Published in final edited form as:

J Pharm Sci. 2015 February ; 104(2): 457–463. doi:10.1002/jps.24134.

Stable-Isotopic Analysis of Porcine, Bovine, and Ovine Heparins

John P. Jasper¹, Fuming Zhang², Russell B. Poe³, and Robert J. Linhardt^{2,4}

¹Molecular Isotope Technologies LLC, 8 Old Oak Lane; Niantic, CT 06357, USA

²Department of Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180, USA

³Birch Biopharmaceutical Consultants LLC, Groton, CT 06340, USA

⁴Departments of Chemistry and Chemical, Biology, Biology, Biomedical Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180, USA

Abstract

The assessment of provenance of heparin is becoming a major concern for the pharmaceutical industry and its regulatory bodies. Batch-specific [carbon ($\delta^{13}\text{C}$), nitrogen ($\delta^{15}\text{N}$), oxygen ($\delta^{18}\text{O}$), sulfur ($\delta^{34}\text{S}$), and hydrogen (δD)] stable-isotopic compositions of five different animal-derived heparins were performed. Measurements readily allowed their differentiation into groups and/or subgroups based on their isotopic provenance. Principle component analysis showed that a bivariate plot of $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ is the best single, bivariate plot that results in the maximum discrimination ability when only two stable isotopes are used to describe the variation in the data set. Stable-isotopic analyses revealed that (i) stable-isotope measurements on these highly-sulfated polysaccharide (MW ~15 kDa) natural products (“biologics”) were feasible; (ii) in bivariate plots, the $\delta^{13}\text{C}$ versus $\delta^{18}\text{O}$ plot reveals a well-defined relationship for source differentiation of hogs raised in the US from hogs raised in Europe and China; (iii) the δD versus $\delta^{18}\text{O}$ plot revealed the most well-defined relationship for source differentiation based on the hydrologic-environmental isotopes of water (D/H and $^{18}\text{O}/^{16}\text{O}$), and (iv) the $\delta^{15}\text{N}$ versus $\delta^{18}\text{O}$ and $\delta^{34}\text{S}$ versus $\delta^{18}\text{O}$ relationships are both very similar, possibly reflecting the food sources used by the different heparin producers.

Keywords

heparin; stable-isotopic analysis; animal-sourced; principle component analysis (PCA)

INTRODUCTION

Heparin is a linear sulfated polysaccharide, consisting of repeating, 1→4-glycosidically linked hexuronic acid and glucosamine residues (Figure 1).¹ Heparin is biosynthesized in the Golgi of mast cells of humans,² other vertebrates³ and invertebrates.⁴ Pharmaceutical

Correspondence to: John P. Jasper (Telephone: (860) 739-1926; Fax: (860) 739-3250. JPJasper@NaturesFingerprint.com) and Robert J. Linhardt (Telephone: (518) 276-3404; Fax: (518) 276-3405; linhar@rpi.edu).

heparin is primarily produced from the intestines and lungs of food animals, primarily pig, bovine cattle, and sheep. It is a critically important anticoagulant drug used in cardiopulmonary bypass, hemodialysis, in the treatment of coagulation abnormalities, as coating materials for implantable devices, and in the preparation of low molecular weight heparins.⁵ Heparin, while commanding a multi-billion dollar worldwide market, has a fragile supply chain that was jeopardized in the past by fears of prion (*i.e.*, bovine spongiform encephalopathy and scrapies) and viral impurities,⁶ was briefly interrupted in 2007–8 during a contamination crisis,⁷ and has been adversely impacted by the recent appearance of the porcine epidemic diarrhea virus.⁸

While only porcine intestinal heparin is currently used in the United States, bovine lung and intestinal heparins^{9,10} have been used in South America and in the Middle East, and ovine intestinal and porcine lung heparins have been used in Asia. Heparins derived from different organisms and tissues display different physical-chemical properties (*i.e.*, molecular weight properties, disaccharide composition and antithrombin III (AT) pentasaccharide binding sites (Figure 1)),^{11–14} different *in vitro* anticoagulant activities,^{12,15–17} different pharmacology (*i.e.*, *in vivo* efficacy, pharmacodynamics, and side effects, *etc.*),^{18–23} and potentially carry different impurities (*i.e.*, polysaccharides, peptides, virus, prions, *etc.*).^{11,24} Regulatory concerns about the substitution of one heparin for another or the blending of heparins coming from two different organisms or tissues have lead to the development of assays to assess the provenance of a given heparin.^{6,24–29} Crude porcine heparins (the unbleached intermediate used to prepare heparin active pharmaceutical ingredient (API)) are currently assessed by quantitative polymerase chain reaction (qPCR), which can sensitively detect the presence of ruminant DNA, suggesting blending of porcine heparin with ovine or bovine heparin.^{6,25–27} When using this qPCR method, heparinase is required to degrade the heparin present in the sample to ensure accurate detection, since heparin-mediated inhibition of PCR as previously described.³⁰ One-dimensional and two-dimensional nuclear magnetic resonance (NMR) spectroscopy of porcine heparin API can detect the presence bovine heparin or ovine heparin but only when present in relatively large amounts (~25%).¹¹ Multivariate analysis of NMR data allows for the detection of smaller levels of bovine heparin API blended into porcine heparin API.¹⁶ The current study examines a novel method relying on natural-abundance stable isotope analysis³¹ to examine the provenance of heparin products. Stable isotope studies on heparin API, requires little sample preparation or pretreatment, and can be rapidly and routinely performed on multiple samples.

