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Mapping of low molecular weight heparins using reversed phase ion pair liquid chromatography–mass spectrometry

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ABSTRACT

Low molecular weight heparins (LMWHs) are structurally complex, highly sulfated and negatively charged, linear carbohydrate polymers prepared by chemical or enzymatic depolymerization of heparin. They are widely used as anticoagulant drugs possessing better bioavailability, longer half-life, and lower side effects than heparin. Comprehensive structure characterization of LMWHs is important for drug quality assurance, generic drug application, and new drug research and development. However, fully characterization of all oligosaccharide chains in LMWHs is not feasible for current available analytical technologies due to their structure complexity and heterogeneity. Fingerprinting profiling is an efficient way for LMWHs' characterization and comparison. In this work, we present a simple, sensitive, and powerful analytical approach for structural characterization of LMWHs. Two different LMWHs, enoxaparin and nadroparin, were analyzed using reversed phase ion pair electrospray ionization mass spectrometry (RPIP-ESI-MS). More than 200 components were identified, including major structures, minor structures, and process related impurities. This approach is robust for high resolution and complementary fingerprinting analysis of LMWHs.

1. Introduction

Heparin is an anticoagulant drug widely used in prevention and treatment of thromboembolic diseases. Belonging to the glycosaminoglycan family, heparin is a negatively charged linear polysaccharide, with repeating disaccharide units composed of glucosamine residue and uronic acid residue. The structural complexity of heparin arises from variable chain lengths, substitutions of sulfo and acetyl groups, as well as epimerization of iduronic acid to glucuronic acid (Cosmi & Palareti, 2012). LMWHs are truncated forms of heparin derived by controlled chemical or enzymatic degradation (Higashi et al., 2012). For example, enoxaparin is prepared by benzylation and alkaline depolymerization, while nadroparin is prepared by nitrous acid depolymerization (Fig. 1). Compared to the unfractionated heparin, LMWHs inherit the repeating disaccharide units and primary chain sequence, but possess lower molecular weights and modified disaccharide units at either or both chain terminals as the result of chemical cleavage. The clinical advantages of LMWHs are their increased bioavailability and minimized side effects, by which LMWHs occupied major share of the overall heparin market (Weitz, 1997; Cosmi & Palareti, 2012; Linhardt & Liu, 2012).

Structural characterization of LMWHs is very important at the aspects of drug safety and generic drug applications. The heparin contamination crisis in 2008 broke out because of the insufficiency of analytical tests used in drug production and regulation at that time (Guerrini et al., 2008). The production of LMWHs from heparin involves of one or more chemical reactions. It is likely to introduce heparin structure related impurities due to the incomplete reactions or side reactions. On the other hand, generic versions of LMWHs can benefit patients by reducing the treatment costs. The Food and Drug Administration (FDA) in the United States approved the first generic enoxaparin based on five criteria to determine the sameness of generic and innovator products in 2010 (Harenberg, 2011; Lee et al., 2013). Among the comparison analytical methods used to meet these criteria, the most critical and challenging one is to provide complementary information through a high-resolution fingerprint profile of oligosaccharide molecular weights. The chain mapping methods mentioned in the FDA guidelines include cetyltrimethylammonium coated strong anion exchange (CTA-SAX), matrix-assisted laser desorption ionization

