

# Heparinase Digestion-Based Disaccharide Composition Analysis of Heparin and Heparinoid Drugs



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**Abstract:** Heparin and low molecular weight heparin have been used widely in clinical therapy as anticoagulants in cardiovascular disease and in hemodialysis. Crude heparin is usually prepared from porcine intestinal mucosa. Purified heparin is a mixture of polysaccharides consisting mainly of repeating GlcNS(6S)-IdoA2S disaccharides and other disaccharides with different GlcNAc/GlcNS±3S±6S-GlcA/IdoA±2S residues. Heparin injections are prepared by purification and then sterilization of ungraded heparin. Low molecular weight heparins are dominant heparin-based drugs used clinically, which are prepared by degrading heparin into smaller molecules. As a result, low molecular weight heparins share the same major disaccharides with heparin, but may have different reducing and non-reducing ends due to different preparation methods. In the current study, we analyzed the disaccharide composition of clinically used heparin and heparin-based drugs. Heparinase I, II and III were used to degrade all heparin and heparin-based drugs including heparin sodium injection, Enoxaparin sodium injection, Nadroparin calcium injection, Dalteparin sodium injection, and Fondaparinux sodium injection into disaccharides. All the degraded products were analyzed by strong anion exchange-high performance liquid chromatography (SAX-HPLC) equipped with an online UV-detector. Commercially available unsaturated disaccharide standards were then used for structural identification. Furthermore, unusual disaccharides present in Nadroparin, Dalteparin and Fondaparinux were confirmed by reversed-phase ion-pair HPLC coupled with mass spectrometry analysis. The developed method produced detailed structural information, which should be useful for quality control of heparin and heparin-based drugs.

**Key Words:** Heparin; Low molecular weight heparin; Heparin lyase; Disaccharide analysis; Mass spectrometry

## 1 Introduction

Heparin is a highly sulfated glycosaminoglycan and an irreplaceable drug in treating thrombosis, cardiovascular, and cerebrovascular diseases<sup>[1,2]</sup>. Heparin sodium injection is a product of heparin active pharmaceutical ingredient (API) that is purified from raw heparin, which is extracted from mammalian intestinal mucosa by a series of strict processes to remove protein, DNA, and ~50% other less sulfated glycosaminoglycans. Low molecular weight heparins are degraded products of heparin that have smaller risks of bleeding, osteoporosis, and heparin-induced thrombocyto-

penia. The four most widely used low molecular weight heparins<sup>[3]</sup> include: (1). Enoxaparin sodium that is prepared by alkaline-eliminative cleavage of the benzyl ester of heparin; (2). Dalteparin sodium which is obtained by nitrous acid cleavage of heparin; (3). Nadroparin which is a calcium salt of nitrous acid-depolymerized heparin; (4). Fondaparinux sodium, a synthetic heparin pentasaccharide.

Quality control of heparin and heparin-based drugs is difficult due to their complexity and heterogeneity in both molecular weight and fine chemical structures. Contaminated heparin in 2008 was linked to at least 149 deaths in the America<sup>[4]</sup> and forced more stringent quality control over

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heparin. Quality control protocols<sup>[5,6]</sup> have been updated and published in the USA and in Europe as USP36 and EP8.0 for heparin sodium where monosaccharide composition analysis, NMR, and ion exchange chromatography methods are added to identify oversulfated chondroitin sulfate, dermatan sulfate, and other potential contaminants. No updated tests have been added for heparin in the latest Chinese pharmacopoeia yet<sup>[7–9]</sup>.

The newly added NMR and ion exchange chromatography methods by the USP and EP are still physical property-based quality control methods for heparin<sup>[5,6]</sup>. Thus, chemical structure-based methods would be desirable for quality control purpose. In the current study, the fine chemical structures of heparin, heparin injections, Enoxaparin, Dalteparin, Nadroparin, and Fondaparinux were assessed by disaccharide composition analysis<sup>[10]</sup>. The disaccharides of all heparin and heparin-based drugs were obtained by combination digestion of heparinase I, II, and III. Anion exchange chromatography was used to separate different disaccharides. Each known disaccharide was identified by using commercially available heparin disaccharide standards, whereas unknown disaccharides present were identified by using reversed-phase ion-pairing chromatography coupled with mass spectrometry. The distinct disaccharide composition profiles of heparin and heparin-based drugs obtained in current study should be useful for chemical structure-based quality control purpose.

