, the GlcNX3S6X peak was integrated from 3.78 ppm to 3.72 ppm, the GlcNY6S peak was integrated from 66.72 ppm to 65.6 ppm and 61.26 ppm to 60.57 ppm on the carbon spectra. The IdoA2S peak was integrated from 5.21 ppm to 5.01 ppm, the IdoA peak was integrated from 4.97 ppm to 4.90 ppm, the GlcA peak was integrated from 3.26 ppm to 3.10 ppm, the ΔUA2S peak was integrated from 6.32 ppm to 6.19 ppm, the ΔUA peak was integrated from 6.13 ppm to 6.06 ppm. These peaks and this temperature were selected to provide minimum spectral overlap, and ensure the most accurate integration values. The uronic acid composition obtained from NMR integration is reported in Fig. 7A. The disaccharide analysis determine by LC–MS (Fig. 7B) can be assessed based on the monosaccharide composition form NMR results (Fig. 7A). Moreover, the application of both LC–MS and NMR on the USP heparin reference standard in Fig. 7 provides very small error bars demonstrating the low level of analytical variability of these methods.

3.5. Statistical analysis

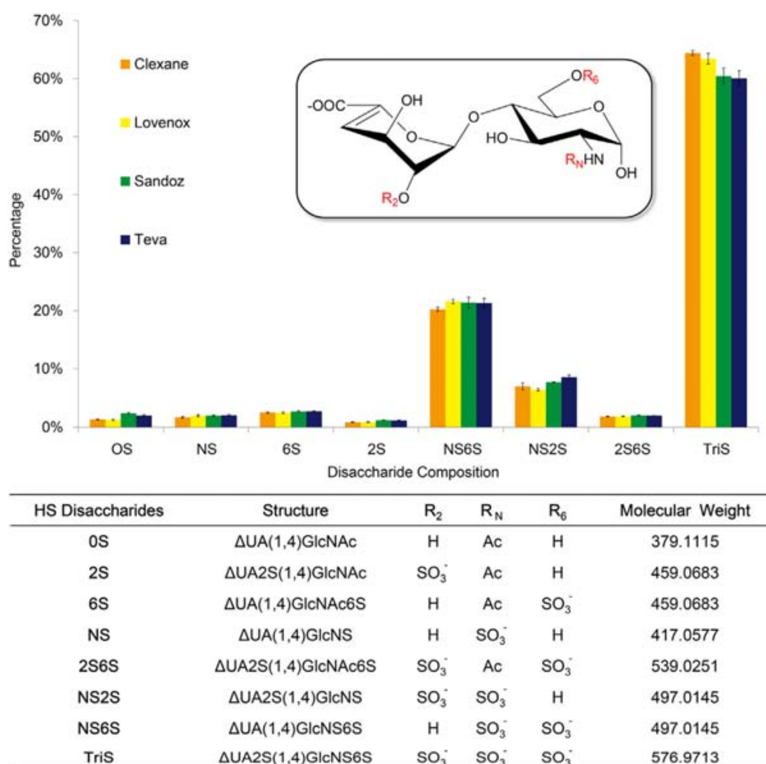
An analysis of variation (ANOVA) was performed to illustrate the similarities and differences observed between four kinds of enoxaparin drugs. The result of top-down analysis was used (Table S2) since it provides the most detailed information of chains of specific length and substitution. As discussed in Section 3.1, differences in *N*-acetylglucosamine content were observed, chains with two acetyl groups ([1,3,4,2,6], [1,4,5,2,6], [1,4,5,2,7], [1,4,5,2,8], [1,4,5,2,9], [1,5,6,2,10], [1,5,6,2,12]) were analyzed to show the specific differences (Table S3). In the highly acetylated chains, $P=0.002<0.01$, showing that the differences are significant.

The P-value of total top-down analysis is 0.99, which indicate the four commercial enoxaparin drugs are highly consistent with each other. Similarly, no significant differences in *N*-acetyl glucosamine content was observed in any of the LMW heparins based on either NMR monosaccharide (detailed ANOVA result shown in Table S4, S5) or LC–MS bottom-up analysis (detailed ANOVA result shown in Table S6). Indeed, no differences were observed in the sulfo group substitution on the glucosamine residues. Since the top-down analysis gives detailed information on each kind of intact chain, these differences could be reduced by the polydispersity of the mixture, and, thus cannot be revealed through statistical analysis. Moreover, most of the chains in enoxaparin are smaller than dp18 and LC–MS top-down analysis gives more sensitive analysis of the smaller chains. Thus, the longer chain length in the parent heparin used to prepare enoxaparin-Teva might simply be explained by a different distribution of GlcNAc instead of a different content of these residues. Top-down LC–MS analysis reveals detailed information while top-down NMR saccharide analysis and the bottom-up analyses can reveal only an overview of the samples.