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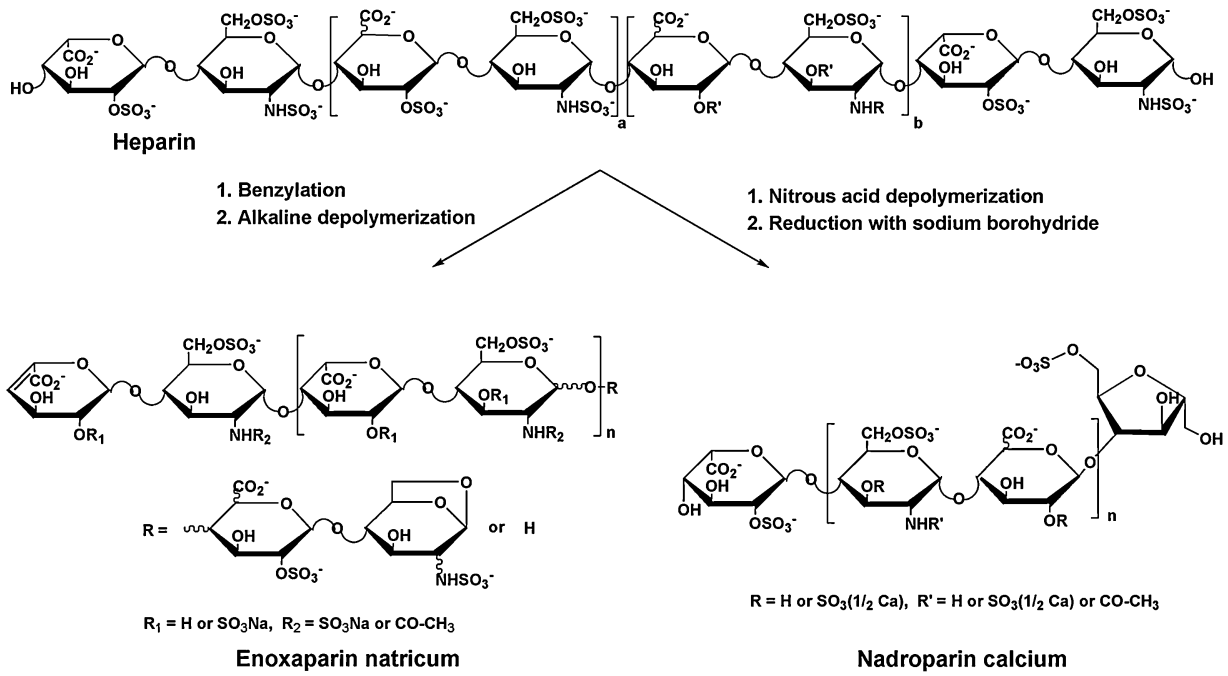

Fig. 1. Structures and depolymerization process of LMWHs. Enoxaparin sodium is prepared by benzylation and alkaline depolymerization, forming unsaturated uroate residue at the non-reducing end and 1,6-anhydro amino sugar residue at the reducing end of some oligosaccharide chains. Nadroparin calcium is prepared by nitrous acid depolymerization followed by reduction with sodium borohydride, forming 2,5-anhydro-D-mannitol at the reducing end.

(MALDI)-MS, gel permeation chromatography (GPC)-ESI-MS, and RPIP-ESI-MS (FDA response to Citizen Petition Docket No. FDA-2003-P-0273).

High performance liquid chromatography (HPLC) methods, such as SAX-HPLC, have been used in heparin analysis for more than two decades (Linhardt et al., 1988). The optimal range of heparin oligosaccharides for SAX-HPLC is from disaccharides (degree of polymerization (dp)2) to decasaccharides (dp10). As the typical oligosaccharide range of LMWHs is from dp2 to dp30, SAX-HPLC method is more suitable for mapping digested LMWH fragments. GPC is a conventional method with inherent low resolution. RPIP and CTA-SAX methods are more recent methods developed to analyze LMWHs with superior resolution and broad molecular weight (MW) range (Patel, Narkowicz, & Jacobson, 2009; Mourier & Viskov, 2004). However, the high resolution chromatographic methods usually provide hundreds of peaks and the identification of each peak is extremely tedious, if possible. The electrophoresis methods, such as capillary electrophoresis (CE) and polyacrylamide gel electrophoresis (PAGE), have the same problems on peak or band identification as chromatographic methods, no matter what resolution they afford (Patel, Narkowicz, Hutchinson, Hilder, & Jacobson, 2008; Volpi, Maccari, Suwan, & Linhardt, 2012).