The stable-isotopic compositions of selected light elements (*e.g.*, C, N, O, S, H) are very useful in determining the natural origin of materials^{32,33} as well as the “manufacturer or batch” origin of the products and materials manufactured from them.^{34,35} Stable carbon isotopes have been particularly useful in differentiating large classes of plant organic matter based on their photosynthetic pathways: *e.g.*, C3-terrestrial versus C3-algal sources, where C3 plants fractionate to a greater degree (using Rubisco) than do C4 plants (using RUBP carboxylase). In addition, the stable-isotopic composition of nitrogen ($\delta^{15}\text{N}$) is highly variable in nature through a number of pathways for inorganic as well as organic species.³³ Furthermore, the stable-isotopic composition of sulfur ($\delta^{34}\text{S}$) has been demonstrated to have

very high dynamic ranges (observed ranges/ 1σ standard deviations > 200) in pharmaceutical studies and typically serves as an excellent tracer of isotopic provenance.³⁶

Stable-isotopic characterization of individual batches of industrial products (“*Nature’s Fingerprint*®”) through their patented synthetic pathways (“*IsoPedigree*®”) provides a highly specific means to identify product batches and to protect chemical process patents.³⁵ In the period between the expiration dates of composition-of-matter patents and of their paired process patents, stable-isotopic analyses permit a novel and efficient means by which to protect the intellectual property of bio/pharmaceutical products^{35,37} through identifying isotopic differences.³⁸

The current study explores the use of isotopic quantification on a limited suite of animal-derived heparin products. In addition to affording information of the organism and/or tissue source of a heparin, these data might even provide a “location fingerprint” and a “manufacturer’s fingerprint” of heparin production.

MATERIALS AND METHODS

Materials

Five samples of animal-derived heparin APIs (heparins #1–5) were examined (Table 1). These samples consisted of porcine intestinal heparin sourced from the US (Celsus Laboratories, Cincinnati, OH, USA, 202 U/mg), China (Dongying Pharmaceutical Inc., Shangdong, China, 200 U/mg), and Spain (Bioiberica SA, Barcelona, Spain, 203 U/mg), bovine lung heparin (150 U/mg) and ovine intestinal heparin (182 U/mg) purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). The heparins were all sodium salts having approximate elemental compositions of $C_{12}H_{15}NO_{19}S_3Na_4$ with molecular weights of ~15 kDa. Detailed chemical structural and biological analysis were performed on all the five heparin APIs.^{11,39} These previous NMR studies, disaccharide analyses, molecular weight analyses and biological analyses demonstrated that all of these heparin samples were of high purity.

Stable-Isotopic Analyses

Five stable-isotope ratios ($\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, $\delta^{18}O$ and δD) were measured on each of the five heparin API samples with singlicate analysis for heparins #2–5 and in one triplicate analysis for heparin #1, to assess analytical precision.^{39,40} Thus, a total of 35 stable-isotopic measurements of these heparin (*i.e.*, 5 batches $\times$ 5 isotope ratios plus replicates) presented in Table 1 were performed in this study.

Carbon and Nitrogen Isotope Analyses

Individual solid samples of ~1.0 mg for $\delta^{13}C$ and ~3.7 mg for $\delta^{15}N$ analysis were weighed and placed into tin boats that were crimped tightly around the analyte. Carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopic analysis were performed with a Carlo Erba 1108 Elemental Analyzer interfaced using a ConFlo III interface to a Thermo Scientific Delta V isotope ratio mass spectrometer (EA/IRMS). The EA operated with an oxidation furnace temperature of 1020°C, reduction furnace temperature of 650°C, and a packed-column temperature of

70°C. All isotopic standards employed here are reported relative to the standards of the International Atomic Energy Agency (IAEA). $\delta^{13}\text{C}$ values are reported relative to the international Vienna Pee Dee Belemnite (VPDB) standard. $\delta^{15}\text{N}$ values are reported relative to the international Air standard.

