Comparative Pharmacokinetic Profile of 3 Batches of Ovine Low-Molecular-Weight Heparin and 1 Batch of Branded Enoxaparin

Walter Jeske, PhD1, Ahmed Kouta, MS1, Rick Duff, BS1, Varun Rangnekar2, Manoj Niverthi2, Debra Hoppensteadt, PhD1, Jawed Fareed, PhD1, and Yiming Yao, MS3

Abstract
Although pharmaceutical grade heparin is obtained almost exclusively from porcine intestinal mucosa, there is interest in diversifying heparin sourcing to address potential supply shortages and economically motivated adulteration. Since ovine-derived heparin is structurally similar to porcine heparin, it is expected that ovine-derived low-molecular-weight heparin (LMWH) will be comparable to porcine-derived LMWH. This study compared the pharmacokinetic (PK) behavior of 3 batches of ovine LMWH with that of enoxaparin in nonhuman primates. Blood samples were collected prior to and at 2, 4, and 6 hours post-administration of a 1 mg/kg subcutaneous dose of LMWH. Circulating drug concentrations determined using anti-Xa and anti-thrombin assays were used to calculate values for PK parameters. Tissue factor pathway inhibitor (TFPI) levels were measured by enzyme-linked immunosorbent assay. The ovine LMWHs tested met pharmacopoeial potency and molecular weight distribution requirements for enoxaparin. In the post-administration samples, comparable levels of branded enoxaparin and ovine enoxaparin were observed using anti-Xa and anti-thrombin assays, with the concentration versus time curves being nearly superimposable. Consistent with this similarity, no significant differences were observed between PK parameters calculated for branded enoxaparin and ovine LMWH. The TFPI levels returned to baseline levels by 6 hours in ovine LMWH-treated animals but remained slightly elevated in animals treated with branded enoxaparin. It is concluded that the pharmacokinetics of ovine enoxaparin were not only comparable between different batches but also similar to the branded product. Thus, LMWH prepared from ovine mucosal heparin is comparable to its porcine-derived counterpart.

Keywords
ovine, low-molecular-weight heparin, pharmacokinetics

Background
Heparin products have been used for more than 75 years for the prevention and treatment of venous and arterial thrombosis.1 Low-molecular-weight forms of heparin offer several pharmacologic advantages over unfractionated heparin including a reduced level of polydispersity, increased subcutaneous bioavailability, and more predictable drug levels.2 Several low-molecular-weight heparins (LMWHs) are marketed in the United States, including Lovenox (enoxaparin sodium), Fragmin (dalteparin sodium), and Innohep (tinzaparin sodium). Each of these products is derived from porcine mucosal heparin.

Direct chemical measurement of heparin levels in plasma is difficult because of the large number of heparin saccharide chains and a lack of sensitive assays. The time course of heparin or LMWH action in circulation is typically described in terms of its anticoagulant activity. For this purpose, anti-Xa and anti-IIa levels are most commonly used, although studies have been carried out using activated partial thromboplastin time (aPTT), activated clotting time (ACT), and other point-of-care assays.3

Approximately 80% of the world supply of porcine heparin originates from China. Following the heparin contamination crisis in 2007 to 2008,4 there is renewed interest in diversifying

1 Cardiovascular Research Institute, Loyola University Chicago Health Sciences Division, Maywood, IL, USA
2 Georgia Thrombosis Forum, Suwanee, Georgia, USA
3 Suzhou Ronnsi Pharma, Suzhou, China

Corresponding Author: Walter Jeske, Cardiovascular Research Institute, Loyola University Chicago Health Sciences Division, 2160 S First Ave, Maywood, IL 60153, USA. Email: wjeske@luc.edu

Creative Commons Non Commercial CC BY-NC: This article is distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 License (http://www.creativecommons.org/licenses/by-nc/4.0/) which permits non-commercial use, reproduction and distribution of the work without further permission provided the original work is attributed as specified on the SAGE and Open Access pages (https://us.sagepub.com/en-us/nam/open-access-at-sage).
the source of heparin.\textsuperscript{5} Heparins derived from different tissues and/or species are known to have subtle differences in chemical structure as related to their degree of sulfation and acetylation. Additionally, isolation conditions can impact the structural and functional characteristics of the final product. The extent to which such structural differences translate to functional differences is not completely clear. Heparin isolated from sheep intestine (ovine) has been shown to have a high degree of structural and functional similarity to porcine-derived heparins.\textsuperscript{6,7}

It was, therefore, hypothesized that low-molecular-weight forms of ovine heparin would also be similar to their porcine-derived counterparts. This study compares the pharmacokinetic (PK) behavior of porcine- and ovine-derived LMWHs following subcutaneous administration to nonhuman primates.