Modern MS techniques led to the revolution in heparin structural analysis by providing high sensitivity, high resolution, and detailed structure information on individual chains. MALDI-MS has been used to sequence heparin oligosaccharides with chain size up to dp12, but is not suitable to analyze complex mixture and the analytical throughput is very low (Venkataraman, Shriver, Raman, & Sasisekharan, 1999). ESI-MS techniques have been successfully used to characterize glycosaminoglycan fragments or intact chains, including chondroitin sulfate and hyaluronan, with size up to dp43 (Chi et al., 2008; Ly et al., 2011; Zhao, Yang, Li, Zhang, & Linhardt, 2013). The structures of heparin and LMWHs are more difficult to analyze, because their sulfation pattern is more complicated and, in addition, the unnatural moieties are present in LWMHs. Therefore, hyphenation of ESI-MS to HPLC is necessary in order to provide complementary chain mapping profiles of LMWHs. SAX- and CTA-SAX-HPLC are not feasible to interface with ESI-MS since they require high concentration of nonvolatile salts in the mobile phases. GPC-ESI-MS has been used to analyze enoxaparin, of which 70 components were identified. But the total ion chromatogram (TIC) afforded relatively low resolution (Zhang et al., 2013). Hydrophilic interaction chromatography (HILIC)–Fourier transform-ESI-MS has been reported to compare innovator and generic enoxaparins. Nearly 300 of oligosaccharide chains were elucidated and semi-quantified. However, this method requires state-of-the-art Fourier transform mass spectrometer (Li, Zhang, Zaia, & Linhardt, 2012). RPIP-ESI-MS techniques have been adapted into heparin oligosaccharide analysis since last decade (Thanawiroon, Rice, Toida, & Linhardt, 2004; Henriksen, Roepstorff, Ringborg, 2006). Various ion pair reagents, including from propylamine to hexylamine, dibutylamine, and tributylamine, were compared for their capabilities of separating LMWHs on LC and facilitating the ionization during ESI-MS. Enoxaparin and tinzaparin (enzymatically depolymerized version LMWH), were tested on ultra performance-LC-ESI-quadruple-time of flight (TOF) instruments. Among these ion pair reagents, pentylamine (PTA) was found to afford the optimal separation as well as efficient ionization (Doneanu, Chen, Gebler, 2009; Langeslay et al., 2013).

In this paper, we described an optimized RPIP-ESI-MS method using capillary HPLC and ion trap (IT)-TOF mass spectrometer for the analysis of enoxaparin and nadroparin. This approach not only provides complementary chain mapping profiles for LWMHs, but also allows sensitive detection of minor contaminants.

2. Materials and methods

2.1. Reagents

Two LMWHs, enoxaparin sodium and nadroparin calcium chemical reference substances, were purchased from European Directorate for the Quality of Medicines & HealthCare. Acetonitrile (HPLC grade), methanol (HPLC grade), water (HPLC grade), and formic acid (certified ACS grade) were purchased from

Fisher-Scientific. PTA (purity > 99%) was purchased from Sigma–Aldrich. All other reagents and chemicals were of the highest quality available.

2.2. RPIP-ESI-MS analysis of LMWHs

A ZORBAX SB-C18 column (0.5 mm × 250 mm, 5 μm, Agilent Technologies) was used to separate LMWHs on Agilent 1100 capillary LC system. Mobile phase A was 15 mM PTA in water. Mobile phase B was 15 mM PTA in 75% aqueous acetonitrile. The pH of both mobile phases was adjusted to pH 7.0 with formic acid. LWMH samples were dissolved in mobile phase A at concentration of 10 μg/μL and the injection volume was 2 μL. For enoxaparin samples, a gradient of 16% mobile phase B for 5 min followed by a linear gradient from 5 to 65 min, 16–56% mobile phase B was used. For nadroparin, the gradient was 20% mobile phase B for 5 min followed by a linear gradient from 5 to 65 min, 20–60% mobile phase B. The flow rate of both separations was 10 μL/min and the column temperature was maintained at 35 °C.

On-line ESI-MS was performed on an IT-TOF hybrid mass spectrometer (LCMS-IT-TOF, Shimadzu, Japan). ESI-MS analysis was set in the positive ion mode and with the following parameters: source voltage at 3.6 kV, nebulizer nitrogen gas flow rate at 0.5 L/min, heat block and curved desolvation line temperature at 200 °C, and detector voltage at 1.8 kV. The mass acquisition range was set at 900–3000.