Sulfur Isotope ($\delta^{34}\text{S}$) Analyses

For $\delta^{34}\text{S}$ analysis, individual solid samples containing 150–300 μg of sulfur within the sample along with approximately ten-times the total sample weight of WO_3 as an oxidant, were weighed into tin boats that were crimped tightly around those materials. Isotopic analysis was performed with an Elementar Vario EL III Elemental Analyzer interfaced via a Finnigan MAT ConFlo III to a Thermo Delta V Advantage Isotope Ratio Mass Spectrometer (EA/IRMS). The EA operated with an oxidation furnace temperature of 1150°C followed by a reduction furnace set at 850° C. Gas flow rates were 200 mL/min for helium and 1 mL/min for oxygen, which is dosed for 90 s directly to the combustion zone. The $\delta^{34}\text{S}$ values are reported in ‰ relative to the international standard Vienna Canyon Diablo Troilite (VCDT) iron meteorite.

Oxygen Isotope ($\delta^{18}\text{O}$) Analyses

Individual samples of ~0.2 mg were weighed and placed into silver boats, which were then crimped tightly around the analyte. Triplicate oxygen ($\delta^{18}\text{O}$) stable-isotopic analyses of one out of the five samples were performed on a Finnigan Thermal Conversion/Elemental Analyzer (TCEA) interfaced to Finnigan Delta V Plus isotope-ratio mass spectrometer (IRMS, thus a TCEA/IRMS). Analogous to a standard Elemental Analyzer/Isotope Ratio Mass Spectrometer (EAMS),⁴¹ the TCEA functions with samples sequentially delivered into a furnace and the effluent gases analyzed by an online IRMS, but with pyrolysis (instead of oxidative combustion as in the EA/IRMS) performed at 1400°C. The TCEA thermally converts analytes to CO rather than combustion into H_2O and CO_2 as in the EAMS. The analyte gas, CO, is chromatographically separated on a packed column at 85°C. The mass spectrometer measures ^{18}O in the form of CO.

Hydrogen (D) Isotopic Analyses

In general, hydrogen that is not bound to carbon in a molecule, readily exchanges with other hydrogen atoms present in ambient moisture (*i.e.* H_2O). This exchange even happens at room temperature and is difficult to control reliably. In order to use δD values to uniquely identify a compound, the exchangeable hydrogen portions must be accounted for or controlled in some way. Samples were weighed and dried under vacuum for one week to remove ambient moisture. The dried samples were then immediately transferred to a Costech Zero Blank autosampler and evacuated to remove moisture.

Samples were weighed and wrapped inside individual 3.5 mm $\times$ 5 mm silver “boats.” All materials were then dried under vacuum in the presence of a desiccant to remove moisture. Samples were then loaded into the autosampler of a Finnigan MAT, Thermal Conversion Elemental Analyzer (TC/EA). Along with each batch of samples, we also included several reference standards, such as a polyethylene standard that has no exchangeable hydrogen and is therefore unaffected by ambient moisture. Samples were then reduced at 1400°C in the

presence of glassy carbon. The resulting hydrogen was then separated from other gases using a gas chromatograph and passed into an isotope ratio mass spectrometer (IRMS) for isotopic analysis to obtain the δD values.

Units of Stable Isotopic Measurement

Carbon (and all other) isotopic results are expressed in δ values (‰ = parts per thousand differences from international standards) defined as:

$$\delta^{13}\text{C}(\text{‰}) = \left(\frac{R_{\text{smp}}}{R_{\text{std}}} \right) - 1 \times (1000)$$

where R_{smp} = the $^{13}\text{C}/^{12}\text{C}$ ratio of the sample material and R_{std} = the $^{13}\text{C}/^{12}\text{C}$ ratio of an International Atomic Energy Authority standard (IAEA, known as “VPDB” whose $^{13}\text{C}/^{12}\text{C}$ ratio has been defined as the official zero point of the carbon-isotopic scale). $^{15}\text{N}/^{14}\text{N}$ values are given relative to the International Air standard. $^{34}\text{S}/^{32}\text{S}$ values are given relative to the IAEA Vienna Canyon Diablo Troilite (VCDT) standard. $^{18}\text{O}/^{16}\text{O}$ and D/H values are given relative to IAEA Vienna Standard Mean Ocean Water (VSMOW) standard.