**Materials and Methods**

**Test Agents**

Enoxaparin sodium (Lovenox) was from Sanofi-Aventis, Bridgewater, New Jersey (Lot #7L010A). Three lots of ovine LMWH (OES L170401D, OES L170402D, OES L170403D) were obtained from Suzhou Ronnsi Pharma, Suzhou, China. The ovine LMWHs were obtained as white powder that were stored at room temperature in a desiccator. These samples were weighed on a Mettler (Columbus, Ohio) balance, and 0.9% NaCl\textsubscript{2} was used as a diluent to make stock solutions of 10 mg/mL for the in vivo and in vitro testing.

**Primates**

Rhesus monkeys (Macaca mulatta) ranging in weight from 6.4 to 10.8 kg were used in this study.\textsuperscript{5} The animals were housed in individual cages in accordance with the Guide for the Care and Use of Laboratory Animals and the National Research Council. All research was conducted under approval granted by the Institutional Animal Care and Use Committee of Loyola University Chicago Health Sciences Division.

**Dosing**

Primates were anesthetized by the intramuscular administration of ketamine (10 mg/kg) and xylazine (1-2 mg/kg) based on their most recent charted weight. Following attainment of the appropriate depth of anesthesia (assessed by a lack of response to foot pinch), primates were freshly weighed to accurately determine the dose of the test agent. The procedure room was maintained at an ambient temperature of 78°F to minimize the chance that primates would become hypothermic while under anesthesia. A baseline blood sample was collected by venipuncture of the saphenous vein. An area in the abdominal region was shaved and cleansed with a combination of betadine and alcohol. The test agent was administered at a dose of 1 mg/kg subcutaneously. Additional blood samples were collected at 2, 4, and 6 hours post-drug administration. Four primates were dosed with each LMWH.

**Sample Collection**

All blood samples were collected using a double syringe technique, employing a 21-gauge butterfly needle. After an initial ~1 mL volume (discard blood) was collected, the syringe was changed and a 2.7 mL sample was drawn and placed into a tube containing 0.3 mL 3.2% sodium citrate. Citrated blood samples were centrifuged at 1100 \( \times \) g for 15 minutes. The supernatant platelet-poor plasma was harvested, and aliquots of plasma were stored frozen at ~70°C until analysis of circulating drug levels and tissue factor pathway inhibitor (TFPI) levels.

**Sample Analysis**

Anti-Xa and anti-IIa activities were determined using in-house amidolytic assays\textsuperscript{9} employing human thrombin or bovine factor Xa (Enzyme Research Laboratories, South Bend, Indiana) and Spectrozyme TH or Spectrozyme Xa (Biomedica Diagnostics, Windsor, Nova Scotia, Canada). Assays were performed on an ACL Elite (Werfen, Bedford, Massachusetts). The aPTT was determined using TriniCLOT reagents (Tcoag, Wicklow, Ireland). The TFPI levels were determined using the Asserachrom Total TFPI Enzyme-Linked Immunosorbent Assay (ELISA; Stago, Parsippany, New Jersey).

In vitro concentration–response curves were made by supplementing the various LMWHs into pooled plasma. Plasma concentrations of various LMWHs were plotted against corresponding optical densities in the anti-Xa or anti-IIa assays using graphing software, SigmaPlot Version 12.0 for Windows (Systat Software, San Jose, California). Best-fit curves were made, and the drug concentration in each primate blood sample was determined by extrapolation.

