Stable Isotopic Analysis of Porcine, Bovine, and Ovine Heparins

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ABSTRACT: The assessment of provenance of heparin is becoming a major concern for the pharmaceutical industry and its regulatory bodies. Batch-specific [carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$), oxygen ($\delta^{18}O$), sulfur ($\delta^{34}S$), 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 $\delta^{13}C$ and $\delta^{18}O$ 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 (1) stable isotope measurements on these highly sulfated polysaccharide (molecular weight ~15 kDa) natural products ("biologics") were feasible; (2) in bivariate plots, the $\delta^{13}C$ versus $\delta^{18}O$ plot reveals a well-defined relationship for source differentiation of hogs raised in the United States from hogs raised in Europe and China; (3) the δD versus $\delta^{18}O$ plot revealed the most well-defined relationship for source differentiation based on the hydrologic environmental isotopes of water (D/H and $^{18}O/^{16}O$); and (4) the $\delta^{15}N$ versus $\delta^{18}O$ and $\delta^{34}S$ versus $\delta^{18}O$ relationships are both very similar, possibly reflecting the food sources used by the different heparin producers. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:457–463, 2015
Keywords: heparin; stable isotopic analysis; animal sourced; principle component analysis; mass spectrometry; analysis

INTRODUCTION

Heparin is a linear sulfated polysaccharide consisting of repeating 1→4-glycosidically linked hexuronic acid and glucosamine residues (Fig. 1).[1] Heparin is biosynthesized in the Golgi of mast cells of humans,[2] other vertebrates,[3] and invertebrates.[4] Pharmaceutical 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 multibillion 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 2007–2008 during a contamination crisis,[7] and has been adversely impacted by the recent appearance of the porcine epidemic diarrhea virus.[8]

Although 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 pentasaccharide binding sites (Fig. 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 PCR 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, as the heparin-mediated inhibition of PCR was previously described.[30] One-dimensional and two-dimensional nuclear magnetic resonance (NMR) spectroscopy of porcine heparin API can detect the presence of 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 analysis[31] to examine the provenance of heparin products. Stable isotope studies on heparin API require little sample preparation or pretreatment

Abbreviations used: EA/IRMS, elemental analyzer/isotope ratio mass spectrometer; TCEA, thermal conversion/elemental analyzer; API, active pharmaceutical ingredient; NMR, nuclear magnetic resonance.

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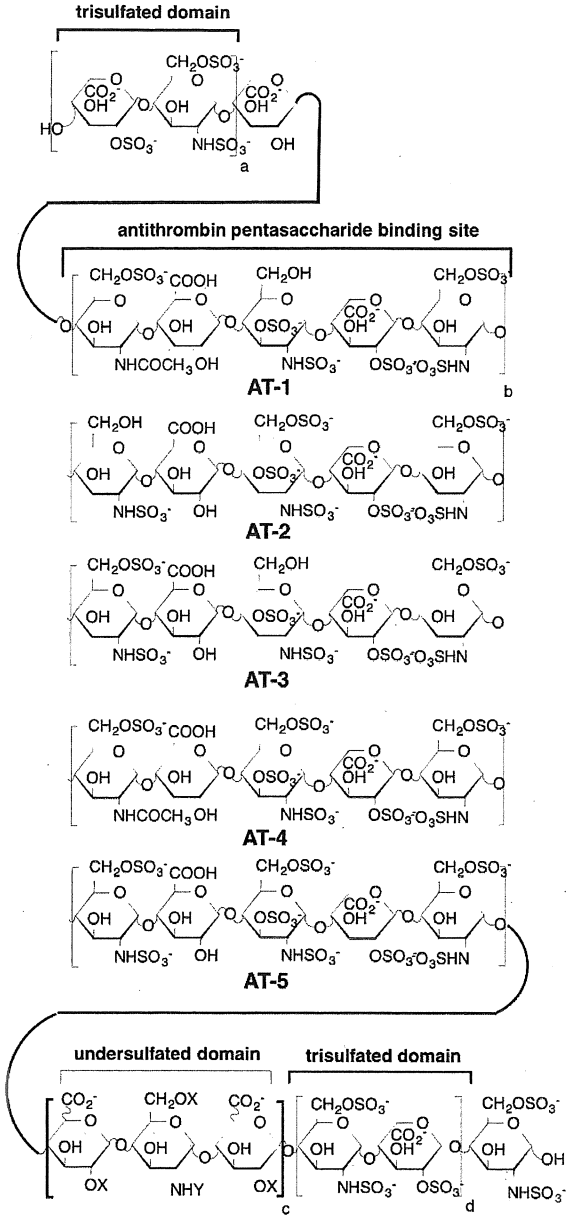