Estimates of Uncertainty

The uncertainty (or precision) of the isotopic measurements in this study was estimated from the pooled standard deviations (SD) of recent studies. In those cases, the pooled SD of raw data were estimated to derive a representative standard deviation from the whole raw data set in which small numbers of replicates (*viz.*, $n = 1-3$) were pooled to generate a representative standard deviation of the whole sample suite.³⁸ Characteristic one sigma (1σ) standard deviations for the isotope values used in this study were: $\delta^{13}\text{C}$ ($\pm 0.06\%$), $\delta^{15}\text{N}$ ($\pm 0.06\%$), $\delta^{18}\text{O}$ ($\pm 0.08\%$), δD ($\pm 1.1\%$) and $\delta^{34}\text{S}$ ($\pm 0.04\%$), as shown in Table 1.

RESULTS AND DISCUSSION

The ^{13}C , ^{18}O , ^{15}N , ^{34}S , and D Analyses of Heparin Samples

The results of stable-isotopic analyses ($\delta^{13}\text{C}$, $\delta^{18}\text{O}$, $\delta^{15}\text{N}$, $\delta^{34}\text{S}$ and δD) of the five-heparin samples given are presented in Table 1 and in Figs. 3–6.

In the following sections, the $\delta^{13}\text{C}$, $\delta^{18}\text{O}$, $\delta^{15}\text{N}$, $\delta^{34}\text{S}$ and δD data from Table 1 are displayed in bivariate plots and in PCA analysis their relationships discussed and assessed.

Principle Component Analysis of the Stable-Isotopic Composition of Heparin Samples

The data set contains results from five stable isotopes and five samples. One sample, (porcine heparin #1), was tested in three replicates to estimate the intrasample variation in the data set. Prior to performing Principle Component Analysis (PCA), the triplicates of the porcine heparin sample #1 were averaged and the average was used in the analysis so that each sample would have equal weight. The data was then autoscaled by mean centering followed by dividing by the standard deviation. Two principle components were empirically selected: principle component 1 accounted for 80% of the variance of the data set, followed by principle component 2, which accounted for 16% of the variance in the data set so that these two principle components accounted for 96% of the total variance of the data set.

The principle component scores and loadings are jointly displayed in the biplot in Fig. 2. From the loadings, the correlation between stable isotopes can be deduced as well as the ability of the stable isotope to differentiate between the heparin samples. The loadings show a strong correlation between $\delta^{15}\text{N}$, $\delta^{34}\text{S}$, and δD and that these three isotopes are strongly, inversely correlated with $\delta^{13}\text{C}$. The isotope, $\delta^{18}\text{O}$, is the most highly-differentiated variable showing less correlation to the other isotopes and also being the only isotope that allows differentiation of Porcine heparin samples from heparin from other sources. The sample scores indicate (i) that the porcine samples are relatively high in $\delta^{13}\text{C}$, (ii) that bovine heparin is relatively low in $\delta^{18}\text{O}$, and (iii) that ovine heparin is relatively high in $\delta^{15}\text{N}$, $\delta^{34}\text{S}$, and δD . Shannon entropy criterion⁴² is related to the amount of information and confirms that a bivariate plot of $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ is the best single, bivariate plot which results in the minimum of $-\log |\text{R}|$ where $|\text{R}|$ is the determinant of the correlation coefficient matrix when only two stable isotopes are used to describe the variation in the data set.

Bivariate Isotope Plots of the Stable-Isotopic Composition of Heparin Samples

Four bivariate plots of the stable-isotopic results collected on the heparin samples are given in Fig. 3–6 to show the interrelationship of the isotopic results and their groupings.

Although there are eleven such plots, PCA shows that the four paired $\delta\text{X}-\delta^{18}\text{O}$ values, shown in in Fig. 3–6s, are probably the most useful (where $\delta\text{X} = \delta^{13}\text{C}$, δD , $\delta^{15}\text{N}$, $\delta^{34}\text{S}$). The seven other optional plots complete the set of eleven (see Supplementary Information Figs. S1–S7).

Four bivariate graphs of the paired $\delta\text{X}-\delta^{18}\text{O}$ values of the heparin samples are shown in Figures 3–6. Significantly, the graphs as a group and the data in Table 1, show high dynamic ranges from the isotopic values (from 43 for $\delta^{15}\text{N}$ to 308 for $\delta^{34}\text{S}$ where dynamic range = observed range/ 1σ standard deviation) and therefore a high specificity ($1.1 * 10^{10}$ = product of the five dynamic ranges) in differentiating this sample suite of biologic pharmaceutical materials (see Supplementary Information Table S1).