## 2 Experimental methods

### 2.1 Instruments and reagents

Agilent 1260 Infinity liquid chromatography system with a ZORBAX SAX chromatography column (250 mm × 4.6 mm) equipped with an on line UV diode array detector (DAD) was used for disaccharide compositional analysis. Liquid chromatography-mass spectrometry system included an Agilent 1290 Infinity capillary liquid chromatography system with a ZORBAX 300SB-C<sub>18</sub> chromatography column (250 mm × 0.5 mm) equipped with an on line DAD, and Thermo Scientific LTQ XL mass spectrometer with electrospray-ionization (Thermo Finnegan Company) was used for mass detection in a negative-ionization mode. Spectramax M5 plate reader (Molecular Devices) was used for monitoring absorbance of reaction mixture at 232 nm. Eight disaccharide standards<sup>[11]</sup> including ΔUA2S-GlcNAc6S (D2A6), ΔUA2S-GlcNS6S (D2S6), ΔUA-GlcNS6S (D0S6), ΔUA2S-GlcNS (D2S0), ΔUA-GlcNAc (D0A0), ΔUA-GlcNS (D0S0), ΔUA-GlcNAc6S (D0A6), ΔUA2S-GlcNAc (D2A0) were purchased from Sigma, USA. Recombinant heparinase I, II, III were from Si Qing Yuan Biotechnology Co Ltd (Beijing, China). The following heparin or heparin-based drugs were used in the experiment: heparin (Sigma), heparin injection (Shanghai Pharma), Enoxaparin (Sanofi-Aventis), Dalteparin (Qilu Pharmaceutical), Nadroparin and Fondaparinux sodium

(GlaxoSmithKline).

## 2.2 Experiment methods

### 2.2.1 Preparation of heparin disaccharides by enzymatic hydrolysis of heparin and heparin-based drugs

Heparin, heparin injection, Enoxaparin, Nadroparin, Dalteparin, and Fondaparinux were dissolved or diluted to a concentration of 10 mg L<sup>-1</sup> respectively. 20 μL of each sample was added in 180 μL of lysis buffer containing 20mM Tris-HCl and 5 mM CaCl<sub>2</sub>, pH 7.6, and then digested respectively with combination of heparinase I, II, and III (20 mU of each enzyme) at 37 °C. All enzymatic hydrolysis reactions were conducted in 96-well plate with no ultraviolet absorption for 24 h. The progression<sup>[12]</sup> of digestion was monitored by recording the absorbance at 232 nm (UV 232) using a Spectramax M5 plate reader. After completion of enzymatic digestion, eg, the UV 232 reached a steady value, the reaction mixture was subjected to further analyses.

### 2.2.2 Strong anion exchange-high performance liquid chromatography (SAX-HPLC) analysis

The disaccharide standards were analyzed by SAX-HPLC equipped with an online UV-detector (detection wavelength 232 nm) to identify the elution time of each disaccharide<sup>[13]</sup>. The heparinase digestion mixtures of heparin and heparin-based drugs were terminated by boiling in 100 °C water bath for 2 min and then centrifuged at 10000 g for 3 min to remove the denatured heparinases. The supernatant was then used for direct SAX-HPLC analysis using a Zorbax SAX column. Gradient elution was used with mobile phase A of 0.2 mM NaH<sub>2</sub>PO<sub>4</sub> solution (adjusted to pH 3.0 by phosphoric acid) and mobile phase B of 1.0 mM sodium perchlorate solution. The gradient elution program was as follows: 0–25 min, 97%–65% A; 25–50 min, 65%–0% A; 50–70 min, 100% B. Then, 3% A was used to equilibrate the chromatography system. The flow rate was 1.0 mL min<sup>-1</sup> and on-line UV detection wavelength was 232 nm. By comparison with the elution time of disaccharide standards, the structures of enzymatic digestion products of each heparin sample were confirmed. The content of each disaccharide was calculated by integrating the peak area at 232 nm.

### 2.2.3 Reversed-phase ion-pairing chromatography coupled with mass spectrometry analysis<sup>[14]</sup>

The enzymatic products of heparin and heparin-based drugs were subjected to reversed-phase ion-pairing chromatography using Agilent 1290 chromatography system with a Zorbax 300SB C<sub>18</sub> column. The mobile phase A was 5% methanol and mobile phase B was 70% methanol aqueous solution.

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