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Liquid chromatography–diode array detection–mass spectrometry for compositional analysis of low molecular weight heparins

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ABSTRACT

Low molecular weight heparins (LMWHs) are important artificial preparations from heparin polysaccharide and are widely used as anticoagulant drugs. To analyze the structure and composition of LMWHs, identification and quantitation of their natural and modified building blocks are indispensable. We have established a novel reversed-phase high-performance liquid chromatography–diode array detection–electrospray ionization–mass spectrometry approach for compositional analysis of LMWHs. After being exhaustively digested and labeled with 2-aminoacridone, the structural motifs constructing LMWHs, including 17 components from dalteparin and 15 components from enoxaparin, were well separated, identified, and quantified. Besides the eight natural heparin disaccharides, many characteristic structures from dalteparin and enoxaparin, such as modified structures from the reducing end and nonreducing end, 3-*O*-sulfated tetrasaccharides, and trisaccharides, have been unambiguously identified based on their retention time and mass spectra. Compared with the traditional heparin compositional analysis methods, the approach described here is not only robust but also comprehensive because it is capable of identifying and quantifying nearly all components from lyase digests of LMWHs.

Heparin is a heterogeneous polysaccharide in terms of variable chain length, sulfo and *N*-acetyl substitutions, and epimerization. It consists of repeating disaccharide units of β-D-glucuronic acid or α-L-iduronic acid (1 → 4) linked to D-glucosamine. The hexuronic acid may be 2-*O*-sulfated, and glucosamine may be 6-*O*-sulfated, *N*-sulfated, *N*-acetylated, or (infrequently) 3-*O*-sulfated. For a long time, heparin has been used extensively as a clinical anticoagulant drug for prevention and treatment of thrombotic diseases [1–3]. However, the negative effects of heparin, including bleeding and inducing thrombocytopenia and osteoporosis, limit its clinical application [4]. Low molecular weight heparins (LMWHs)[1] are overtaking the market share of heparin as new anticoagulant and antithrombotic drugs in particular due to their improved bioavailability and reduced bleeding risk [5,6]. Several types of LMWHs have been produced by either controlled enzymatic or chemical depolymerization of heparin, including heparinase depolymerization, nitrous acid degradation, β-elimination, and hydrolytic cleavage with hydrogen peroxide [7]. The enzymatic and chemical reactions usually result in modified structures at the cleavage sites, that is, the reducing end (RE) and nonreducing end (NRE) of LMWHs. For example, enoxaparin is manufactured by alkaline treatment of benzyl ester derivative of heparin. The NRE becomes an unsaturated hexuronic acid, and the RE is a 1,6-anhydro structure for 15 to 25% of the constituents [8]. Dalteparin is produced by nitrous acid depolymerization. The NRE is a 2-*O*-sulfo-α-L-idopyranosuronic acid, and the RE is a 6-*O*-sulfo-2,5-anhydro-d-mannitol structure [9]. Besides the natural disaccharides and modified structures at the RE and NRE, LMWHs also contain 3-*O*-sulfated pentasaccharide structures, which are responsible for their anticoagulant activity [10]. Peeling reaction is a common side reaction during preparation of heparin and LMWHs, which results in glucosamine structures at the RE [11].

Because heparin and LMWHs are heterogeneous macro biomolecules, they are usually degraded completely into basic building blocks and then analyzed using chromatographic or electrophoretic techniques. The cocktail of three types of heparinase (heparinases I, II, and III) isolated from *Flavobacterium heparinum* is

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[1] *Abbreviations used:* LMWH, low molecular weight heparin; RE, reducing end; NRE, nonreducing end; HPLC, high-performance liquid chromatography; SAX, strong anion exchange; UV, ultraviolet; HILIC, hydrophilic interaction liquid chromatography; RPIP, reversed-phase ion pairing; RP, reverse-phase; CE, capillary electrophoresis; AMAC, 2-aminoacridone; MS, mass spectrometry; ESI, electrospray ionization; GPC, gel permeation chromatography; LC, liquid chromatography; DAD, diode array detection; DMSO, dimethyl sulfoxide; IT–TOF, ion trap–time-of-flight; TIC, total ion chromatogram.

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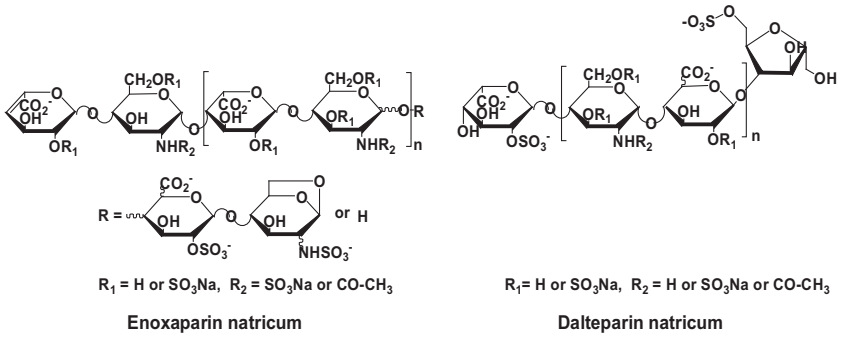
generally used to deconstruct heparin and LMWHs [12,13]. In the case of enoxaparin and dalteparin, the possible structures from exhaustive digestion include eight natural disaccharides, modified oligosaccharides from both termini, 3-*O*-sulfated tetrasaccharides, and trisaccharides derived from peeling reaction (Table 1).