2.3. Mass spectrum interpretation

A database containing all possible oligosaccharide structures with different chain lengths, compositions (number of sulfation and number of acetylation), unnatural terminal structures, and PTA adductions was generated. The monoisotopic *m*/*z* corresponding to each oligosaccharide chain in LMWH RPIP-ESI-MS analysis was used to search for matches in the theoretical structure database. When the oligosaccharide size was larger than dp14, an isotopic shift function was incorporated because the monoisotopic peak became too low. The peak assignment was achieved by retrieving the theoretical structure corresponding to the matched *m*/*z*.

2.4. Two dimensional nuclear magnetic resonance analysis (2D-NMR)

2D-NMR was performed on a Bruker Avance 600 spectrometer equipped with 5 mm cryoprobe. The heteronuclear single-quantum correlation (HSQC) spectrum was acquired at 298 K. All spectra were processed using Topspin 2.1.

3. Results and discussion

3.1. RPIP separation of enoxaparin and nadroparin

According to the United States Pharmacopeia and the European Pharmacopeia, the characteristic weight average MWs are 4500 Da for enoxaparin and 4300 Da for nadroparin, with the ranges being between 3800 Da and 5000 Da for enoxaparin, 3600 Da and 5000 Da for nadroparin, respectively. Two separate research groups compared many of ion pair reagents and reported PTA to be most efficient for the separation and ionization of large size oligosaccharides (Doneanu et al., 2009; Langeslay et al., 2013). In addition, PTA is relatively MS friendly compared to other ion pair reagents since it contaminants the instrument least. Doneanu et al. used hexafluoroisopropanol accompanied with PTA and detected LMWHs in both the positive and negative ion modes. Langeslay et al. avoided the use of hexafluoroisopropanol since it is expensive and difficult to remove after use. They focused solely on the negative ion mode. In our RPIP separation, we optimized the method by using PTA in acetonitrile gradients, adjusted pH with formic acid. The LMWHs were still able to be ionized in the positive ion mode in the absence of hexafluoroisopropanol. The low flow rate of capillary LC at 10 μL/min minimized the amount of ion pair reagent injected to MS instrument and facilitated the electrospray.

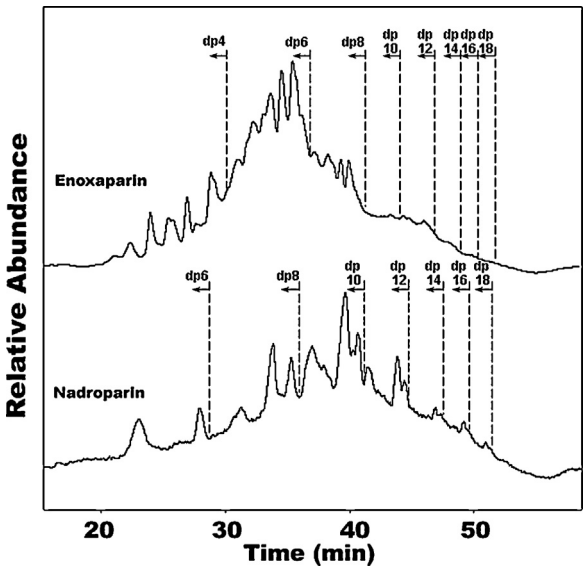

Fig. 2. TICs of RPIP-ESI-MS analysis of LMWHs. The fingerprinting profile of enoxaparin is on top and nadroparin is on bottom.

The oligosaccharides in enoxaparin were separated and detected with 15 mM PTA in a aqueous acetonitrile gradient of 16% mobile B for 5 min followed by a linear gradient from 5 to 65 min, 16–56% mobile phase B (Fig. 2). Although nadropain possesses very close weight average MW and MW distribution to enoxaparin, the separation took much longer if using the same gradient (Data not shown). We increased the starting mobile phase B composition to 20% and elevated the followed linear gradient correspondingly in order to shorten the total separation time for nadroparin. It is likely that the different counter ions, calcium for nadroparin and sodium for enoxaparin, affected the retention times of oligosaccharides with similar chain sizes on RPIP separation.

