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# Fragment profiling of low molecular weight heparins using reversed phase ion pair liquid chromatography-electrospray mass spectrometry

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## ABSTRACT

Low molecular weight heparins (LMWHs) are linear and highly charged carbohydrate polymers prepared by chemical or enzymatic depolymerization of heparin. Compared to unfractionated heparin (UFH), LMWHs are prevalently used as clinical anticoagulant drugs due to their lower side effects and better bioavailability. The work presented herein provides a rapid and powerful fragment mapping method for structural characterization of LMWHs. The chain fragments of two types of LMWHs, enoxaparin and nadroparin, were generated by controlled enzymatic digestion with each of heparinase I (Hep I, Enzyme Commission (EC) # 4.2.2.7), heparinase II (Hep II, no EC # assigned) and heparinase III (Hep III, EC # 4.2.2.8). Reversed phase ion pair high performance liquid chromatography (RPIP-HPLC) coupled with electrospray ion trap time-of-flight mass spectrometry (ESI-IT-TOF-MS) was used to profile the oligosaccharide chains ranging from disaccharides to decasaccharides. A database containing all theoretical structural compositions was established to assist the mass spectra interpretation. The six digests derived by three enzymes from two types of LMWHs exhibited distinguishable fingerprinting patterns. And a total of 94 enoxaparin fragments and 109 nadroparin fragments were detected and identified. Besides the common LMWH oligosaccharides, many components containing characteristic LMWH structures such as saturated L-idopyranosuronic acid, 2,5-anhydro-D-mannitol, 1,6-anhydro-D-aminopyranose, as well as odd number oligosaccharides were also revealed. Quantitative comparison of major components derived from innovator and generic nadroparin products was presented. This approach to profile LMWHs' fragments offers a highly reproducible, high resolution and information-rich tool for evaluating the quality of this category of anticoagulant drugs or comparing structural similarities among samples from various sources.

## 1. Introduction

Heparin is a negatively charged, highly sulfated linear polysaccharide. It plays numerous significant roles in biological activities and physiologic processes through its interaction with multiple proteins.[1] LMWHs are derived from the UFH by either controlled enzymatic digestion with heparinase or different chemical degradations.[2] For example, enoxaparin is produced by alkaline degradation through chemical β-elimination reaction, while nadroparin is obtained by nitrous acid degradation.

Belonging to the family of glycosaminoglycans (GAG), heparin is composed of a repeating disaccharide building block of alternating 1,4-linked hexuronic acid (HexA) and glucosamine residue (GlcN). The HexA, either β-D-glucuronic acid (GlcA) or α-L-iduronic acid (IdoA), can be modified with 2-O-sulfo group. The GlcN may be N-acetylated (GlcNAc), N-sulfated (GlcNS), or unsubstituted, and its 3- and/or 6- positions can be substituted with O-sulfo group or unsubstituted.[3] LMWHs have the same repeating disaccharide unit with heparin but different terminal structures altered by depolymerization processes. The non-reducing end (NRE) of enoxaparin is an unsaturated 4-enopyranose uronate structure, making ultraviolet (UV) absorption at 232 nm a versatile method to detect enoxaparin chains. Meanwhile, this kind of LMWH contains a specific 1,6-anhydro structure at the reducing end (RE) accounting

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for 15%–25% of the overall oligosaccharide chains.[4] The characteristic terminal structures of nadroparin include a 2-O-sulfo-α-L-idopyranosuronic acid structure at the NRE and a 6-O-sulfo-2,5-anhydro-D-mannitol structure at the RE (Fig. 1).

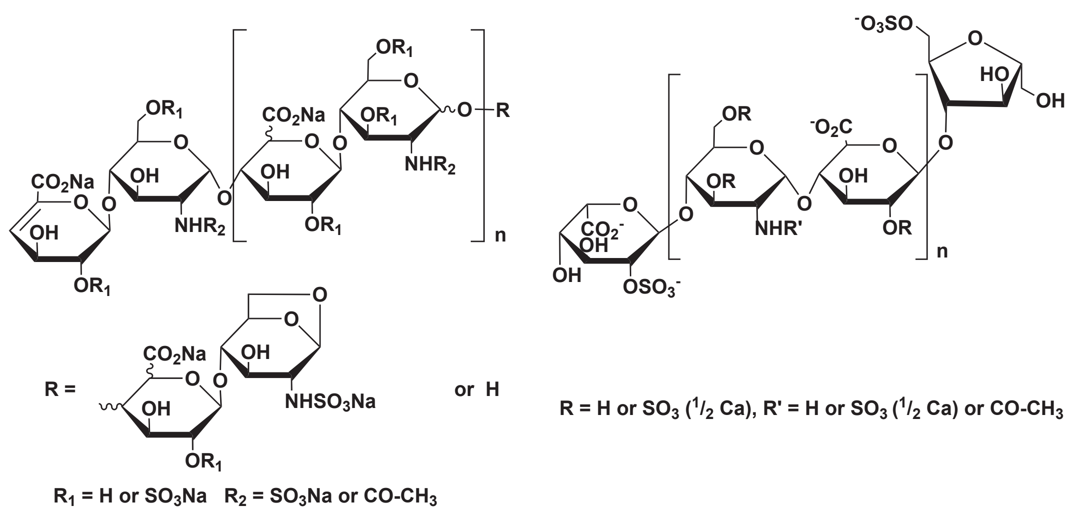
Heparin and LMWHs have been widely utilized as anticoagulant drugs for prevention and treatment of thromboembolic diseases.[5] Compared with UFH, LMWHs are currently more favorable drugs administrated to patients due to better bioavailability, longer biological half-lives and lower adverse effects.[6] The occurrence of pharmaceutical heparin contamination with oversulfated chondroitin sulfate (OSCS) in 2008 aroused the introduction of sophisticated analytical methods to assure the quality and safety of pharmaceutical agent.[7,8] On the other hand, the U.S. Food and Drug Administration (FDA) developed five criteria to demonstrate the active ingredient sameness between generic and innovator LMWH products for an Abbreviated New Drug Application.[9] Among these criteria, fragment mapping of LMWHs' partial digests from various enzymes provides global information on the sequences of oligosaccharides within the LMWHs' structures.