AT binding site variant (mole %)					
	AT-1	AT-2	AT-3	AT-4	AT-5
Porcine intestinal	14	7	4	62	7
Bovine lung	9	6	2	71	12
Ovine intestinal	4	5	4	75	11

a + d ~ 20, b = 0 or 1, c ~ 8 for MW_W average 20,000
X = SO_3^-, H; Y= SO_3^-, $COCH_3$, H

Figure 1. Typical structure of heparins (viz., relative elemental composition of $C_{12}H_{15}NO_{19}S_3Na_4$; molecular weight: 10–15 kDa). The structural variability of their antithrombin III (AT) binding sites has been previously determined.[11]

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: for example, C3-terrigenous versus C3-algal sources, where C3 plants fractionate to a greater degree (using Rubisco) than do C4 plants (using ribulose-1,5-bisphosphate (RUBP) carboxylase). In addition, the stable isotopic composition of nitrogen ($\delta^{15}N$) 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 ($\delta^{34}S$) has been demonstrated to have very high dynamic ranges (observed ranges/1σ SD > 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 biopharmaceutical/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 United States (Celsus Laboratories, Cincinnati, Ohio, 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 Corporation (St. Louis, Missouri). 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

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Table 1. Stable Isotopic Compositions of Heparin Samples

Sample Number/Name	Source	$\delta^{13}C$ (‰)	PSE ^{13}C (‰)	$\delta^{15}N$ (‰)	PSE ^{15}N (‰)	$\delta^{18}O$ (‰)	PSE ^{18}O (‰)	δD (‰)	PSE D (‰)	$\delta^{34}S$ (‰)	PSE ^{34}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

batches × 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 analyses 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). $\delta^{13}C$ values are reported relative to the international Vienna Peedee Belemnite (VPDB) standard. $\delta^{15}N$ values are reported relative to the international air standard.

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

For $\delta^{34}S$ analysis, individual solid samples containing 150–300 μg of sulfur within the sample along with approximately 10 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 $\delta^{34}S$ values are reported in percentage relative to the international standard Vienna Canyon Diablo Troilite (VCDT) iron meteorite.

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

Individual samples of ~0.2 mg were weighed and placed into silver boats, which were then crimped tightly around the analyte. Triplicate oxygen ($\delta^{18}O$) stable isotopic analyses of one 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 (δD) 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 1 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 × 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 (TCEA). 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 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:

$$\delta^{13}C(‰) = ([(R_{smpl})/(R_{std})] - 1) \times (1000)$$

where R_{sample} = the $^{13}C/^{12}C$ ratio of the sample material and R_{std} = the $^{13}C/^{12}C$ ratio of an IAEA standard (known as "VPDB" whose $^{13}C/^{12}C$ ratio has been defined as the official zero point of the carbon isotopic scale). $^{15}N/^{14}N$ values are given relative to the international air standard. $^{34}S/^{32}S$ values are given relative to the IAEA VCDT standard. $^{18}O/^{16}O$ and D/H values are given relative to the IAEA Vienna Standard Mean Ocean Water standard.

Estimates of Uncertainty

The uncertainty (or precision) of the isotopic measurements in this study was estimated from the pooled SD of recent studies. In those cases, the pooled SD of raw data were estimated to derive a representative SD from the whole raw data set in

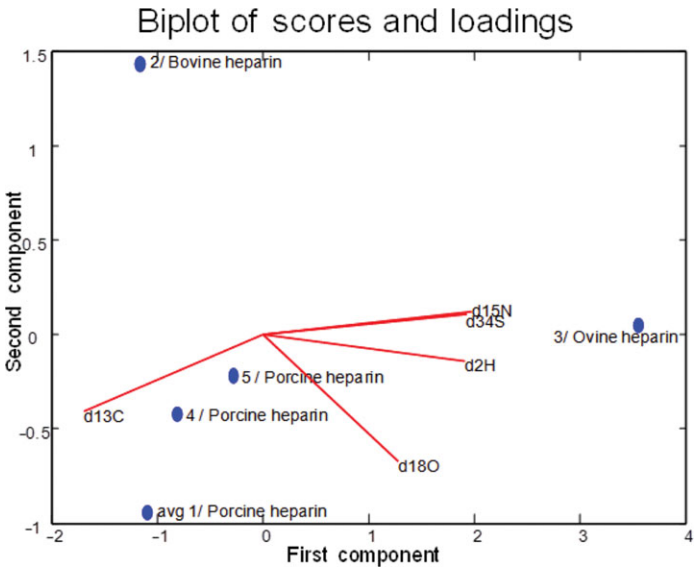

Figure 2. Principle component analysis of the stable isotopic composition of heparin samples: biplot of scores and loadings.