3.2. Mass spectra peak assignment

The mass spectra of RPIP-ESI-MS analysis of LMWHs are extremely complicated because of the heterogeneous LMWHs' structures, multiple charge states, as well as variable PTA adductions. Example mass spectra were shown in Fig. 3. Manual interpretation of these data is incredibly time-consuming. Furthermore, it may easily cause misinterpretation or overlooking minor structures. Bioinformatics tools, for example, the GlycReSoft software package, have been developed to extract glycan structures from LC–MS data (Maxwell et al., 2012). The process involves deconvoluting the raw data firstly, and then matching the experimental MWs to the theoretical MWs derived from the database containing all possible LMWH structures. However, automatic deconvolution of the raw data is not practical when the variable PTA adducts are present. It may also exemplify the errors when deconvoluting the data acquired by a regular TOF mass analyzer, whose resolution and mass accuracy cannot compete with a Fourier transform mass analyzer. We developed a semi-automatic data processing approach. By inputting the *m*/*z* values directly into the calculation system, the matched structures were generated with the information on chain lengths, terminal

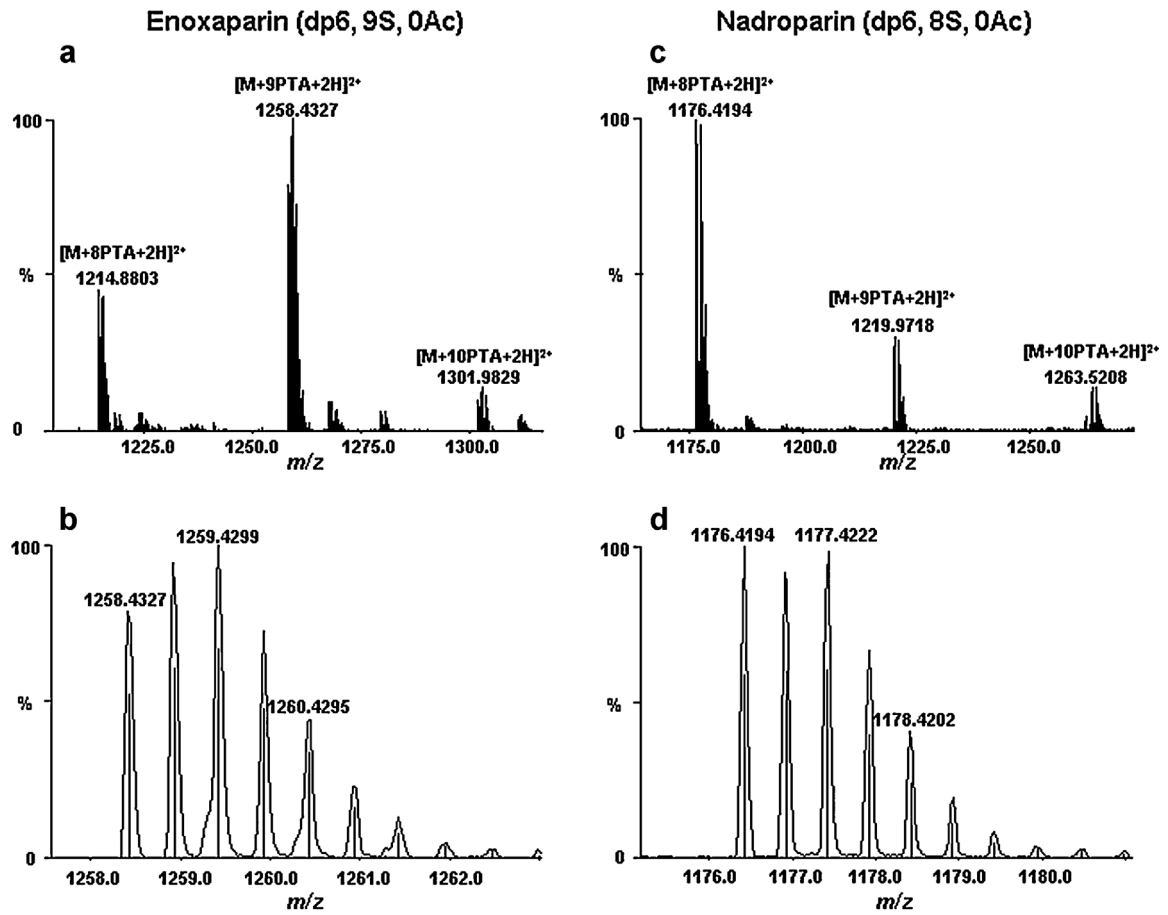

Fig. 3. Example mass spectra of LMWHs. (a) Mass spectrum of enoxaparin component dp6 with 9 sulfo groups showing various PTA adductions. (b) The zoomed spectrum of isotopic peaks with 9 PTA adductions. (c) Mass spectrum of nadroparin component dp6 with 8 sulfo groups showing various PTA adductions. (d) The zoomed spectrum of isotopic peaks with 8 PTA adductions.

structures, sulfation and acetylation substitution compositions, charge states, PTA adduction numbers, as well as errors.