As indicated by the PCA, the $\delta^{13}\text{C}-\delta^{18}\text{O}$ graph (Fig. 3) shows a significant differentiation of the three types of heparin (porcine, ovine, bovine) as well as a distinct differentiation of the three sources of porcine heparin (USA, China, Spain). Further, PCA indicates that the $\delta\text{D}-\delta^{18}\text{O}$ graph (Fig. 4) differentiates the three types of heparin with maximum resolving power. Finally, PCA analysis indicates a close similarity of $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$. This similarity is manifested in the $\delta^{15}\text{N}-\delta^{18}\text{O}$ and $\delta^{34}\text{S}-\delta^{18}\text{O}$ graphs (Figs. 5 and 6, respectively), which also strongly resolve the three types of heparin (also see Supplementary Information Figures S1–S7).

This study only demonstrates differences in the stable isotope distribution of a small number of heparin APIs and does not provide any data supporting the underlying mechanism for these differences. It is, however, possible to speculate on the cause of such differences in isotopic distribution. The major animal sources of heparin API, pig, sheep and bovine cattle, have different diets, digestive systems and gut (or lung) flora. Pigs are naturally omnivorous but are commercially raised on grains and legumes and have a gut flora similar to that of humans and other monogastrics. In contrast, sheep and cattle are grazing ruminants eating a

diet rich in fiber cellulotics with a complex microbial flora consisting of bacteria, methanogens, yeast, molds and protozoans. Studies using stable isotopes have shown differences in carbon isotope fractionation by ruminants and non-ruminants⁴³ as well as isotope incorporation differences between different ruminants, including ovine and bovine species.⁴⁴ The diet of animals can also impact C and N isotope ratios⁴⁵ as can differences in their gut microbiome.⁴⁶ The triplicate analysis of a single porcine intestinal heparin (heparin #1, hogs raised in the United States) shows very little analytical variation in the measurement of stable isotope distributions. The analyses of porcine intestinal heparins show similar stable isotope distribution with some differences based on the geographic area where the animals were raised. These differences might be attributable to different strains of pigs, different diets, different environmental factors, different seasons of animal harvesting, different microbiomes or different factory processing of these heparins.^{5,47} Differences were observed between heparins derived from ovine intestine and bovine lung (as compared to porcine intestinal heparin) but no firm conclusions can be drawn from these differences because of the limited number of samples studied.

CONCLUSIONS

Thirty-five measurements of the carbon- ($\delta^{13}\text{C}$), nitrogen- ($\delta^{15}\text{N}$), sulfur- ($\delta^{34}\text{S}$), oxygen- ($\delta^{18}\text{O}$) and deuterium (δD) isotope ratios of five samples of animal-derived heparins and presumably other biologic molecules readily allow differentiation of the samples into groups and/or subgroups based on their stable-isotopic provenance.

In particular, the stable-isotopic analyses revealed that (i) stable-isotopic measurements of biologic molecules, such as heparin (MW ~ 10–15 kDa), were feasible; (ii) in bivariate plots, the $\delta^{13}\text{C}$ versus $\delta^{18}\text{O}$ plot reveals a well-defined relationship for source differentiation, separating USA-porcine heparin from non-USA heparin; (iii) the δD versus $\delta^{18}\text{O}$ plot reveals the most well-defined relationship for source differentiation based on the hydrologic-environmental isotopes of water (D/H and $^{18}\text{O}/^{16}\text{O}$), and (iv) the $\delta^{15}\text{N}$ versus $\delta^{18}\text{O}$ and $\delta^{34}\text{S}$ versus $\delta^{18}\text{O}$ relationships are both very similar, suggesting that the food sources used for animals by different heparin producers may play a role.

Heparin products that are also chemically (*i.e.*, Arixtra) or chemoenzymatically (*i.e.*, bioengineered heparin)⁴⁷ synthesized. While these heparins are not natural products, their manufacturing provenance might also be monitored using stable isotope methods.^{35,37,38} Such monitoring might be also be useful in preventing the introduction of counterfeit synthetic heparins onto the world market.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was supported by grants from the funded by the National Institutes of Health HL101721 and HL096972. We thank Mark Swift and Peter Farina for their early guidance in the stable-isotopic analysis of biologics.

Abbreviations used

EA/IRMS	elemental analyzer / isotope ratio mass spectrometer
TCEA	thermal conversion / elemental analyzer
LC-MS	liquid chromatography mass spectrometry
API	active pharmaceutical ingredient
NMR	nuclear magnetic resonance.