The minor differences observed in major component composition (Section 3.3), which suggests a longer parent heparin chain for Teva enoxaparin, were not significant based on ANOVA analysis (Table S7, S8). Similarly, in disaccharide compositional analysis, showing that Sandoz and Teva had a relatively higher 0S and TriS content (Section 3.4) were also not significant based on ANOVA analysis (Table S9).

4. Conclusion

The integrated application of top-down and bottom-up analysis relying on both LC–MS and NMR has provides a comprehensive assessment of the enoxaparin class of LMW heparins. The lot-to-lot variability of an enoxaparin product coming from a single manufacturer is quite low on the same order as the analytical variability of the methods used (see analytical variability in Fig. 7 for NMR



HS Disaccharides	Structure	R_2	R_N	R_6	Molecular Weight
0S	ΔUA(1,4)GlcNAc	H	Ac	H	379.1115
2S	ΔUA2S(1,4)GlcNAc	SO_3^-	Ac	H	459.0683
6S	ΔUA(1,4)GlcNAc6S	H	Ac	SO_3^-	459.0683
NS	ΔUA(1,4)GlcNS	H	SO_3^-	H	417.0577
2S6S	ΔUA2S(1,4)GlcNAc6S	SO_3^-	Ac	SO_3^-	539.0251
NS2S	ΔUA2S(1,4)GlcNS	SO_3^-	SO_3^-	H	497.0145
NS6S	ΔUA(1,4)GlcNS6S	H	SO_3^-	SO_3^-	497.0145
TriS	ΔUA2S(1,4)GlcNS6S	SO_3^-	SO_3^-	SO_3^-	576.9713

Fig. 6. Disaccharide composition comparison by RPIP-HPLC-ITMS. Disaccharide structure information is listed below The error bars indicate the standard deviation of 3 lots of LMW heparins.

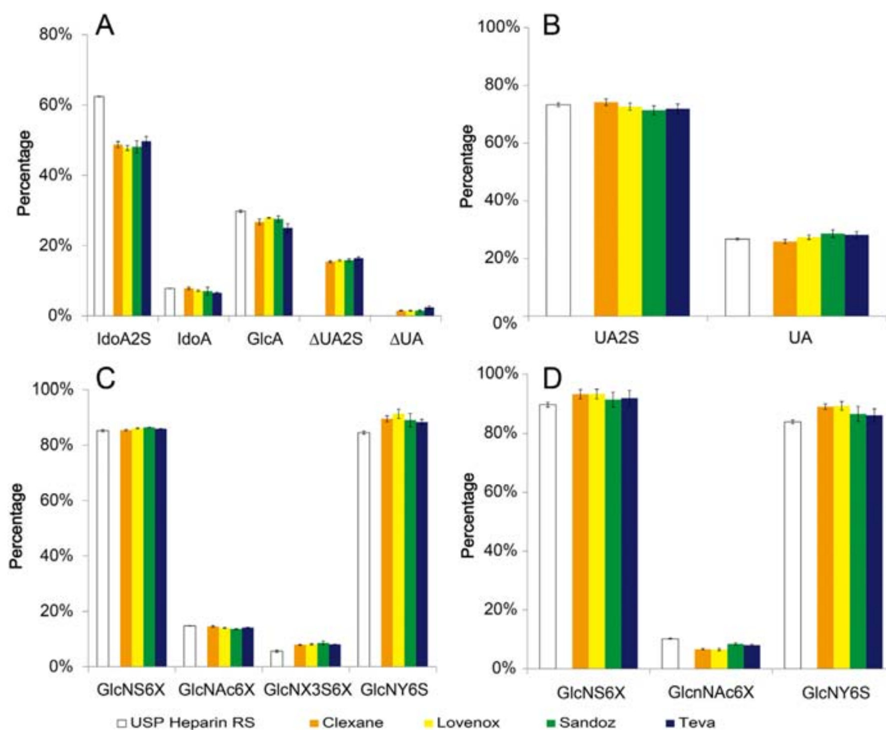


Fig. 7. Uronic acid composition and N-substituted glucosamine composition comparison. A. Uronic acid composition based on NMR integration. B. Uronic acid composition based on disaccharide analysis. C. N-substituted glucosamine composition based on NMR integration. D. N-substituted glucosamine composition based on disaccharide analysis. The error bar on the USP heparin sample indicates the analytical variability of the method. The bars on the LMWHs correspond to batch variability.

