Comparison of Low-Molecular-Weight Heparins Prepared From Ovine Heparins With Enoxaparin

Jianle Chen, BS¹,², Yanlei Yu, PhD², Jawed Fareed, PhD³, Debra Hoppensteadt, PhD³, Walter Jeske, PhD³, Ahmed Kouta, BS³, Caijuan Jin, MS⁴, Yongsheng Jin, MS⁴, Yiming Yao, MS⁴, Ke Xia, PhD², Fuming Zhang, PhD⁵, Shiguo Chen, PhD¹, Xingqian Ye, PhD¹, and Robert J. Linhardt, PhD²,⁵,⁶,⁷

Abstract
Heparin and its low-molecular-weight heparin derivatives are widely used clinical anticoagulants. These drugs are critical for the practice of medicine in applications, including kidney dialysis, cardiopulmonary bypass, and in the management of venous thromboembolism. Currently, these drugs are derived from livestock, primarily porcine intestine and less frequently bovine intestine and bovine lung. The worldwide dependence on the pig as a single dominant animal species has made the supply chain for this critical drug quite fragile, leading to the search for other sources of these drugs, including the expanded use of bovine tissues. A number of laboratories are now also examining the similarities between heparin and low-molecular-weight heparins prepared from porcine and ovine tissues. This study was designed to compare low-molecular-weight heparin prepared from ovine heparin through chemical β-elimination, a process currently used to prepare the low-molecular-weight heparin, enoxaparin. Using top-down, bottom-up, and compositional analyses as well as bioassays, low-molecular-weight heparin derived from ovine intestine was shown to closely resemble enoxaparin. Moreover, the compositions of daughter low-molecular-weight heparins prepared from three unfractionated ovine parent heparins were compared. Ovine enoxaparins had similar molecular weight and in vitro anticoagulant activities as Lovenox. Some disaccharide compositional, oligosaccharide composition at the reducing and nonreducing ends and intact chain compositional differences could be observed between porcine enoxaparin and ovine low-molecular-weight heparin. The similarity of these ovine and porcine heparin products suggests that their preclinical evaluation and ultimately clinical assessment is warranted.

Keywords
ovine heparin, enoxaparin, chemical β-elimination, mass spectrometry, nuclear magnetic resonance spectroscopy, bioassay

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Introduction
Heparin is a critically important anticoagulant–antithrombotic drug without which the practice of modern medicine would not be possible.¹ Heparin, a natural product, is extracted in ton quantities from the tissues of food animals.² Porcine intestinal tissue is the principal source of heparin worldwide.²,³ The worldwide dependence on the pig as a single dominant animal species has made the supply chain for this critical drug quite fragile, leading to the search for other sources of these drugs, including the expanded use of bovine tissues.⁴ Moreover, the production of bioengineered heparins prepared through a combination of microbial fermentation and chemoenzymatic synthesis⁵ or using metabolic engineering⁶ is now under extensive study.

¹ Department of Food Science and Nutrition, Zhejiang University, Hangzhou, China
² Department of Chemistry and Chemical Biology, Rensselaer Polytechnic Institute, Troy, NY, USA
³ Department of Pathology, Loyola University Medical Center, Maywood, IL, USA
⁴ Ronni Pharma Co, Ltd, Suzhou Industrial Park, Suzhou, China
⁵ Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, Troy, NY, USA
⁶ Department of Biology, Rensselaer Polytechnic Institute, Troy, NY, USA
⁷ Department of Biomedical Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY, USA

Corresponding Author:
Robert J. Linhardt, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, 110 Eghree Street, Troy, NY 12180, USA. Email: linhar@rpi.edu

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This reexamination of the sourcing of heparin is largely motivated by the 2007 to 2008 heparin contamination crisis in which much of the world’s supply of porcine intestinal heparin was adulterated with oversulfated chondroitin sulfate. Oversulfated chondroitin sulfate resulted in the biochemical activation of multiple pathways in the circulation leading to hypotension and death of a number of patients receiving contaminated heparin. This crisis resulted in the introduction of new analytical methodology for heparin as well as revised, more rigorous pharmacopeial monographs for this class of drugs.

Heparin is a linear, polydisperse, microheterogenous, polysaccharide comprised repeating disaccharide units of heparin.

Table 1. Preparation of Low-Molecular-Weight Heparin Analog of Enoxaparin From Ovine Heparins.