Molecular weight determinations were made using gel permeation chromatography in a high-performance liquid chromatography (HPLC) system as reported previously.\textsuperscript{10} Briefly, the system was equilibrated using freshly degassed mobile phase (0.3 M sodium sulfate) until a stable baseline was obtained. Analysis was carried out by injecting 20 \( \mu \)L of sample (10 mg/mL in 0.3 M sodium sulfate) into the HPLC system. The flow rate for the mobile phase was 0.5 mL/min, and the run time for each sample was 65 minutes. The internal temperature for the refractive index (RI) detector was set at 35°C, and UV determination was made at 205 nm. All analyses were made at room temperature. The elution profile of each sample was analyzed in relation to a calibration curve prepared using 13 heparin fractions ranging in molecular weight from 3.0 to 40 kDa. The molecular weight profile consists of parameters such as weight average molecular weight, number average molecular weight, peak molecular weight, and polydispersity.

**Pharmacokinetic/Pharmacodynamic Calculations**

The extrapolated plasma concentrations were used to calculate values for PK parameters such as elimination half-life (\( t_{1/2} \), area under the plasma concentration–time curve (AUC), systemic clearance (Cl), and volume of distribution (Vd). The PK
parameters were calculated by noncompartmental analysis using PKSolver add-in software for Microsoft Excel 2013.11

**Statistical Analysis**

Statistical analysis was performed using SigmaPlot Version 12.0 for Windows. Circulating drug levels were compared using 2-way repeated measures analysis of variance. Differences with \( P \) values ≤.05 were considered to be statistically significant.

**Results**

The elution profiles of the 3 lots of ovine LMWH were observed to be nearly superimposable; however, the elution profile of Lovenox appeared to have a slightly larger tail at the early elution times (higher molecular weight; Figure 1A). The values for number average molecular weight and weight average molecular weight were slightly higher for Lovenox (3.82 and 4.49 kDa, respectively) compared to ovine LMWH (3.72 and 4.24 kDa, respectively; Figure 1B).

The anticoagulant and antiprotease activities of Lovenox and ovine LMWH were compared following in vitro supplementation to plasma. Comparable activities were observed in terms of the inhibition of factor Xa and thrombin as well as for aPTT prolongation (Figure 2).

Primates were administered Lovenox or ovine LMWH at a 1 mg/kg subcutaneous dose. Blood samples were subsequently drawn at 2, 4, and 6 hours post-administration. Circulating drug levels were determined using amidolytic anti-Xa and anti-IIa assays (Figure 3). By anti-Xa assay, peak drug levels of approximately 10 \( \mu \)g/mL were observed following administration of each LMWH, with only minor differences observed between batches of ovine LMWH.

The TFPI levels in all plasma samples were measured by ELISA (Figure 4). Some variability in TFPI release was observed between lots of ovine LMWH. As a whole, peak TFPI levels at 2 hours were the same following administration of ovine LMWH and Lovenox. Differences were observed, however, at later time points, where TFPI levels were observed to remain elevated for a longer time following Lovenox treatment \[ \text{AUC}_{0-6} \text{ hours Lovenox} = 104.75 (\text{ng} \times \text{h})/\text{mL} \text{ vs } \text{AUC}_{0-6} \text{ hours ovine LMWH} = 87.50 (\text{ng} \times \text{h})/\text{mL} \].

Values for PK parameters were derived by applying a noncompartmental model to circulating drug levels determined by anti-Xa and anti-IIa assays (Figure 5). The PK profiles of ovine LMWH and Lovenox were observed to be comparable, with no statistically significant differences observed.

**Discussion**

Despite the introduction of small-molecule direct oral anticoagulants, the usage of LMWHs continues to increase. Currently, commercially available LMWHs are made exclusively from porcine mucosal heparin. Owing to a possible shortage of porcine heparin starting material, it is of interest to evaluate the feasibility of using other common heparin sources to produce LMWHs. Heparins can be obtained in commercial quantities from bovine lung, bovine intestine, and ovine intestine. Bovine unfractionated heparins have been shown to differ from porcine heparin in terms of molecular weight distribution, the degree of sulfation, and the biologic potency.12 Unfractionated ovine heparin more closely matches porcine heparin in terms of both structure and activity.6,7

There have been several previous studies to evaluate LMWHs derived from non-porcine heparins using either \( \beta \)-elimination/alkaline hydrolysis or nitrous acid-based depolymerization processes. In one study, LMWHs derived from
Figure 2. In vitro activity of Lovenox and ovine low-molecular-weight heparin (LMWH). (A) Anticoagulant activity was determined by measuring the activated partial thromboplastin time (aPTT). (B) Anti-Xa and (C) anti-IIa activities were measured using amidolytic assays. Lovenox and ovine LMWH produced comparable anticoagulant and antiprotease activities in all of these assays.