analysis, Fig. S4 for bottom-up analysis and Fig. S5 for top-down analysis). This demonstrates that each manufacturer is preparing a consistent product. Furthermore these comprehensive analyses demonstrate that the generic enoxaparins, prepared by both Sandoz and Teva, are very similar to the innovator products. The most prominent differences in top-down analyses come in minor chains and appear to arise from differences in the structure of the parent heparins being used by different manufacturers. The bottom-up analyses seem to confirm these observations with the largest differences between LMW heparins from different manufacturers and even differences in lots of LMW heparin from a single manufacturer being observed in the linkage region domains associated with the parent precursor heparins. Finally some differences are observed, which are greater than either analytical error or lot-to-lot variability, between Lovenox™ and Clexane™, both prepared by the same manufacturer and presumably the same process but under US and European regulations, suggest that some regulatory drift has taken place in products that were once identical. More research will be required to compare matched lots of parent heparins with lots of daughter LMW heparins undergoing identical manufacturing processes to better understand the reason for differences in supposedly identical enoxaparin products.

Conflict of interest

The authors declare no competing financial interest.

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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.chroma.2016.12.021.

References

[1] R.J. Linhardt, Heparin: an important drug enters its seventh decade, Chem. Ind. (1991).
[2] R.J. Linhardt, N.S. Gunay, Production and chemical processing of low molecular weight heparins, in: Semin Thromb. Hemost, Stratton Intercontinental Medical Book Corporation, New York, 1999, pp. 5–16, c1974.
[3] U. Bhaskar, E. Sterner, A.M. Hickey, A. Onishi, F. Zhang, J.S. Dordick, R.J. Linhardt, Engineering of routes to heparin and related polysaccharides, Appl. Microbiol. Biotechnol. 93 (2012) 1–16.
[4] R.J. Linhardt, Claude S. Hudson award address in carbohydrate chemistry. Heparin: structure and activity, J. Med. Chem. 46 (2003) 2551–2564.
[5] A. Onishi, K. St Ange, J.S. Dordick, R.J. Linhardt, Heparin and anticoagulation, Glycosaminoglycans Relat. Disord. Front. Biosci. 21 (2015) 1372–1392.
[6] D.C. Sabiston Jr., Mysterious heparin: the key to open heart surgery, Ann. Surg. 214 (1991) 89.
[7] Y. Xu, C. Cai, K. Chandarajoti, P.-H. Hsieh, L. Li, T.Q. Pham, E.M. Sparkenbaugh, J. Sheng, N.S. Key, R. Pawlinski, Homogeneous low-molecular-weight heparins with reversible anticoagulant activity, Nat. Chem. Biol. 10 (2014) 248.
[8] A.Y. Szajek, E. Chess, K. Johansen, G. Gratzl, E. Gray, D. Keire, R.J. Linhardt, J. Liu, T. Morris, B. Mulloy, The US regulatory and pharmacopeia response to the global heparin contamination crisis, Nat. Biotechnol. 34 (2016) 625–630.
[9] R.J. Linhardt, J. Liu, Synthetic heparin, Curr. Opin. Pharmacol. 12 (2012) 217–219.
[10] B. Wang, L.F. Buhse, A. Al-Hakim, M.T.B. Ii, D.A. Keire, Characterization of currently marketed heparin products: analysis of heparin digests by RPIP-UHPLC–QTOF–MS, J. Pharm. Biomed. Anal. 67 (2012) 42–50.
[11] M. Cohen, W.P. Jeske, J.C. Nicolau, G. Montalescot, J. Fareed, US Food and drug administration approval of generic versions of complex biologics: implications for the practicing physician using low molecular weight heparins, J. Thromb. Thrombolysis 33 (2012) 230–238.
[12] Z. Zhang, J. Xie, H. Liu, J. Liu, R.J. Linhardt, Quantification of heparan sulfate disaccharides using ion-pairing reversed-phase microflow high-performance liquid chromatography with electrospray ionization trap mass spectrometry, Anal. Chem. 81 (2009) 4349–4355.
[13] Z. Xiao, B.R. Tappen, M. Ly, W. Zhao, L.P. Canova, H. Guan, R.J. Linhardt, Heparin mapping using heparin lyases and the generation of a novel low molecular weight heparin, J. Med. Chem. 54 (2010) 603–610.
[14] B. Yang, A. Weyers, J.Y. Baik, E. Sterner, S. Sharfstein, S.A. Mousa, F. Zhang, J.S. Dordick, R.J. Linhardt, Ultra-performance ion-pairing liquid chromatography with on-line electrospray ion trap mass spectrometry for heparin disaccharide analysis, Anal. Biochem. 415 (2011) 59–66.
[15] Y. Chang, B. Yang, X. Zhao, R.J. Linhardt, Analysis of glycosaminoglycan-derived disaccharides by capillary electrophoresis using laser-induced fluorescence detection, Anal. Biochem. 427 (2012) 91–98.
[16] N. Volpi, F. Maccari, J. Suwan, R.J. Linhardt, Electrophoresis for the analysis of heparin purity and quality, Electrophoresis 33 (2012) 1531–1537.
