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 (δ13C), nitrogen (δ15N), oxygen (δ18O), sulfur (δ34S), 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 δ13C and δ18O 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 δ13C versus δ18O 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 δ18O plot revealed the most well-defined relationship for source differentiation based on the hydrologic environmental isotopes of water (D/H and 18O/16O); and (4) the δ15N versus δ18O and δ34S versus δ18O 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). Heparin is biosynthesized in the Golgi of mast cells of humans, other vertebrates, and invertebrates. 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, and potentially carry different impurities (i.e., polysaccharides, peptides, virus, prions, etc.). 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. 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. 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. 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%). Multivariate analysis of NMR data allows for the detection of smaller levels of bovine heparin API blended into porcine heparin API. The current study examines a novel method relying on natural-abundance stable isotope analysis 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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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 as well as the "manufacturer or batch" origin of the products and materials manufactured from them. Stable carbon isotopes have been particularly useful in differentiating large classes of plant organic matter based on their photosynthetic pathways: for example, C3-terrestrial versus C3-algal sources, where C3 plants fractionate to a greater degree (using Rubisco) than do C4 plants (using ribulose-1,5-bisphosphate carboxylase). In addition, the stable isotopic composition of nitrogen (δ¹⁵N) is highly variable in nature through a number of pathways for inorganic as well as organic species. Furthermore, the stable isotopic composition of sulfur (δ³⁴S) has been demonstrated to have very high dynamic ranges (observed ranges/10 SD > 200) in pharmaceutical studies and typically serves as an excellent tracer of isotopic provenance.

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. 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 through identifying isotopic differences.

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/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 (Celsius Laboratories, Cincinnati, Ohio, 202 U/mg), China (Dongying Pharmaceutical Inc., Shangdong, China, 200 U/mg), and Spain (Biobureau 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 C₁₂H₁₅NO₉S₅Na₄ with molecular weights of ~15 kDa. Detailed chemical structural and biological analysis were performed on all the five heparin APIs. 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 (δ¹³C, δ¹⁵N, δ³⁴S, δ¹⁸O, and δD) were measured on each of the five heparin API samples with single-crate analysis for heparins #2–5 and in one triplicate analysis for heparin #1 to assess analytical precision. Thus, a total of 35 stable isotopic measurements of these heparin (i.e., 5

Figure 1. Typical structure of heparins (viz., relative elemental composition) of C₁₂H₁₅NO₉S₅Na₄: molecular weight: 10–15 kDa). The structural variability of their antithrombin III (AT) binding sites has been previously determined.
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 δ13C and ~3.7 mg for δ15N analysis were weighed and placed into tin boats that were crimped tightly around the analyte. Carbon (δ13C) and nitrogen (δ15N) 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). δ13C values are reported relative to the international Vienna PeeDee Belemnite (VPDB) standard. δ15N values are reported relative to the international air standard.

**Sulfur Isotope (δ34S) Analyses**

For δ34S analysis, individual solid samples containing 150–300 µg of sulfur within the sample along with approximately 10 times the total sample weight of WO3 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 δ34S values are reported in percentage relative to the international standard Vienna Canyon Diablo Troilite (VCDT) iron meteorite.

**Oxygen Isotope (δ18O) Analyses**

Individual samples of ~0.2 mg were weighed and placed into silver boats, which were then crimped tightly around the analyte. Triplicate oxygen (δ18O) 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 H2O and CO2 as in the EAMS.

The analyte gas, CO, is chromatographically separated on a packed column at 85°C. The mass spectrometer measures 18O 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., H2O). 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 (%ε = parts per thousand differences from international standards) defined as:

\[
\delta^{13}C(\% \epsilon) = \left(\frac{R_{\text{sampl}}}{R_{\text{std}}} \right) - 1 \times (1000)
\]

where \(R_{\text{sampl}}\) = the 13C/12C ratio of the sample material and \(R_{\text{std}}\) = the 13C/12C ratio of an IAEA standard (known as “VPDB” whose 13C/12C ratio has been defined as the official zero point of the carbon isotopic scale). 15N/14N values are given relative to the international air standard. 34S/32S values are given relative to the IAEA VCDT standard. 18O/16O 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

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**Table 1. Stable Isotopic Compositions of Heparin Samples**