<table>
<thead>
<tr>
<th>Species / organs</th>
<th>Purified Ovine Heparin Sodium</th>
<th>Ovine Enoxaparin Sodium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Batch no./Lot#</td>
<td>Batch no./Lot#</td>
</tr>
<tr>
<td>Parent, Heparin</td>
<td>OES-L170404-A</td>
<td>OES-L170401-D</td>
</tr>
<tr>
<td>80-ALR-011-089-02</td>
<td>OES-L170402-D</td>
<td></td>
</tr>
<tr>
<td>80-ALR-011-092</td>
<td>OES-L170403-D</td>
<td></td>
</tr>
</tbody>
</table>

This table summarizes the preparation of low-molecular-weight heparin from ovine heparins. Ovine intestinal mucosa was used to prepare 3 batches of ovine enoxaparin (Table 1). These batches were then used to prepare 3 batches of ovine enoxaparin (Table 1).
ovine-derived heparin products were chemically analyzed using state-of-the-art methods and their biological/pharmacological activities were evaluated. The structure and activity of unfractionated ovine intestinal heparin and ovine enoxaparin were compared to the porcine-derived products.

**Materials and Methods**

**Materials**

The ovine heparins and ovine-derived LMWHs were obtained from Romnss (Jiangsu, China). Porcine intestinal heparin reference standard was obtained from the US Pharmacopeia Convention (USP). Lovenox from Sanofi-Aventis (Bridgewater, New Jersey) was obtained from commercial suppliers. All samples were analyzed prior to their expiration dates. Heparin disaccharide standards (0S: ΔUA [1 → 4] GlcNAc; NS: ΔUA [1 → 4] GlcNS; 6S: ΔUA [1 → 4] GlcNAc6S; 2S: ΔUA2S [1 → 4] GlcNAc; NS2S: ΔUA2S [1 → 4] GlcNS; NS6S: ΔUA [1 → 4] GlcNS6S; 2S6S: ΔUA2S [1 → 4] GlcNAc6S; and TriS: ΔUA2S [1 → 4] GlcNS6S, where ΔUA is 4-deoxy-β-threo-hex-4-enopyranosiduronic acid, GlcN is glucosamine, Ac is acetyl, and S is sulfo) were from Iduron (Manchester, United Kingdom). Tributylamine was from Sigma Chemical (St Louis, Missouri). Ammonium acetate, calcium chloride, acetic acid, water, and acetonitrile were of high-performance liquid chromatography grade (Fisher Scientific, Springfield, New Jersey). Microcon YM-3 centrifugal filter units were obtained from Millipore (Bedford, Massachusetts). *Escherichia coli* expression and purification of recombinant flavobacterial heparin lyase I, II, and III (Enzyme Commission #s 4.2.2.7, 4.2.2.X, and 4.2.2.8) were performed in our laboratory as described previously.\(^2\) The LMWHs were desalted by dialysis using 1-kDa MW cutoff dialysis tube (Spectrum Laboratories, Rancho Dominguez, California), lyophilized before NMR analysis, and redissolved in distilled water into stock solution (20 mg/mL) for liquid chromatography (LC)-mass spectrometry (MS) analysis.

**Chemical β-Elimination of Ovine Heparin**

Chemical β-elimination of porcine and ovine heparins to prepare LMWHs has been described in detail previously.\(^2\) Sodium heparin was converted into benzethonium salt and was recovered by precipitation. The heparin benzethonium salt was dissolved in methylene chloride and benzylated using benzyl chloride. The benzyl ester of ovine heparin was recovered and treated with aqueous sodium hydroxide resulting in its alkaline depolymerization and debenzylation. Recovery by methanol precipitation and dialysis provided the ovine enoxaparin.

**Potency Evaluation**

Ovine heparin and enoxaparin were supplemented in pooled normal human plasma and tested in a concentration range of 0 to 10 μg/mL to determine potency. The amidolytic anti-IIa and anti-Xa assays were performed on an ACL ELITE (Instrumentation Laboratory, Lexington, Massachusetts) using bovine Xa and human thrombin (TH) from Enzyme Research Laboratories (South Bend, Indiana). Chromogenic substrates, Spectrozyme Xa, and TH were obtained from American Diagnostica (Stamford, Connecticut) or using kits from Aniara Diagnostica (West Chester, Ohio). The potency of the ovine heparin was calculated using the USP standard for heparin (lot FOI 187). The potency of each heparin was calculated based on the calibration curve prepared with the USP heparin standard. The potency of the enoxaparin was calculated using the National Institute for Biological Standards and Control (01/608; Potters Bar, England) standard.