[17] L. Li, F. Zhang, J. Zaia, R.J. Linhardt, Top-down approach for the direct characterization of low molecular weight heparins using LC–FT–MS, Anal. Chem. 84 (2012) 8822–8829.
[18] G. Li, J. Steppich, Z. Wang, Y. Sun, C. Xue, R.J. Linhardt, L. Li, Bottom-up low molecular weight heparin analysis using liquid chromatography-Fourier transform mass spectrometry for extensive characterization, Anal. Chem. 86 (2014) 6626–6632.
[19] D. Li, L. Chi, L. Jin, X. Xu, X. Du, S. Ji, L. Chi, Mapping of low molecular weight heparins using reversed phase ion pair liquid chromatography–mass spectrometry, Carbohydr. Polym. 99 (2014) 339–344.
[20] Z. Wang, D. Li, X. Sun, X. Bai, L. Jin, L. Chi, Liquid chromatography–diode array detection–mass spectrometry for compositional analysis of low molecular weight heparins, Anal. Biochem. 451 (2014) 35–41.
[21] X. Xu, D. Li, L. Chi, X. Du, X. Bai, L. Chi, Fragment profiling of low molecular weight heparins using reversed phase ion pair liquid chromatography-electrospray mass spectrometry, Carbohydr. Res. 407 (2015) 26–33.
[22] X. Sun, L. Li, K.H. Overdier, L.A. Ammons, I.S. Douglas, C.C. Burlew, F. Zhang, E.P. Schmidt, L. Chi, R.J. Linhardt, Analysis of total human urinary glycosaminoglycan disaccharides by liquid chromatography–tandem mass spectrometry, Anal. Chem. 87 (2015) 6220–6227.
[23] Y. Guan, X. Xu, X. Liu, A. Sheng, L. Jin, R.J. Linhardt, L. Chi, Comparison of low molecular weight heparins prepared using bovine lung heparin and porcine intestine heparin as starting materials, J. Pharm. Sci. 105 (2016) 1843–1850.
[24] R.P. Patel, C. Narkowicz, G.A. Jacobson, Effective reversed-phase ion pair high-performance liquid chromatography method for the separation and characterization of intact low-molecular-weight heparins, Anal. Biochem. 387 (2009) 113–121.
[25] A.M. Brustkern, L.F. Buhse, M. Nasr, A. Al-Hakim, D.A. Keire, Characterization of currently marketed heparin products: reversed-phase ion-pairing liquid chromatography mass spectrometry of heparin digests, Anal. Chem. 82 (2010) 9865–9870.
[26] F. Galeotti, N. Volpi, Online reverse phase-high-performance liquid chromatography-fluorescence detection-electrospray ionization-mass spectrometry separation and characterization of heparan sulfate heparin, and low-molecular weight-heparin disaccharides derivatized with 2-ami, Anal. Chem. 83 (2011) 6770–6777.
[27] Q. Zhang, X. Chen, Z. Zhu, X. Zhan, Y. Wu, L. Song, J. Kang, Structural analysis of low molecular weight heparin by ultraperformance size exclusion chromatography/time of flight mass spectrometry and capillary zone electrophoresis, Anal. Chem. 85 (2013) 1819–1827.
[28] F. Galeotti, N. Volpi, Novel reverse-phase ion pair-high performance liquid chromatography separation of heparin, heparan sulfate and low molecular weight-heparins disaccharides and oligosaccharides, J. Chromatogr. A 1284 (2013) 141–147.
[29] J. Henriksen, L.H. Ringborg, P. Roepstorrf, On-line size-exclusion chromatography/mass spectrometry of low molecular mass heparin, J. Mass Spectrom. 39 (2004) 1305–1312.
[30] G.O. Staples, M.J. Bowman, C.E. Costello, A.M. Hitchcock, J.M. Lau, N. Leymarie, C. Miller, H. Naimy, X. Shi, J. Zaia, A chip-based amide-HILIC LC/MS platform for glycosaminoglycan glycomics profiling, Proteomics 9 (2009) 686–695.
[31] H. Su, F. Blain, R.A. Musil, J.J. Zimmermann, K. Gu, D.C. Bennett, Isolation and expression in Escherichia coli of hepB and hepC genes coding for the glycosaminoglycan-degrading enzymes heparinase II and heparinase III, respectively, from Flavobacterium heparinum, Appl. Environ. Microbiol. 62 (1996) 2723–2734.
[32] E. Maxwell, Y. Tan, Y. Tan, H. Hu, G. Benson, K. Aizikov, S. Conley, G.O. Staples, G.W. Slysz, R.D. Smith, GlycReSoft: a software package for automated recognition of glycans from LC/MS data, PLoS One 7 (2012) e45474.
[33] G. Mascellani, M. Guerrini, G. Torri, L. Liverani, F. Spelta, P. Bianchini, Characterization of di-and monosulfated, unsaturated heparin disaccharides with terminal N-sulfated 1, 6-anhydro-β-D-glucosamine or N-sulfated 1, 6-anhydro-β-D-mannosamine residues, Carbohydr. Res. 342 (2007) 835–842.
[34] M. Guerrini, S. Elli, D. Gaudesi, G. Torri, B. Casu, P. Mourier, F. Herman, C. Boudier, M. Lorenz, C. Viskov, Effects on molecular conformation and anticoagulant activities of 1, 6-anhydrosugars at the reducing terminal of antithrombin-binding octasaccharides isolated from low-molecular-weight heparin enoxaparin, J. Med. Chem. 53 (2010) 8030–8040.
[35] D. Beccati, S. Roy, F. Yu, N.S. Gunay, I. Capila, M. Lech, R.J. Linhardt, G. Venkataraman, Identification of a novel structure in heparin generated by potassium permanganate oxidation, Carbohydr. Polym. 82 (2010) 699–705.