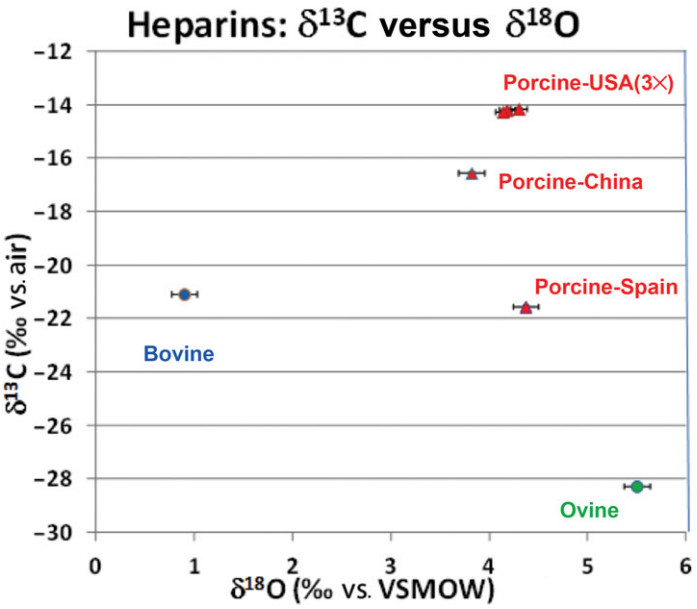

Figure 3. An isotopic bivariate ($\delta^{13}C$–$\delta^{18}O$) plot of the three (ovine, bovine, porcine) heparin samples. Most important graph for source differentiation: separates USA-porcine heparin from non-USA heparin.

which small numbers of replicates (viz., $n = 1$–3) were pooled to generate a representative SD of the whole sample suite.[38] Characteristic one sigma (1σ) SD for the isotope values used in this study were $\delta^{13}C$ (±0.06%), $\delta^{15}N$ (±0.06%), $\delta^{18}O$ (±0.08%), δD (±1.1%), and $\delta^{34}S$ (±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 ($\delta^{13}C$, $\delta^{18}O$, $\delta^{15}N$, $\delta^{34}S$, and δD) of the five heparin samples given are presented in Table 1 and Figures 3–6.

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

PCA 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 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 were then autoscaled by mean centering followed by dividing by the SD. 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 Figure 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 $\delta^{15}N$, $\delta^{34}S$, and δD and that these three isotopes are strongly, inversely correlated with $\delta^{13}C$. The isotope, $\delta^{18}O$, 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 (1) that the porcine samples are relatively high in $\delta^{13}C$, (2) that bovine heparin is relatively low in $\delta^{18}O$, and (3) that ovine heparin is relatively high in $\delta^{15}N$, $\delta^{34}S$, and δD. Shannon entropy criterion[42] is related to the amount of information and confirms that a bivariate plot of $\delta^{13}C$ and $\delta^{18}O$ is the best single, bivariate plot, which results in the minimum of –log|R|, where |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 from the heparin samples are given in Figures 3–6 to show the interrelationship of the isotopic results and their groupings.

Although there are 11 such plots, PCA shows that the four paired δX–$\delta^{18}O$ values, shown in Figures 3–6, are probably the most useful (where δX = $\delta^{13}C$, δD, $\delta^{15}N$, $\delta^{34}S$). The seven other optional plots complete the set of 11 (see Supplementary Figs. S1–S7).

Four bivariate graphs of the paired δX–$\delta^{18}O$ 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 $\delta^{15}N$ to 308 for $\delta^{34}S$ where dynamic range = observed range/1σ SD), 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 Table S1).

As indicated by the PCA, the $\delta^{13}C$–$\delta^{18}O$ graph (Fig. 3) shows a significant differentiation of the three types of heparin (porcine, ovine, and bovine) as well as a distinct differentiation of the three sources of porcine heparin (USA, China, and

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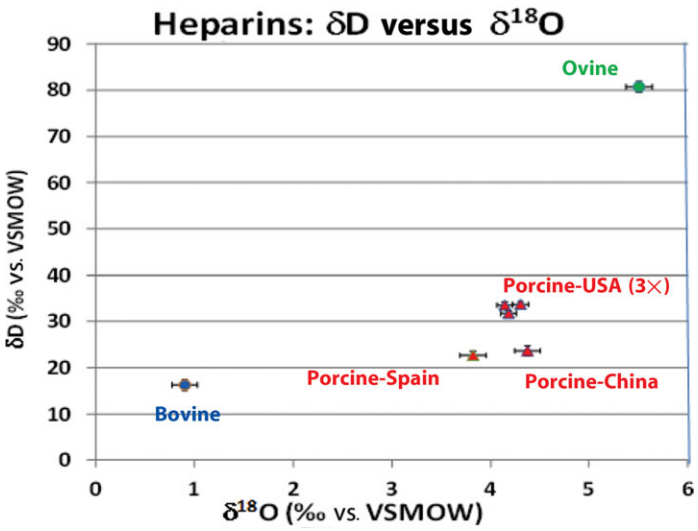

Figure 4. An isotopic bivariate (δD–$\delta^{18}O$) plot of the three (ovine, bovine, porcine) heparin samples. Maximum resolving power for the three types of heparin.