**Activity and MW Assays**

The activity and MW assays for heparins and LMWHs were comparable to USP methods.\(^2\)

**Disaccharide Analysis**

Each sample (100 μg) was dissolved in 100 μL digestion buffer (50 mmol/L NH₄OAc, 2 mmol/L CaCl₂, pH 7.0). A mixture of heparin lyase I, II, and III (10 mU each in Tris–HCl buffer, pH 7.0) was added and the samples were digested in 37°C water bath for 12 hours. The lyase enzymes were removed using a YM-3 centrifugal filter unit (Millipore, Billerica, Massachusetts) to terminate the reaction. The filtrates were lyophilized and redissolved in distilled water at a concentration of 1 mg/mL. Reversed-phase, ion-pairing LC with online electrospray ion (ESI)-trap MS analysis relied on an Agilent 1200 LC/MSD instrument (Agilent Technologies, Wilmington, Delaware) equipped with a 6300 ion-trap and a binary pump. A solution of disaccharide standards was prepared (each disaccharide at 100 μg/μL) for relative quantification.

**Bottom-Up Analysis of Heparins and LMWHs**

Each sample (100 μg) was added to 100 μL digestion buffer (50 mmol/L pH 7.0). Heparin lyase II (10 mU in Tris–HCl buffer, pH 7.0) was added and the sample was digested in 37°C water bath for 12 hours to produce fragments. Enzymatic digestion was terminated by removing the enzymes using an YM-3 centrifugal filter unit. The filtrates were lyophilized and redissolved in 100 μL of distilled water at a concentration of 1 μg/mL. Online hydrophilic interaction chromatography (HILIC) Fourier transform mass spectrometry (FTMS) was used to analyze the resulting oligosaccharides.\(^2\) A Luna HILIC column (2.0 mm² × 50 mm², 200 Å; Phenomenex, Torrance, California) was connected online to the standard ESI source of LTQ-Orbitrap XL FTMS (Thermo Fisher Scientific, San Jose, California). Mass spectra were acquired at a resolution 60 000 with 200 to 1800 m/z range.

**Top-Down Analysis of LMWHs**

Online HILIC-FTMS was applied to analyze the intact chains.\(^2\) A Luna HILIC column (2.0 mm² × 150 mm², 200 Å;
Phenomenex, Torrance, California) was used. Mass spectra were acquired at a resolution 60 000 with 200 to 2000 Da mass range.

Bioinformatics Used in Processing Bottom-Up and Top-Down Data

Charge deconvolution was autoprocessed by DeconTools software (web source from PNNL at http://omics.pnl.gov/). The LMWH structural assignment was done by automatic processing using GlycReSoft 1.0 software developed at Boston University (http://code.google.com/p/glycresoft/downloads/list). A theoretical database was generated by GlycReSoft 1.0 as described previously. All of the relative quantitative data were normalized to the total identified oligosaccharides peak area (in the format of percentage).

Nuclear Magnetic Resonance Analysis

One-dimensional (1D) $^1$H and $^{13}$C NMR spectra were obtained at 600 MHz on $^2$H$_2$O exchanged heparin (20 mg/mL in $^2$H$_2$O) using a Bruker Advance II 600 MHz spectrometer (Bruker BioSpin, Billerica, Massachusetts) with Toppspin 3.2 software (Bruker BioSpin) for signal integration. Two-dimensional HSQC-NMR spectra were obtained at 800 MHz on $^2$H$_2$O exchanged heparin (20 mg/mL in $^2$H$_2$O) using a Bruker Advance II 800 MHz spectrometer.

Results and Discussion

Three batches of ovine heparin were prepared from ovine intestinal mucosa (Table 1). The MWs of the ovine heparins were somewhat lower than those for USP heparin produced from porcine intestine (Table 2).

Characterization of the MW and Activity Properties

The USP porcine heparin had a weight average MW of 18 900 with a polydispersity index (PI) of 1.23, and the ovine heparins had an average MW of 13 800 and an average PI of 1.38. Each parent ovine heparin was then converted to their benzyl esters and chemically β-eliminated to afford 3 daughter ovine exoaparins. The ovine enoxaparin had an average MW of 4200 and an average PI of 1.14 very similar to porcine enoxaparin MW of 4300 and a PI of 1.19 (Table 2 and SI 1-4). The anticoagulant activities, as determined using antifactor Xa and IIa assays, of unfractionated USP porcine heparin and ovine heparins and the porcine and ovine enoxaparins were very similar showing no major differences (Table 2 and SI 5-6).

Table 2. Molecular Weight and Potency of Ovine Heparin Sodium and Ovine Enoxaparin Sodium.

