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Comparison of Low-Molecular-Weight Heparins Prepared From Bovine Lung Heparin and Porcine Intestine Heparin

Yudong Guan [1], Xiaohui Xu [1], Xinyue Liu [1, 2], Anran Sheng [1], Lan Jin [1], Robert J. Linhardt [2, 3, 4, 5], Lianli Chi [1, *]

[1] National Glycoengineering Research Center, Shandong Provincial Key Laboratory of Carbohydrate Chemistry and Glycobiology, Shandong University, Jinan, Shandong, China
[2] Department of Chemistry and Chemical Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180
[3] Department of Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180
[4] Department of Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180
[5] Department of Biomedical Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180

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ABSTRACT

Currently porcine intestine is the only approved source for producing pharmaceutical heparin in most countries. Enoxaparin, prepared by benzylation and alkaline depolymerization from porcine intestine heparin, is prevalent in the anticoagulant drug market. It is predicted that porcine intestine heparin–derived enoxaparin (PIE) will encounter shortage, and expanding its production from heparins obtained from other animal tissues may, therefore, be inevitable. Bovine lung heparin is a potential alternative source for producing enoxaparin. Critical processing parameters for producing bovine lung heparin–derived enoxaparin (BLE) are discussed. Three batches of BLEs were prepared and their detailed structures were compared with PIEs using modern analytical techniques, including disaccharide composition, intact chain mapping by liquid chromatography-mass spectrometry and 2-dimensional nuclear magnetic resonance spectroscopy. The results suggested that the differences between PIEs and BLEs mainly result from *N*-acetylation differences derived from the parent heparins. In addition, bioactivities of BLEs were about 70% of PIEs based on anti-factor IIa and Xa chromogenic assays. We conclude that BLE has the potential to be developed as an analogue of PIE, although some challenges still remain.

Introduction

Heparin has been used as an anticoagulant drug to treat thrombosis for about 80 years.[1] It was originally isolated from dog liver and demonstrated to possess anticoagulant activity in 1916.[2] During the 1930s, heparin was successfully prepared from bovine lung, and heparin from this source was later developed as a pharmaceutical product in the United States.[3,4] The bovine lung was the primary source of heparin until the end of 1950s, when porcine intestinal mucosa became the preferred alternative due to a simpler extraction process and a higher yield. In 1986, the first bovine spongiform encephalopathy case was reported in Europe, and certain bovine tissues were consequently banned for food and human consumption in many countries.[5,6] The global production and utilization of heparin from cattle decreased significantly. Currently, the only approved animal species for manufacturing pharmaceutical heparin in the United States is pig. However, there are potential risks with using porcine intestinal mucosa as the sole source of heparin. First, if a major outbreak of a pig infectious disease occurs in the future, heparin would be in severe shortage. Second, China accounts for more than 50% of pig livestock and exports most raw heparin around the world. The over-sulfated chondroitin sulfate contamination of heparin in China caused the heparin crisis in 2008.[7] Third, due to the increasing demand from developing countries, heparin will eventually be in shortage if no other alternative anticoagulant agents emerge. Thus, the

Abbreviations used: ATIII, antithrombin III; BLE, bovine lung heparin–derived enoxaparin; ESI, electrospray ionization; GlcA, glucuronic acid; GlcN, glucosamine; GPC, gel permeation chromatography; HexA, hexuronic acid; HILIC, hydrophilic interaction chromatography; HSQC, heteronuclear single quantum coherence; IdoA, iduronic acid; LMWH, low-molecular-weight heparin; MALLS, multi-angle laser light scattering; MWCO, molecular-weight cutoff; TIC, total ion chromatogram; PIE, porcine intestine heparin–derived enoxaparin.

* *Correspondence to:* Lianli Chi (Telephone: +86-531-88363200; Fax: +86-531-88363002).
E-mail address: lianlichi@sdu.edu.cn (L. Chi).

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reintroduction of bovine heparin to the United States market is currently under active investigation.[8]

Heparin is a linear polysaccharide composed of a repeating disaccharide building block of alternating β-1,4-linked hexuronic acid (HexA) and glucosamine residue (GlcN). The HexA can be either β-D-glucuronic acid (GlcA) or α-L-iduronic acid (IdoA), at which the C2 position can be substituted by an *O*-sulfo group. The GlcN may be modified by an *N*-acetyl group (GlcNAc), an *N*-sulfo group (GlcNS), or can be unsubstituted, whereas *O*-sulfo group substitution can occur at its C3 and/or C6 positions (Fig. 1a).[9,10] A pentasaccharide sequence of GlcNAc/NS(6S)-GlcA-GlcNS(3S,6S)-IdoA(2S)-GlcNS(6S) (Fig. 1b) is the structural motif for heparin that specifically binds to antithrombin III (ATIII) and inactivates the blood clotting process.[11] Heparin is polydisperse with a molecular weight (MW) range from 5000 to 40,000 Da, in which most components have a MW of 12,000-25,000 Da.[12] The heparins produced from different animal species and organs usually possess variable structural characteristics, physicochemical properties, and biological properties, such as MW, disaccharide composition, oligosaccharide sequence, anti-factor IIa, and anti-factor Xa activities.[13] Although unfractionated heparin is directly used as a drug, over 60% of heparin is used as the starting material to produce low-molecular-weight heparins (LMWHs), which demonstrate improved bioavailability, longer half-life, and more controllable anticoagulation.[14,15] Enoxaparin, the most widely used LMWH is produced through esterification followed by alkaline depolymerization.[16] The weight-averaged MW (M_w) of enoxaparin ranges from 3800 to 5000 Da. It inherits some structural characteristics from the parent heparin, including the natural disaccharide building blocks and oligosaccharide sequences. The terminal structure of enoxaparin is modified by the chemical process used in its preparation. Unsaturated uronate residues are commonly found at the nonreducing end of enoxaparin chains, and 1,6-anhydro groups are found at 15%-25% of the reducing ends of enoxaparin chains (Fig. 1a). Structural characterization of a LMWH and its parent heparin is fundamentally important for evaluating the efficacy and safety of these drugs. It also provides critical data for the development and approval of alternative anticoagulant drugs.[17] Modern analytical techniques, such as top-down and bottom-up mass spectrometric chain mapping approaches, 2-dimensional nuclear magnetic resonance (NMR) methods and novel liquid chromatographic methods, provide powerful tools for determining the fine structure of these complicated polysaccharides.[18-23]