<table>
<thead>
<tr>
<th>Sample Number/Name</th>
<th>Source</th>
<th>δ34S (%)</th>
<th>PSE δ34S (%)</th>
<th>δ34S (%)</th>
<th>PSE δ34S (%)</th>
<th>δ34S (%)</th>
<th>PSE δ34S (%)</th>
<th>δ34S (%)</th>
<th>PSE δ34S (%)</th>
<th>δ34S (%)</th>
<th>PSE δ34S (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/Porcine heparin</td>
<td>USA</td>
<td>-14.19</td>
<td>0.03</td>
<td>-2.69</td>
<td>0.03</td>
<td>4.18</td>
<td>0.05</td>
<td>31.8</td>
<td>0.6</td>
<td>-5.28</td>
<td>0.02</td>
</tr>
<tr>
<td>1/Porcine heparin</td>
<td>USA</td>
<td>-14.15</td>
<td>0.03</td>
<td>-2.80</td>
<td>0.03</td>
<td>4.30</td>
<td>0.05</td>
<td>33.8</td>
<td>0.6</td>
<td>-5.35</td>
<td>0.02</td>
</tr>
<tr>
<td>1/Porcine heparin</td>
<td>USA</td>
<td>-14.26</td>
<td>0.03</td>
<td>-2.75</td>
<td>0.03</td>
<td>4.14</td>
<td>0.05</td>
<td>33.6</td>
<td>0.6</td>
<td>-5.34</td>
<td>0.02</td>
</tr>
<tr>
<td>2/Bovine heparin</td>
<td>Unknown</td>
<td>-21.08</td>
<td>0.06</td>
<td>-2.49</td>
<td>0.06</td>
<td>0.90</td>
<td>0.13</td>
<td>16.3</td>
<td>1.1</td>
<td>-3.57</td>
<td>0.04</td>
</tr>
<tr>
<td>3/Ovine heparin</td>
<td>Unknown</td>
<td>-28.27</td>
<td>0.06</td>
<td>-0.43</td>
<td>0.06</td>
<td>5.50</td>
<td>0.13</td>
<td>80.8</td>
<td>1.1</td>
<td>6.30</td>
<td>0.04</td>
</tr>
<tr>
<td>4/Porcine heparin</td>
<td>China</td>
<td>-16.55</td>
<td>0.06</td>
<td>-2.68</td>
<td>0.06</td>
<td>3.82</td>
<td>0.13</td>
<td>22.7</td>
<td>1.1</td>
<td>-2.24</td>
<td>0.04</td>
</tr>
<tr>
<td>5/Porcine heparin</td>
<td>Spain</td>
<td>-21.55</td>
<td>0.06</td>
<td>-2.37</td>
<td>0.06</td>
<td>4.37</td>
<td>0.13</td>
<td>23.7</td>
<td>1.1</td>
<td>-3.60</td>
<td>0.04</td>
</tr>
</tbody>
</table>
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 (1\( \sigma \)) SD for the isotope values used in this study were \( \delta^{13}\text{C} (\pm 0.06\%\text{)}, \delta^{15}\text{O} (\pm 0.06\%\text{)}, \delta^{18}\text{O} (\pm 0.08\%\text{)},\) \( \delta\text{D} (\pm 1.1\%\text{)},\) and \( \delta^{34}\text{S} (\pm 0.04\%\text{)},\) as shown in Table 1.

### RESULTS AND DISCUSSION

#### The \( ^{13}\text{C}, ^{18}\text{O}, ^{15}\text{N}, ^{34}\text{S}, \) and \( \text{D} \) 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 \( \delta\text{D} \)) of the five heparin samples given are presented in Table 1 and Figures 3–6.

In the following sections, the \( \delta^{13}\text{C}, \delta^{18}\text{O}, \delta^{15}\text{N}, \delta^{34}\text{S}, \) and \( \delta\text{D} \) 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 \( \delta^{15}\text{N}, \delta^{34}\text{S}, \) and \( \delta\text{D} \) 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 (1) that the porcine samples are relatively high in \( \delta^{13}\text{C}, \) (2) that bovine heparin is relatively low in \( \delta^{18}\text{O}, \) and (3) that ovine heparin is relatively high in \( \delta^{15}\text{N}, \delta^{34}\text{S}, \) and \( \delta\text{D}. \)

Shannon entropy criterion \(^{42} \) 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 \( -\log|R|, \) 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 \( \delta\text{X}–\delta^{18}\text{O} \) values, shown in Figures 3–6, are probably the most useful (where \( \delta\text{X} = \delta^{13}\text{C}, \delta\text{D}, \delta^{15}\text{N}, \delta^{34}\text{S}. \) The seven other optional plots complete the set of 11 (see Supplementary 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/1 SD), and therefore a high specificity (1.1 \( \times \) \( 10^{10} \) = 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 \( \delta^{13}\text{C}–\delta^{18}\text{O} \) 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 China).
Figure 4. An isotopic bivariate (δD–δ18O) plot of the three (ovine, bovine, porcine) heparin samples. Maximum resolving power for the three types of heparin.

Figure 5. An isotopic bivariate (δ15N–δ18O) plot of the three (ovine, bovine, porcine) heparin samples.

Spain). Furthermore, PCA indicates that the δD–δ18O graph (Fig. 4) differentiates the three types of heparin with maximum resolving power. Finally, PCA analysis indicates a close similarity of δ15N and δ34S. This similarity is manifested in the δ15N–δ18O and δ34S–δ18O 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 as well as isotope incorporation differences between different ruminants, including ovine and bovine species. The diet of animals can also impact C and N isotope ratios as can differences in their gut microbiome. 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 microorganisms, or different factory processing of these heparins. 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 δ13C, δ15N, δ34S, δ18O, 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 δ13C versus δ18O plot reveals a well-defined relationship for source differentiation, separating
USA-porcine heparin from non-USA heparin; (3) the δD versus δ18O plot reveals the most well-defined relationship for source differentiation based on the hydrologic environmental isotopes of water (D/H and 18O/16O); and (4) the δ15N versus δ18O and δD versus δ18O 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) synthesized. Although these heparins are not natural products, their manufacturing provenance might also be monitored using stable isotope methods. Such monitoring might also be useful in preventing the introduction of counterfeit synthetic heparins onto the world market.

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