[36] D. Beccati, S. Roy, M. Lech, J. Ozug, J. Schaeck, N.S. Gunay, R. Zouaoui, I. Capila, G.V. Kaundinya, Identification of a novel structure in heparin generated by sequential oxidative–reductive treatment, Anal. Chem. 84 (2012) 5091–5096.
[37] C.J. Jones, S. Beni, J.F.K. Limtiaco, D.J. Langeslay, C.K. Larive, Heparin characterization: challenges and solutions, Annu. Rev. Anal. Chem. 4 (2011) 439–465.
[38] H. Ye, T.K. Toby, C.D. Sommers, H. Ghasriani, M.L. Trehy, W. Ye, R.E. Kolinski, L.F. Buhse, A. Al-Hakim, D.A. Keire, Characterization of currently marketed heparin products: key tests for LMWH quality assurance, J. Pharm. Biomed. Anal. 85 (2013) 99–107.
[39] L. Fu, G. Li, B. Yang, A. Onishi, L. Li, P. Sun, F. Zhang, R.J. Linhardt, Structural characterization of pharmaceutical heparins prepared from different animal tissues, J. Pharm. Sci. 102 (2013) 1447–1457.
[40] J.J. Wolff, L. Chi, R.J. Linhardt, I.J. Amster, Distinguishing glucuronic from iduronic acid in glycosaminoglycan tetrasaccharides by using electron detachment dissociation, Anal. Chem. 79 (2007) 2015–2022.
[41] F.E. Leach III, M. Ly, T.N. Laremore, J.J. Wolff, J. Perlow, R.J. Linhardt, I.J. Amster, Hexuronic acid stereochemistry determination in chondroitin sulfate glycosaminoglycan oligosaccharides by electron detachment dissociation, J. Am. Soc. Mass Spectrom. 23 (2012) 1488–1497.
[42] R.J. Linhardt, D. Loganathan, A. Al-Hakim, H.M. Wang, J.M. Walenga, D. Hoppensteadt, J. Fareed, Oligosaccharide mapping of low-molecular-weight heparins: structure and activity differences, J. Med. Chem. 33 (1990) 1639–1645.
[43] K.G. Rice, R.J. Linhardt, Study of structurally defined oligosaccharide substrates of heparin and heparan monosulfate lyases, Carbohydr. Res. 190 (1989) 219–233.
[44] U.R. Desai, H.M. Wang, R.J. Linhardt, Substrate specificity of the heparin lyases from Flavobacterium heparinum, Arch. Biochem. Biophys. 306 (1993) 461–468.