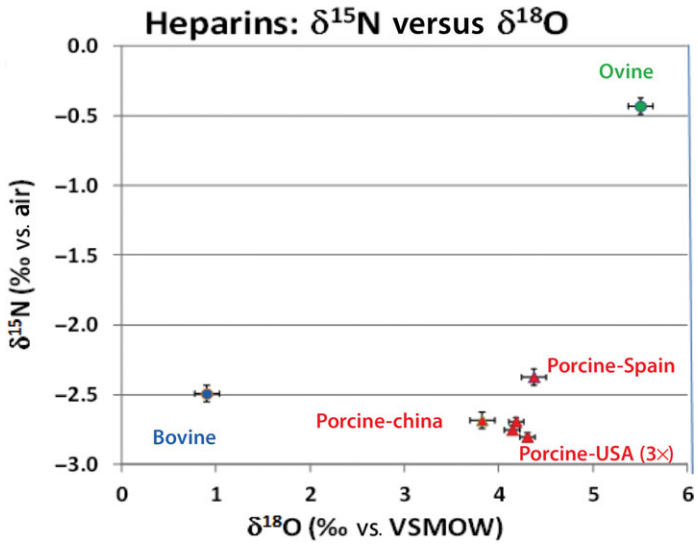

Figure 5. An isotopic bivariate ($\delta^{15}N$–$\delta^{18}O$) plot of the three (ovine, bovine, porcine) heparin samples.

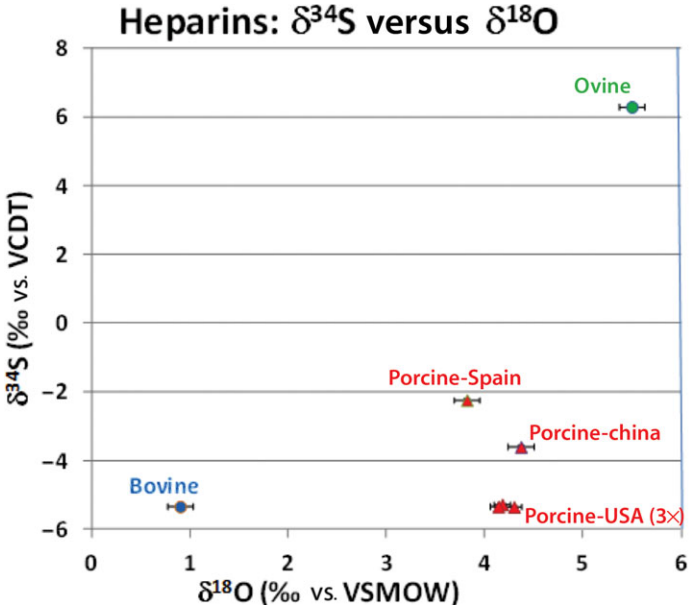

Figure 6. An isotopic bivariate ($\delta^{34}S$–$\delta^{18}O$) plot of the three (ovine, bovine, porcine) heparin samples.

Spain). Furthermore, PCA indicates that the δD–$\delta^{18}O$ graph (Fig. 4) differentiates the three types of heparin with maximum resolving power. Finally, PCA analysis indicates a close similarity of $\delta^{15}N$ and $\delta^{34}S$. This similarity is manifested in the $\delta^{15}N$–$\delta^{18}O$ and $\delta^{34}S$–$\delta^{18}O$ graphs (Figs. 5 and 6, respectively), which also strongly resolve the three types of heparin (also see Supplementary 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 cellulosics 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 nonruminants[43] 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 ratios[45] 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 with 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 $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, $\delta^{18}O$, and 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 (1) stable isotopic measurements of biologic molecules, such as heparin (molecular weight ~10–15 kDa), were feasible; (2) in bivariate plots, the $\delta^{13}C$ versus $\delta^{18}O$ plot reveals a well-defined relationship for source differentiation, separating

USA-porcine heparin from non-USA heparin; (3) the δD versus $\delta^{18}O$ plot reveals the most well-defined relationship for source differentiation based on the hydrologic environmental isotopes of water (D/H and $^{18}O/^{16}O$); and (4) the $\delta^{15}N$ versus $\delta^{18}O$ and $\delta^{34}S$ versus $\delta^{18}O$ 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. Although these heparins are not natural products, their manufacturing provenance might also be monitored using stable isotope methods.[35,37,38] Such monitoring might also be useful in preventing the introduction of counterfeit synthetic heparins onto the world market.

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