<table>
<thead>
<tr>
<th>Lot#</th>
<th>Ovine Heparin Sodium</th>
<th>HP std</th>
<th>Ovine Enoxaparin Sodium</th>
<th>LMWH std</th>
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</thead>
<tbody>
<tr>
<td>80-ALR-089</td>
<td>Medefil Hep</td>
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<td></td>
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<tr>
<td>80-ALR-092</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>OES-404A</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>MW (Da)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MW (UV)</td>
<td>13 624</td>
<td></td>
<td>18 853</td>
<td>3778</td>
</tr>
<tr>
<td>MW (RI)</td>
<td>13 407</td>
<td></td>
<td>18 856</td>
<td>4245</td>
</tr>
<tr>
<td>Mn (UV)</td>
<td>10 113</td>
<td></td>
<td>14 781</td>
<td>3260</td>
</tr>
<tr>
<td>Mn (RI)</td>
<td>9794</td>
<td></td>
<td>15 405</td>
<td>3725</td>
</tr>
<tr>
<td>USP potency</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Xa (U/mg)</td>
<td>201</td>
<td></td>
<td>190</td>
<td>105</td>
</tr>
<tr>
<td>Ila (U/mg)</td>
<td>201</td>
<td></td>
<td>191</td>
<td>42</td>
</tr>
<tr>
<td>Xa/Ila ratio</td>
<td>1.00</td>
<td></td>
<td>0.99</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Abbreviations: MW, molecular weight; RI, refractive index.

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Top-Down Analysis

Top-down analysis uses LC-MS to examine intact oligosaccharide chains found within an LMWH. The top-down analysis of LMWHs showed that ovine enoxaparin contains 227 oligosaccharide species (Figure 3), while 225 oligosaccharide species were identified in Lovenox, and 130 of the same oligosaccharide species were present in both ovine enoxaparin and Lovenox (Figure 4). The highly sulfated oligosaccharides, [1, 2, 3, 0 8], [1, 4, 5, 0, 12], [1, 5, 6, 0, 16], [1, 5, 6, 0, 17], and [1, 6, 7, 0, 17], in ovine enoxaparin were in higher abundance than in Lovenox. The less sulfated N-acetylated oligosaccharides, [1, 4, 5, 1, 10], [1, 4, 5, 1, 11], and [1, 4, 5, 1, 12], was in higher abundance in ovine enoxaparin than in Lovenox. These analyses were in agreement with the disaccharide analysis,
Figure 3. Top-down analysis of low-molecular-weight heparins (LMWHs). For the 3 ovine enoxaparin intact chains detected in top-down analysis whose (A) reducing ends are 1, 6-anhydro, (B) nonreducing ends are unsaturated, and (C) nonreducing ends are saturated. The error bars indicate sample variability.
Figure 4. Top-down analysis of low-molecular-weight heparins (LMWHs). Comparison of ovine enoxaparin and Lovenox about intact chains detected in top-down analysis whose (A) reducing ends are 1,6-anhydro, (B) nonreducing ends are unsaturated, and (C) nonreducing ends are saturated.
showing that ovine enoxaparins were richer in IdoA2S(1→4)GlcNS6S sequences (Figure 2).

**Bottom-Up Analysis**

Treatment of LMWH sample with a single heparin lyase, heparin lyase II, affords resistant oligosaccharides that provide a bottom-up analysis. Ovine enoxaparins and Lovenox were compared to one another using this approach (Figure 5A and B).

Lovenox had a higher normalized abundance of linkage region oligosaccharide than did the ovine enoxaparins. The 1,6-anhydro at reducing end in ovine enoxaparin (30.7 ± 0.3%) was higher than Lovenox, 23.2 ± 0.7%, as determined by mass spectrometry (Figures 3–5). The nonreducing end saturated oligosaccharides in ovine enoxaparins (25.6% ± 1.7%) was slightly higher in ovine enoxaparins than in Lovenox (20.6 ± 1.4%) and the unsaturated oligosaccharides of ovine enoxaparin (74.4 ± 1.7%) was lower than Lovenox (79.4 ± 1.4%; Figure 5C). These differences were expected based on the lower MW of the ovine intestinal heparins.

**Conclusion**

There is a clear need to develop new sources for heparin and LMWH to diversify the supply of these critical life-saving drugs. The current study examines ovine intestinal heparin and ovine enoxaparin as potential replacements for porcine intestinal heparin and porcine enoxaparin or Lovenox. Unfractionated ovine intestinal heparin, while having some compositional differences and MW differences from unfractionated porcine heparin, has a similar profile of in vitro anticoagulant activities. Ovine enoxaparins had similar MW and in vitro anticoagulant activities as Lovenox. Some disaccharide compositional, oligosaccharide composition at the reducing and nonreducing ends and intact chain compositional differences could be observed between porcine enoxaparin and ovine LMWH. The similarity of these ovine and porcine heparin products suggests that their preclinical evaluation and ultimately clinical assessment is warranted.

**Authors’ Note**

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**Author Contribution**

Jianle Chen and Yanlei Yu contributed equally to this work.

**Declaration of Conflicting Interests**

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Caijuan Jin, Yongsheng Jin, and Yiming Yao are employees of Ronnsi Pharma Co, Ltd.

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**Supplemental Material**

Supplemental material for this article is available online.

**References**