REFERENCES

- Linhardt RJ. Heparin: Structure and activity. *J Med Chem.* 2003; 46:2551–2554. [PubMed: 12801218]
- Linhardt RJ, Ampofo SA, Fareed J, Hoppensteadt D, Mulliken JB, Folkman J. Isolation and characterization of human heparin. *Biochemistry.* 1992; 31:12441–12445. [PubMed: 1463730]
- Loganathan D, Wang HM, Mallis LM, Linhardt RJ. Structural variation in the antithrombin III binding site region and its occurrence in heparin from different sources. *Biochemistry.* 1990; 29:4362–4368. [PubMed: 2350542]
- Luppi E, Cesaretti M, Volpi N. Purification and characterization of heparin from the Italian clam *Callista chione*. *Biomacromol.* 2005; 6:1672–1678.
- Linhardt RJ, Gunay NS. Production and chemical processing of low molecular weight heparins. *Sem Thromb Hemostas.* 1999; 25:5–16.
- Concannon SP, Wimberley PB, Workman WE. A quantitative PCR method to quantify ruminant DNA in porcine crude heparin. *Anal Bioanal Chem.* 2011; 399:757–762. [PubMed: 21058016]
- Liu H, Zhang Z, Linhardt RJ. Lessons learned from the contamination of heparin. *Nat Prod Rep.* 2009; 26:313–321. [PubMed: 19240943]
- Wang L, Byrum B, Zhang Y. New variant of porcine epidemic diarrhea virus, United States. *Emerg Infect Dis.* 2014; 20:917–919. [PubMed: 24750580]
- Junqueira DR, Viana TG, Peixoto ER, Barros FC, Carvalho MD, Perini E. Heparin pharmacovigilance in Brazil. *Rev Assoc Med Bras.* 2011; 57:322–326. [PubMed: 21691698]
- Gomes WJ, Braile DM. The troubled heparin issue in the Brazilian market and the search for solutions. *Rev Bras Cir Cardiovasc.* 2009; 24:3–4. [PubMed: 19768285]
- Fu L, Li G, Yang B, Onishi A, Li L, Sun P, Zhang F, Linhardt RJ. Structural characterization of pharmaceutical heparins prepared from different animal tissues. *J Pharm Sci.* 2013; 102:1447–1457. [PubMed: 23526651]
- Tovar AM, Capillé NV, Santos GR, Vairo BC, Oliveira SN, Fonseca RJ, Mourão PA. Heparin from bovine intestinal mucosa: glycans with multiple sulfation patterns and anticoagulant effects. *Thromb Haemost.* 2012; 107:903–915. [PubMed: 22437650]
- Mascellani G, Liverani L, Bianchini P. Analysis of heparin origin by HPLC quantitation of disaccharide components. *Farmaco.* 1996; 51:247–254. [PubMed: 8645412]
- Casu B, Guerrini M, Naggi A, Torri G, De-Ambrosi L, Boveri G, Gonella S, Ronzoni G. Differentiation of beef and pig mucosal heparins by NMR spectroscopy. *Thromb Haemost.* 1995; 74:1205. [PubMed: 8560442]
- Tovar AM, Teixeira LA, Rembold SM, Leite M Jr, Lugon JR, Mourão PA. Bovine and porcine heparins: different drugs with similar effects on human haemodialysis. *BMC Res Notes.* 2013; 6:230. [PubMed: 23763719]
- Gotti R, Parma B, Spelta F, Liverani L. Affinity capillary electrophoresis in binding study of antithrombin to heparin from different sources. *Talanta.* 2013; 105:366–371. [PubMed: 23598032]
- Aquino RS, Pereira MS, Vairo BC, Cinelli LP, Santos GR, Fonseca RJ, Mourão PA. Heparins from porcine and bovine intestinal mucosa: Are they similar drugs? *Thromb Haemost.* 2010; 103:1005–1015. [PubMed: 20216993]