The fragment mapping analysis of LMWHs is in analogy to peptide mapping analysis of proteins. The enzymes including Hep I, Hep II and Hep III isolated from *Flavobacterium heparinum* are commonly used separately or as a cocktail to partially digest LMWHs.[10,11] Hep I shows a substrate specificity for α-L-IdoA2S (1→4) α-D-GlcNS. Hep II displays a broad selection for substrates comprised of either α-L-IdoA or β-D-GlcA. Hep III is selective for glycosidic bonds between α-D-GlcNS or α-D-GlcNAc and β-D-GlcA.[12,13] The fragments generated by various enzymatic reagents with diversely specific cleavage modes reflect global sequence of parent LMWHs and provide adequate evidence for sequence equivalence between generic and innovator LMWHs.

The mapping analysis can be achieved through various analytical techniques such as polyacrylamide gel electrophoresis (PAGE), capillary electrophoresis (CE), gel permeation chromatography (GPC), HPLC and MS. PAGE with relatively low resolution and MS incompatibility is a versatile method for the separation of heparin derived oligosaccharides in terms of size, conformation and charge.[14] CE based on charge-to-size ratio provides high sensitivity and high separation resolution but still faces challenges on day-to-day reproducibility and CE/MS interface technique.[15] GPC with relatively insufficient resolution is normally used as the first step to isolate a partially digested sample differing in size by disaccharide units.[16] Strong anion exchange (SAX)-HPLC, a traditional approach for the separation and preparation of heparin components, usually possesses high resolution for low mass components ranging from disaccharides to decasaccharides.[17,18] Cetyltrimethylammonium (CTA) coated SAX-HPLC is powerful to separate highly sulfated heparin components with superior resolution.[18] However, the identification of numerous peaks in SAX and CTA-SAX chromatograms is rather time-consuming and burdensome. Since both of them rely on high concentration nonvolatile salts in mobile phases, desalting of each peak is required prior to further structural analysis.

RPIP-HPLC is an increasingly popular method for heparin derived oligosaccharides analysis according to the size, isomerization and the number of sulfo groups.[19,20] RPIP-HPLC is performed on a hydrophobic reversed phase column (typically C18) with volatile ion pairing reagents to improve the retention of heparin chains on the column. Recent efforts demonstrate the combination of on-line separation techniques with ESI-MS offers an obvious advantage in understanding heparin structures.[21,22] RPIP-ESI-MS technique has been established for GAG analysis by adding volatile ion pairing reagents for improved separation performance and sufficient MS response.[23,24] A series of ion pairing reagents ranging from propylamine (PPA) to hexylamine (HXA), and tributylamine (TrBA) were evaluated in previous studies.[25,26] With a post-column addition of acetonitrile/TrBA to improve the volatility of electrosprayed solution in the ion source, TrBA is applied for heparin derived disaccharides/oligosaccharides analysis at the cost of severe instrument contamination problem.[27,28] MS ion counts from mobile phases containing HXA are less than that from pentylamine (PTA) on the condition that both mobile phases are added 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) as an organic modifier to control solution pH and improve MS signal.[26] However, similar to TrBA, the addition of HFIP also results in instrument contamination. In general, PTA is an optimal ion pairing reagent with efficient separation and ionization performance as well as MS friendly characteristic.

This paper described a RPIP-ESI-MS technique coupling a capillary HPLC with an IT-TOF mass spectrometer for analysis of partial enzymatic depolymerization products of enoxaparin and nadroparin. PTA avoiding the addition of HFIP was used in the



**Enoxaparin sodium** **Nadroparin calcium**

**Fig. 1.** Structures of enoxaparin and nadroparin.