Figure 3. Circulating drug levels in primates treated with Lovenox or ovine low-molecular-weight heparin (LMWH). Circulating drug levels were determined using amidolytic anti-Xa (A) and anti-IIa (B) assays relative to batch-specific calibration curves. Good batch-to-batch consistency was observed between drug levels obtained with different batches of ovine enoxaparin. Drug levels in ovine LMWH-treated animals were comparable to those observed following Lovenox administration.
Figure 4. Plasma tissue factor pathway inhibitor (TFPI) levels in primates treated with Lovenox or ovine low-molecular-weight heparin (LMWH). Circulating TFPI levels were determined by enzyme-linked immunosorbent assay (ELISA). *P < .02 OES-402D versus OES-401D, OES-403D, and Lovenox at 4 hours.

Figure 5. Pharmacokinetic profile of subcutaneously administered ovine low-molecular-weight heparin (LMWH) and Lovenox. Values for pharmacokinetic parameters were calculated using circulating drug levels determined by anti-IIa and anti-Xa assays.
bovine lung and bovine intestinal heparin were produced by β-
elimination reaction and subjected to compositional and
potency analysis.13 Despite the fact that the starting bovine
heparins were different than porcine heparin, depolymerized
versions of bovine lung and intestinal heparins met the speci-
fications for Lovenox in terms of molecular weight and
potency. In terms of their disaccharide composition, the parent
heparins and daughter LMWHs were highly correlated.
Another comparison of LMWHs produced by benzylation/
alkaline hydrolysis of porcine mucosal heparin and bovine lung
heparin demonstrated that while such products are comparable
in terms of molecular weight and 1,6-anhydro content, bovine-
derived LMWHs have lower degrees of N-acetylation and a
lower content of antithrombin (AT)-binding sites leading to
an anti-Xa potency that was approximately 70% that of
porcine-derived LMWHs.14 In another study, LMWHs were
produced from ovine, bovine, and porcine heparins by nitrous
acid and hydrogen peroxide–induced depolymerization.15
Here, 13C nuclear magnetic resonance (NMR) spectra and
disaccharide analyses indicated that LMW ovine heparin shows
a higher degree of similarity to porcine- and bovine-derived
LMWHs than the parent heparins. The LMWHs were produced
from porcine and ovine intestinal heparin and bovine lung
heparin by nitrous acid depolymerization and compared in
terms of their composition and functional activity.16 Overall,
current literature suggests that bioequivalent LMWHs can be
generated when appropriate modifications to the production
process are employed.

In addition to a demonstration of structural comparability
and bioequivalent potencies, Food and Drug Administration
guidance for the development of biosimilar LMWHs also
requires demonstration of bioequivalence in terms of pharma-
codynamic profile.17 The current study compares the pharma-
codynamic profile of LMWH derived from ovine heparin with
that of Lovenox following subcutaneous administration to pri-
mates. Primates have previously been shown to respond simi-
larly to humans to the administration of heparin-like
anticoagulants.

The current study assessed plasma drug levels in terms of
anti-Xa and anti-IIa activities. By both assays, the time course
of circulating LMWH levels was comparable for Lovenox and
the ovine LMWHs, and the ratio of anti-Xa to anti-IIa activities
as assessed by area under the curve were as expected for
LMWHs. Heparin administration results in an increase in the
circulating level of TFPI. In the current study, comparable peak
levels of TFPI were observed following administration of
Lovenox and ovine LMWH; however, the increase in TFPI
levels was more prolonged at later time points in Lovenox-
treated primates. This may result from subtle differences in
chemical composition of the tested LMWHs as properties such
as charge density and chain length have previously been shown
to impact circulating TFPI levels.18

The studies described here demonstrate that LMWH derived
from ovine heparin and Lovenox exhibit comparable anticoa-
gulant, antiprotease, and PK profiles and suggest that these
LMWHs will exhibit similar PK profiles when administered
to humans.

Acknowledgments
The authors are grateful to Dr Atul Laddu, CEO of the Georgia
Thrombosis Forum, for facilitating research fellowships for Mr Rang-
nekar and Mr Niverthi.