18. Warkentin TE. Pork or beef? *Ann Thorac Surg.* 2003; 75:15–16. [PubMed: 12537185]
19. Schmitt BP, Adelman B. Heparin-associated thrombocytopenia: a critical review and pooled analysis. *Am J Med Sci.* 1993; 305:208–215. [PubMed: 8475945]
20. Boldt J, Zickmann B, Ballesteros M, Scholz D, Dapper F, Hempelmann G. Does the preparation of heparin influence anticoagulation during cardiopulmonary bypass? *J Cardiothorac Vasc Anesth.* 1991; 5:449–453. [PubMed: 1932649]
21. Bailey RT Jr, Ursick JA, Heim KL, Hilleman DE, Reich JW. Heparin-associated thrombocytopenia: a prospective comparison of bovine lung heparin, manufactured by a new process, and porcine intestinal heparin. *Drug Intell Clin Pharm.* 1986; 20:374–378. [PubMed: 3519142]
22. Silverglade A. Biological equivalence of beef lung and hog musosal heparins. *Curr Ther Res Clin Exp.* 1975; 18:91–103. [PubMed: 808395]
23. Harada A, Tatsuno K, Kikuchi T, Takahashi Y, Sai S, Murakami Y, Takada K. Use of bovine lung heparin to obviate anaphylactic shock caused by porcine gut heparin. *Ann Thorac Surg.* 1990; 49:826–827. [PubMed: 2339944]
24. Rivera V, Levieux A, Levieux D. Immunochemical characterisation of species-specific antigens in bovine crude heparin. *J Pharm Biomed Anal.* 2002; 29:431–441. [PubMed: 12062644]
25. Huang Q, Xu T, Wang GY, Huang JF, Xia H, Yin R, Tang A, Fu WL. Species-specific identification of ruminant components contaminating industrial crude porcine heparin using real-time fluorescent qualitative and quantitative PCR. *Anal Bioanal Chem.* 2012; 402:1625–1634. [PubMed: 22147273]
26. Houiste C, Auguste C, Macrez C, Dereux S, Derouet A, Anger P. Quantitative PCR and disaccharide profiling to characterize the animal origin of low-molecular-weight heparins. *Clin Appl Thromb Hemost.* 2009; 15:50–58. [PubMed: 18805847]
27. Huang Q, Yao CY, Chen B, Wang F, Huang JF, Zhang X, Fu WL. Species-specific identification by inhibitor-controlled PCR of ruminant components contaminating industrial crude porcine heparin. *Mol Cell Probes.* 2006; 20:250–258. [PubMed: 16563696]
28. Ruiz-Calero V, Saurina J, Galceran MT, Hernández-Cassou S, Puignou L. Estimation of the composition of heparin mixtures from various origins using proton nuclear magnetic resonance and multivariate calibration methods. *Anal Bioanal Chem.* 2002; 373:259–265. [PubMed: 12110977]
29. Levieux A, Rivera V, Levieux D. Immunochemical control of the species origin of porcine crude heparin and detection of ovine and caprine materials. *J Pharm Biomed Anal.* 2002; 27:305–313. [PubMed: 11682238]
30. Satsangi J, Jewell DP, Welsh K, Bunce M, Bell JI. Effect of heparin on polymerase chain reaction. *Lancet.* 1994; 343:1509–1510. [PubMed: 7911214]
31. Carter J, Meirer-Augenstein W. Stable isotope analysis of drugs. *Wiley Encyclopedia of Forensic Science.* 2012
32. Smith BN, Epstein S. Two categories of $^{13}\text{C}/^{12}\text{C}$ ratios for higher plants. *Plant Physiol.* 1971; 47:38–384. [PubMed: 16657576]
33. Fogel, ML. *Encyclopedia of Mass Spectrometry.* Vol. 5. UK: Elsevier, Oxford; 2010. Variations in abundances of nitrogen isotopes in nature.
34. Jasper JP. Pharmaceutical security: Using stable isotopes to authenticate pharmaceutical materials. *Tablets and Capsules.* 2004; 2:37–42.
35. Jasper JP, Weaner LE, Hayes JM. Process Patent Protection: Characterizing synthetic pathways by stable-isotopic measurements. *Pharm Technol.* 2007; 31:68–73.
36. Jasper JP. The increasing use of stable isotopes in the pharmaceutical industry. *Pharm Technol.* 1999; 23:106–114.
37. Jasper JP, Pavane M, Eyler D, Sharma I, Lee A. Process Patent Protection: Protecting intellectual property via natural-abundance stable isotopes. *Am Intel Prpty Law Assoc webinar.* 2012 Nov 13. 2012.
38. Jasper JP, Lyon RC, Weaner LE. Stable isotopes provide a new PAT [Process Analytical Technology] tool. *Pharm. Mfg.* 2005; 4:28–33.