In this study, we prepared enoxaparin-like LWMHs from bovine lung heparin to evaluate as a potential analogue of porcine intestine heparin–derived enoxaparin (PIE). Key chemical reaction parameters were controlled to make bovine enoxaparin with MW meeting the requirements of the United States Pharmacopeia (USP). The structural and biological properties between bovine- and porcine-derived enoxaparins were then examined using several state-of-the-art analytical techniques.

Materials and Methods

Materials

Porcine intestine heparin and bovine lung heparin were gifts from Innokare Bio-pharmaceutical Tech Co., Ltd (Suzhou, China). Enoxaparin reference standard (PIE-S) and heparin reference standard were obtained from the USP Convention (Rockville, MD). Benzethonium chloride, dichloromethane, benzyl chloride, methanol, and hydrochloric acid were purchased from J & K Chemical Technology (Beijing, China). Sodium acetate and sodium hydroxide were purchased from Amersco (Solon, Ohio). Heparinase I, II, III and reagents for bioactivity analysis, including antithrombin, factor IIa, factor Xa, chromogenic substrate S-2238, S-2222, and S-2765 were purchased from Adhoc International Technologies (Beijing, China).

Preparation of Enoxaparin Using Porcine Intestine Heparin

Two batches of LMWH samples (PIE-1 and PIE-2) were prepared using porcine intestine heparin as starting materials according to

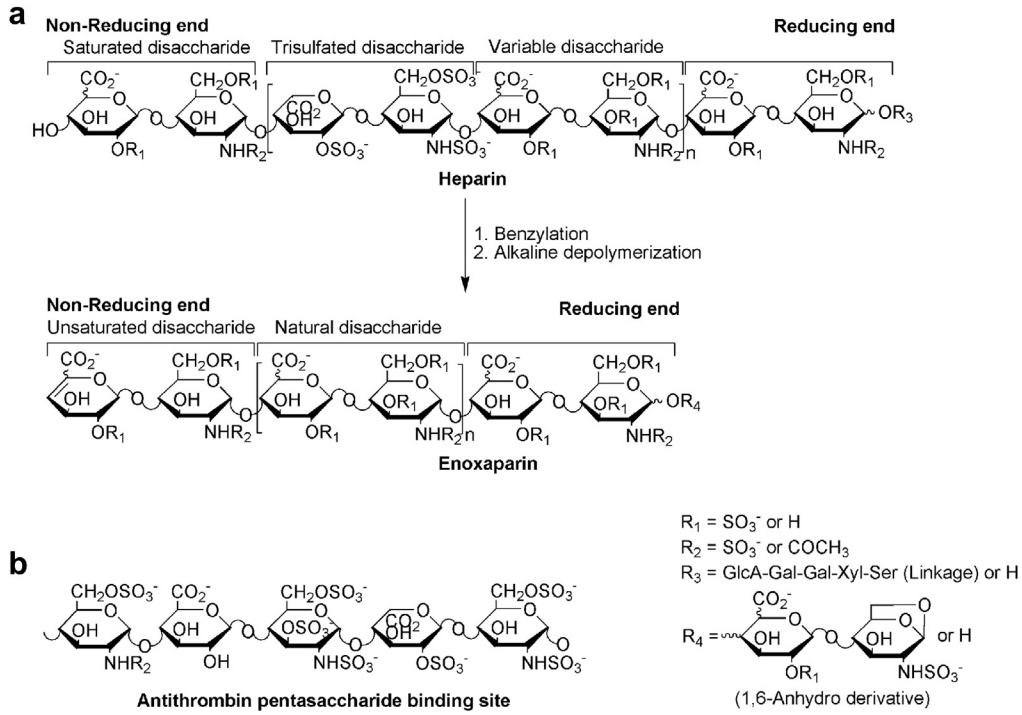

Figure 1. Structures of heparin and enoxaparin. (a) The structure modification from heparin to enoxaparin. (b) The ATIII-binding pentasaccharide sequence of heparin and enoxaparin.