Compositional analysis is a fundamental approach to characterize heparin and LMWHs. It is required by the U.S. Food and Drug Administration to demonstrate the compositional sameness between generic and innovators' LMWH products for an abbreviated new drug application [14]. High-performance liquid chromatography (HPLC) is a conventional method and has been used extensively to determine the composition of heparin and LMWHs. Strong anion exchange (SAX)–HPLC, regarded as a general analytic method, has been used largely to identify and characterize heparin- and LMWH-derived oligosaccharides based on their acidic properties and characteristic ultraviolet (UV) absorption at 232 nm [15]. Hydrophilic interaction liquid chromatography (HILIC) is capable of separating hydrophilic analytes and has been reported to separate heparin- and other glycosaminoglycan-derived disaccharides [16]. During recent years, reversed-phase ion pairing (RPIP)–HPLC has drawn increasing attention. The ion pairing reagents modify the hydrophobicity of hydrophilic heparin-derived disaccharides and oligosaccharides, which can then be well resolved on traditional reverse-phase (RP) columns [17]. Capillary electrophoresis (CE) has also been employed to analyze LMWHs' composition with relatively high resolution and low limit of detection (10–20 pg) [18,19]. However, most of the HPLC and CE methods mentioned above are limited to detecting only eight natural heparin disaccharides. The exhaustive digestion of LMWHs results in more complicated disaccharide and oligosaccharide compositions. Unlike the natural heparin disaccharides, there are no commercially available reference standards for modified disaccharides and oligosaccharides. Moreover, some of these disaccharides and oligosaccharides lack unsaturated structures at the NRE and cannot be monitored by a UV detector in HPLC or CE analysis.

Fluorescence derivatization techniques are frequently used to facilitate the separation and detection of heparin-derived oligosaccharides. A variety of fluorophores, such as 2-aminopyridine and

Table 1
Structures of dalteparin, enoxaparin, and corresponding building blocks.



	Name	Structure
Common heparin disaccharides		
	ΔIS	ΔUA2S-GlcNS6S
	ΔIIS	ΔUA-GlcNS6S
	ΔIIIS	ΔUA2S-GlcNS
	ΔIVS	ΔUA-GlcNS
	ΔIA	ΔUA2S-GlcNAc6S
	ΔIIA	ΔUA-GlcNAc6S
	ΔIIIA	ΔUA2S-GlcNAc
	ΔIVA	ΔUA-GlcNAc
Dalteparin characteristic structures		
	Δdp4(4OS,1NS) RE	ΔUA2S-GlcNS6S-IdoA2S-Mnt6S-2,5-anhydro
	Δdp2(2OS) RE	ΔUA2S-Mnt6S-2,5-anhydro
	Δdp2(1OS) RE	ΔUA-Mnt6S-2,5-anhydro
	dp2(2OS,1NS) NRE	IdoA2S-GlcNS6S
	dp2(1OS,1NS) NRE	IdoA2S-GlcNS
	ΔIIA-IVS$_{glu}$	ΔUA-GlcNAc6S-GlcA-GlcNS3S
	ΔIIA-IIS$_{glu}$	ΔUA-GlcNAc6S-GlcA-GlcNS3S6S
	Δdp3(3OS,1NS)	ΔUA2S-GlcNS6S-IdoA2S
	Δdp3(1OS)	ΔUA-GlcNAc-IdoA (1OS)
Enoxaparin characteristic structures		
	1,6-anhydro ΔIS-IS^{epi}	ΔUA2S-GlcNS6S-IdoA2S-ManNS-1,6-anhydro
	1,6-anhydro ΔIS 1,6-anhydro ΔIIS	ΔUA2S-GlcNS-1,6-anhydro ΔUA-GlcNS-1,6-anhydro
	dp2(2OS,1NS) NRE	IdoA2S-GlcNS6S
	ΔIIA-IVS$_{glu}$	ΔUA-GlcNAc6S-GlcA-GlcNS3S
	ΔIIA-IIS$_{glu}$	ΔUA-GlcNAc6S-GlcA-GlcNS3S6S
	Δdp3(3OS,1NS)	ΔUA2S-GlcNS6S-IdoA2S
	Δdp3(1OS)	ΔUA-GlcNAc-IdoA (1OS)
Synthetic heparin disaccharide	ΔIP	ΔUA2S-GlcNCOEt6S

Note. ΔUA, unsaturated uronic acid; GlcA, glucuronic acid; IdoA, iduronic acid; GlcN, glucosamine; ManN, mannosamine; Ac, acetyl group, S, sulfo group; COEt, ethoxycarbonyl; Mnt, mannitol.