Majority of enoxaparin components consist 4-enopyranose uronate at the non-reducing end. And 15–25% of enoxaparin contain 1,6-anhydro derivative at the reducing end as minor structures. The terminal structures of nadroparin chains are uniform, consisting of a 2-*O*-sulfo-α-L-idopyranosuronic acid at the non-reducing end and a 6-*O*-sulfo-2,5-anhydro-D-mannitol at the reducing end. About 150 enoxaparin and 80 nadroparin oligosaccharide structures were revealed from our RPIP-ESI-MS analysis. The majority of these oligosaccharides ranged from dp4 to dp26, with no or one *N*-acetyl group, and variable sulfation degrees. Besides the oligosaccharides having the defined enoxaparin and nadroparin structures, some minor components, such as odd number oligosaccharides and saturated enoxaparin, were also detected. All the major oligosaccharide structures are summarized in Table 1 and the comprehensive structures are listed in Supporting Information (Tables 1S and 2S). The RPIP-ESI-MS analysis with semi-automatic data processing was able to detect and identify LMWHs with MW up to nearly 8000 Da, with high mass accuracy of less than 20 ppm. It provides high resolution fingerprinting profiles for LMWH samples.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.08.074.

Table 1
Summary of oligosaccharide structures identified in RPIP-ESI-MS analysis of LMWHs.

Chain Size	Enoxaparin		Nadroparin
	Major	Minor	
dp3	3–4		
dp4	2–6	2–6	
dp6	2–9	3–9	5–8
dp8	4–12	4–12	3–11
dp10	5–15		6–14
dp12	10–18		11–17
dp14	16–21		15–20
dp16	21–24		18–23
dp18	21–24		23–26
dp20	28		27–29
dp22	27		29–31
dp24			32–33
dp26			32

3.3. Reproducibility

Both of enoxaparin and nadroparin were tested for three times using RPIP-ESI-MS in order to evaluate the reproducibility of this method (Fig. 4). The run-to-run TIC fingerprinting profiles are highly reproducible, while different LMWHs displayed characteristic patterns. The components identified from each run were also examined. The same sets of oligosaccharide chains were found from run-to-run with all mass errors within 20 ppm.

3.4. Impurity identification using RPIP-ESI-MS

The LWMH preparation process involves multiple chemical reactions, and sensitive analytical approach is necessary to detect any possible minor impurities. NMR provides detailed structure