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: Yiming Yao is an employee of Ronssi Pharma.

Funding
The author(s) disclosed receipt of the following financial support for
the research, authorship, and/or publication of this article: Yiming Yao
is an employee of Ronssi Pharma, the company that produced the
ovine LMWHs that were used in this study.

References
1. Mulloy B, Hogwood J, Gray E, Lever R, Page CP. Pharmacol-
ology of heparin and related drugs. Pharmacol Rev. 2016;68(1):
76-141.
2. Del Bono R, Martini G, Volpi R. Update on low molecular weight
heparins at the beginning of the third millennium. Focus on revi-
parin. Eur Rev Med Pharmacol Sci. 2011;15(8):950-959.
3. Brunet P, Simon N, Opris A, et al. Pharmacodynamics of unfraction-
ated heparin during and after a hemodialysis session. Am J
Kidney Dis. 2008;51(5):789-795.
4. Kishimoto TK, Viswanathan K, Ganguly T, et al. Contami-
inated heparin associated with adverse clinical events and activi-
tion of the contact system. N Engl J Med. 2008;358(23):
2457-2467.
5. Tremblay JF. Making heparin safe. Chem Eng News. 2016;
94(40):30-34.
6. Hoppensteadt D, Maia P, Silva A, et al. Resourcing of heparin and
low molecular weight heparins from bovine, ovine and porcine
origin. Studies to demonstrate the biosimilarities. Blood. 2015;
126(23):4733.
7. Monakhova YB, Diehl BWK, Fareed J. Authentication of animal
origin of heparin and low molecular weight heparin including
ovine, porcine and bovine species using 1D NMR spectroscopy
and chemometric tools. J Pharm Biomed Anal. 2018;149:
114-119.
8. Fareed J, Kumar A, Rock A, Walenga JM, Davis P. A primate
model (Macaca mulatta) to study the pharmacokinetics of
heparin and its fractions. Semin Thromb Hemost. 1985;11(2):
138-154.
9. Hoppensteadt DA, Walenga JM, Fareed J. Validity of serine pro-
 tease inhibition tests in the evaluation and monitoring of the effect
of heparin and its fractions. Semin Thromb Hemost. 1985;11(2):
112-120.
10. Ahsan A, Jeske W, Hoppensteadt D, Lormeau JC, Wolf H, Fareed
J. Molecular profiling and weight determination of heparins and
depolymerized heparins. J Pharm Sci. 1995;84(6):724-727.
11. Zhang Y, Huo M, Zhou J, Xie S. PKSolver: an add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel. *Comput Methods Programs Biomed.* 2010;99(3):306-314.

12. Tovar AM, Santos GR, Capillé NV, et al. Structural and haemostatic features of pharmaceutical heparins from different animal sources: challenges to define thresholds separating distinct drugs. *Sci Rep.* 2016;6:35619. doi:10.1038/srep35619.

13. Liu X, St Ange K, Fareed J, et al. Comparison of low-molecular-weight heparins prepared from bovine heparins with enoxaparin. *Clin Appl Thromb Hemost.* 2017;23(6):542-553.

14. Guan Y, Xu X, Liu X, et al. Comparison of low-molecular-weight heparins prepared from bovine lung heparin and porcine intestine heparin. *J Pharm Sci.* 2016;105(6):1843-1850.

15. Watt DK, Yorke SC, Slim GC. Comparison of ovine, bovine and porcine mucosal heparins and low molecular weight heparins by disaccharide analyses and 13C NMR. *Carbohydr Polym.* 1997;33(1):5-11.

16. Xie S, Guan Y, Zhu P, et al. Preparation of low molecular weight heparins from bovine and ovine heparins using nitrous acid degradation. *Carbohydr Polym.* 2018;197:83-91.

17. Lee S, Raw A, Yu L, et al. Scientific considerations in the review and approval of generic enoxaparin in the United States. *Nat Biotech.* 2013;31(3):220-226.

18. Valentin S, Larnkjaer A, Ostergaard P, Neilsen JI, Nordfang O. Characterization of the binding between tissue factor pathway inhibitor and glycosaminoglycans. *Thromb Res.* 1994;75(2):173-184.