39. Zhang F, Yang B, Ly M, Solakyildirim K, Xiao Z, Wang Z, Chang Y, Dordick JS, Linhardt RJ. Chemical structure characterization of commercial heparin from different commercial sources, *Anal. Bioanal. Chem.* 2011; 401:2793–2803.
40. Jasper JP. Quantitative estimates of precision for molecular isotopic measurements. *Rap. Comm. Mass Spec.* 2001; 15:1554–1557.
41. Habfast, K. Advanced Isotope Ratio Mass Spectrometry I: Magnetic Isotope Ratio Mass Spectrometers. In: Platzner, IT., editor. *Modern Isotope Ratio Mass Spectrometry*. New York: J. Wiley and Sons, Ltd; 1997. p. 11-82.
42. Jin R, Chen W, Sudjianto A. An efficient algorithm for constructing optimal design of computer experiments. *J Stat Plan Infer.* 2005; 134:268–287.
43. Cerling TE, Harris JM. Carbon isotope fractionation between diet and bioapatite in ungulate mammals and implications for ecological and paleoecological studies. *Oecologia.* 1999; 120:347–363.
44. Evershed RP, Dudd SN, Copley MS, Mutherjee A. Identification of animal fats via compound specific $\delta^{13}\text{C}$ values of individual fatty acids: assessments of results for reference fats and lipid extracts of archaeological pottery vessels. *Documenta Praehistorica.* 2002; 29:73–96.
45. Kuitens M, van Kolfshoten T, van der Plicht J. Elevated $\delta^{15}\text{N}$ values in mammoths: a comparison with modern elephants. *Archaeol Anthropol Sci.* 2012
46. Harris CI, Fuller MF, Torrallardona D. Pigs' Gastrointestinal microflora provide them with essential amino acids. *J Nutr.* 2003; 133:1127–1131. [PubMed: 12672930]
47. Bhaskar U, Sterner E, Hickey AM, Onishi A, Zhang F, Dordick JS, Linhardt RJ. Engineering of routes to heparin and related polysaccharides. *Appl Microbiol Biotechnol.* 2012; 93:1–16. [PubMed: 22048616]

a + d ~ 20, b = 0 or 1, c ~ 8 for MW_W average 20,000, X = SO₃⁻, H; Y = SO₃⁻, COCH₃, H

Figure 1.

Typical structure of heparins (*viz.*, relative elemental composition of C₁₂H₁₅NO₁₉S₃Na₄; MW: 10–15 kDa). The structural variability of their antithrombin III (AT) binding sites has been previously determined.¹¹

Figure 2. Principle component analysis of the stable-isotopic composition of heparin samples: biplot of scores and loadings.

Figure 3.

An isotopic bivariate ($\delta^{13}\text{C}$ - $\delta^{18}\text{O}$) plot of the three (ovine, bovine, porcine) heparin samples. Most important graph for source differentiation: separates USA-porcine heparin from non-USA heparin.

Figure 4.

An isotopic bivariate (δD - $\delta^{18}O$) plot of the three (ovine, bovine, porcine) heparin samples. Maximum resolving power for the three types of heparin.

Figure 5.

An isotopic bivariate ($\delta^{15}\text{N}$ - $\delta^{18}\text{O}$) plot of the three (ovine, bovine, porcine) heparin samples.

Figure 6.

An isotopic bivariate ($\delta^{34}\text{S}$ - $\delta^{18}\text{O}$) plot of the three (ovine, bovine, porcine) heparin samples.

Table 1

Stable-Isotopic Compositions of Heparin Samples

Sample #	Name	Source	$\delta^{13}\text{C}$ ‰	PSE $\delta^{13}\text{C}$ ‰	$\delta^{15}\text{N}$ ‰	PSE $\delta^{15}\text{N}$ ‰	$\delta^{18}\text{O}$ ‰	PSE $\delta^{18}\text{O}$ ‰	δD ‰	PSE δD ‰	$\delta^{34}\text{S}$ ‰	PSE $\delta^{34}\text{S}$ ‰
1	Porcine heparin	USA	-14.19	0.03	-2.69	0.03	4.18	0.05	31.8	0.6	-5.28	0.02
1	Porcine heparin	USA	-14.15	0.03	-2.80	0.03	4.30	0.05	33.8	0.6	-5.35	0.02
1	Porcine heparin	USA	-14.26	0.03	-2.75	0.03	4.14	0.05	33.6	0.6	-5.34	0.02
2	Bovine heparin	Unknown	-21.08	0.06	-2.49	0.06	0.90	0.13	16.3	1.1	-3.57	0.04
3	Ovine heparin	Unknown	-28.27	0.06	-0.43	0.06	5.50	0.13	80.8	1.1	6.30	0.04
4	Porcine heparin	China	-16.55	0.06	-2.68	0.06	3.82	0.13	22.7	1.1	-2.24	0.04
5	Porcine heparin	Spain	-21.55	0.06	-2.37	0.06	4.37	0.13	23.7	1.1	-3.60	0.04