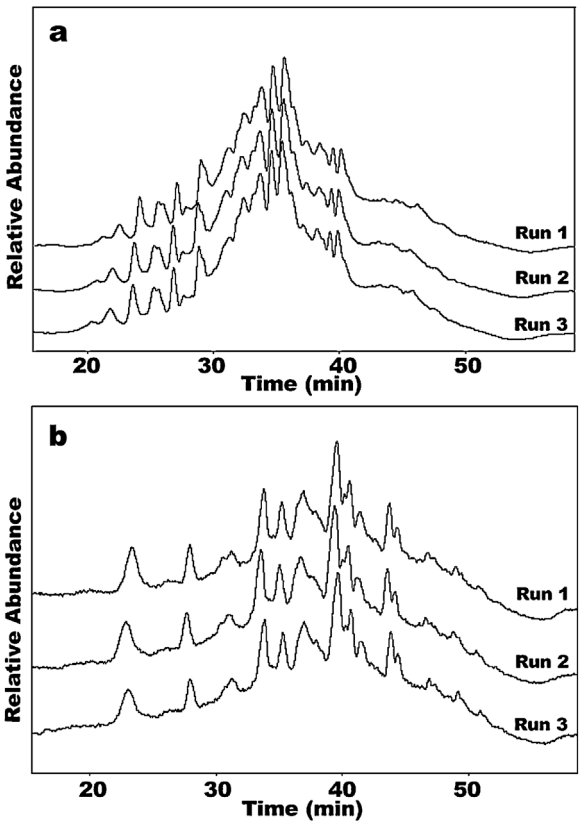

Fig. 4. Reproducibility of RPIP-ESI-MS analysis in 3 runs. (a) TICs of enoxaparin and (b) TICs of nadroparin.

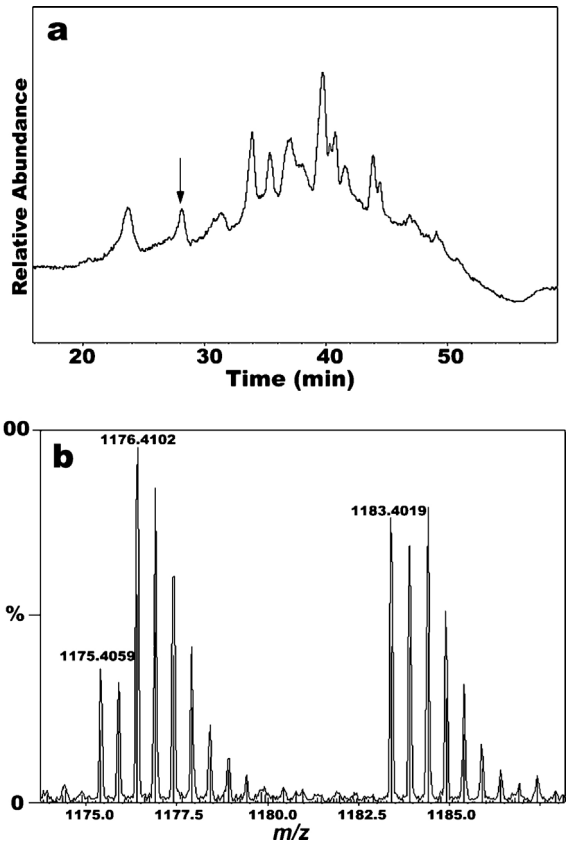

Fig. 5. Identification of impurities in nadroparin. (a) TIC of one in-house nadroparin sample. The TIC profile is similar to the TIC of nadroparin chemical reference substance. However, their mass spectra are significantly different. For example, averaged mass spectrum of the arrow pointed peak is shown in (b). *m*/*z* 1176.4102 is the doubly charged monoisotopic peak of nadroparin oligosaccharide dp6 with 8 sulfo groups, peaks with −2 Da (*m*/*z* 1175.4059) and +14 Da (1183.4019) deconvoluted MW difference are also observed.

features of LMWHs, but it is low through-put and requires relative large quantity of samples. RPIP-ESI-MS not only affords ion chromatographic profiles for LMWHs, but also provides comprehensive and sensitive information on oligosaccharide structures. Some minor impurities were noticed when an in-house process control nadroparin was tested. Its TIC profile is similar with the nadroparin reference substance. However, minor components with structures different from nadroparin oligosaccharide chains were detected in RPIP-ESI-MS analysis. Fig. 5b showed an example averaged mass spectrum from 26.5 to 27.5 min. Besides the ions of oligosaccharide dp6 with 8 sulfo groups, some doubly charged ions with *m*/*z* values 1 unit less and 7 units more were observed. The -2 Da and +14 Da MWs shifting suggests the alditol structure at the reducing end may be converted to aldehyde and acid due to the incomplete reduction reaction and followed by oxidation side

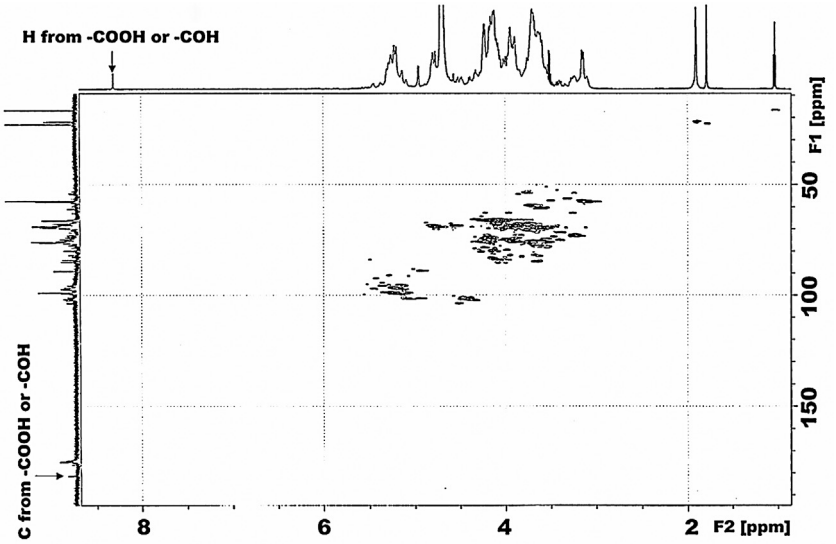

Fig. 6. 2D-CH-HSQC NMR analysis of nadroparin impurities. The signals with δ_H = 8.3 ppm and δ_C = 181.4 ppm suggest the presence of carbonyl group at the reducing end.

reaction. The hypothesis was partially confirmed by 2D-CH-HSQC NMR analysis (Fig. 6). The signal with a chemical shift of δ_H = 8.3 ppm suggests the presence of possible proton from carbonyl group at the reducing end. This signal could be either from acid (—COOH) or aldehyde (—COH). The signal with a chemical shift of δ_C = 181.4 ppm is also the evidence for the presence of carbonyl group at the reducing end. The aldehyde signals supposed to appear as a cross peak showing the one-bond linkage in the HSQC spectrum was not detected in NMR, possibly due to its low concentration.

The minor impurities bring risk on LWMH drug safety, since they may easily overlooked by other analytical approaches that lack the same sensitivity and specificity as the RPIP-ESI-MS method.

4. Conclusions

LMWHs contain very complicated micro-heterogeneous oligosaccharide chains. It is neither possible nor necessary to sequence all the carbohydrate chains in the LMWHs for quality assurance or generic drug application. RPIP-ESI-MS offers a high resolution fingerprinting method for characterization, comparison, and trouble-shooting of LMWHs. PTA effectively separated oligosaccharides on capillary HPLC and formed positively charged ions accompany with sulfated LMWH chains during ESI process. PTA is also more MS friendly than other ion pair reagents. The MS instrument can be used to analyze peptides and other compounds with minimal clean-up after RPIP-ESI-MS analysis of LMWHs. More than two hundred oligosaccharides were revealed for enoxaparin and nadropain with the help of computer assisted semi-automatic data process. Minor components in enoxaparin and process related impurities in nadroparin were sensitively detected and identified. Unlike the very expensive Fourier transform-MS mass instruments, TOF mass analyzers, including quadruple-TOF and IT-TOF, are mid-cost and widely used in biopharmaceutical industries. This analytical approach presented herein can be easily adapted for research, development, and production of LMWH drugs. Furthermore, the high sensitivity of this method on detecting minor structures made it potentially useful for structure–activity relationship study of heparin, heparan sulfate, and other important glycosaminoglycans.

Acknowledgements

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