Characterization of currently marketed heparin products: Analysis of heparin digests by mass spectrometry.

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Disclaimer

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CDER Scientific Research

“What will really improve drug safety is for us to improve the science of safety, and we are working on that. We are working on better ways to detect safety signals, and we’re working on the mechanistic side to figure out what causes drug safety problems and how they could be prevented.”

Dr. Janet Woodcock
Director of the Center for Drug Evaluation and Research
from an interview with Life Science Leader

Knowledge gained from CDER science and research increases the certainty and consistency of regulatory decisions, and contributes to the development of regulatory guidance documents and best practice standards for pharmaceutical companies.
Heparin

Epimerization and Sulfation activity in ER and Golgi

\( I_{2S}-(1,4)-A_{NS,6S} \)

\(~70\%\) of heparin

\(~20\%\) of heparin

\(~15\%\) of heparin

\( G_{2OH}-(1,4)-A_{NAC} \)

Heparin starts out as

\( M_w \sim 17kDa \)
Low Molecular Weight Heparin (LMWH)

- **Dalteparin** is produced through controlled nitrous acid depolymerization of UFH heparin
- **Tinzaparin** is obtained by controlled enzymatic depolymerization of UFH heparin using heparinase from *Flavobacterium heparinum*
- **Enoxaparin** is obtained by alkaline depolymerization of UFH heparin benzyl ester
Dalteparin: Nitrous Acid Deaminative Cleavage

2,5-anhydromannitol (AM.0l6S)
Tinzaparin (Enoxaparin) Ends

**Tinzaparin:** (beta-elimination by heparinase)

\[ \Delta U_{20H} \]

\[ \Delta U_{2S}: \]

\[ A_{NS-\alpha}\text{-reducing end} \]

\[ A_{NSred}: \]

\[ A_{NAc\text{red}}: \]
Enoxaparin Ends

\(\text{1,6-\text{anA}}\): 2-amino-1,6-anhydro-2-deoxy-\(\beta\)-D-glucopyranose

\(\text{1,6-\text{anM}}\): 2-amino-1,6-anhydro-2-deoxy-\(\beta\)-D-mannopyranose

\(\text{GaLA}\): galacturonic acid

\(\text{Epo-I}\): Alkaline treatment of I2S

\(\text{MNa}\): 2-deoxy-2-sulfoamino-\(\beta\)-D-mannopyranose

Miscellaneous Odds and Ends:
Marketplace Heparin Samples

- 19 heparin sodium (a.k.a. unfractionated heparin, UFH) samples from 6 DMF holders of heparin sodium and 10 samples of LMWH (dalteparin, tinzaparin and enoxaparin).
- Plus the USP Heparin Sodium and Enoxaparin identification standards.
- These samples were collected in the summer of 2009 (after the “heparin crisis”).
A well characterized sample set

Intact heparin

• We were unable to resolve mixture of heparin sodium chains present in the drug on high resolution mass spectrometers.
  – 12 Tesla FT-MS system at Wash Univ.-NCRR
  – Orbitrap systems in our lab.
  – Issues were sulfate decomposition (loss of $SO_3$) and the multiple acidic groups leading to adducts with metal cations.

• Thus we used a “bottom up” approach by digesting heparin to the disaccharide level.
Reverse phase ion paring liquid chromatography-mass spectrometry (RPIP-LC-MS)
The heparin family of possible disaccharides:

N-sulfated glucosamine species

- I-S: SO$_3^-$, SO$_3^-$, SO$_3^-$
- II-S: SO$_3^-$, SO$_3^-$, H
- III-S: SO$_3^-$, H, SO$_3^-$
- IV-S: SO$_3^-$, H, H

Internal standard

I-P: COEt, SO$_3^-$, SO$_3^-$

N-Acetyl glucosamine species

- I-A: Ac, SO$_3^-$, SO$_3^-$
- II-A: Ac, SO$_3^-$, H
- III-A: Ac, H, SO$_3^-$
- IV-A: Ac, H, H

Amine glucosamine species

- I-H: H, SO$_3^-$, SO$_3^-$
- II-H: H, SO$_3^-$, H
- III-H: H, H, SO$_3^-$
- IV-H: H, H, H
IUHPLC of UFH disaccharides

**UFH digest products**

**Standard mixture of 12 possible disaccharides**

Chromatography: Agilent 1290 system using a Waters BEH C<sub>18</sub> column (2.1 x 100 mm). Buffer A: 30 mM HA in water at a pH of 5.3 adjusted with formic acid, Buffer B: 75% ACN, 25% water with 30 mM HA

II-S and III-S are isobaric and only differ in the position of a SO<sub>4</sub> group.
Enzymatic Digestion Optimization

A. Mainly I-S, no X-A

B. I-S+good yield of other species

C. No I-S; good for IV-A

D. I + III

E. II + III

F. I + II + III

Retention Time (min)
II-S in {+} vs. {-} modes

{+}  
[M+HA]⁺  519.1923  
[M+HA-SO₃]⁺  418.0716  

{-}  
[M-H-SO₃]⁻  496.0101  
[M-H]⁻  416.0530  

Response \{+\} vs. \{-\}

\[ y = 37951x + 1 \times 10^6 \]
\[ R^2 = 0.9769 \]

\[ y = 13295x + 19613 \]
\[ R^2 = 0.9997 \]

\{-\} Better dynamic range and greater linear working range
Response factors

• Disaccharide standard mixture solutions containing 12 disaccharides and an internal standard (IP) ranging from 1.1 to 50 µM in 5 steps were analyzed to obtain individual response factors.
  – EIC chromatograms for observed disaccharide masses were obtained, summed and divided by the EIC I-P response and plotted vs. pmol injected.

• In general, RF values are higher for species with more sulfate groups.
LMWH Digests

Enoxaparin or tinzaparin digests: look like UFH.

Dalteparin digest: Has other disaccharide components.
LMWH Composition

Dalteparin: Lots #1-4
Tinzaparin: Lots #5-7
Enoxaparin: Lots #8-10
### RPIP-LC-MS Heparin Metrics

<table>
<thead>
<tr>
<th>Type</th>
<th>I-S ±</th>
<th>II-S ±</th>
<th>III-S ±</th>
<th>IV-S ±</th>
<th>I-A ±</th>
<th>II-A ±</th>
<th>III-A ±</th>
<th>IV-A ±</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin sodium (n=20)</td>
<td>61 ± 3</td>
<td>14 ± 2</td>
<td>8 ± 0</td>
<td>5 ± 1</td>
<td>1 ± 0</td>
<td>4 ± 1</td>
<td>1 ± 0</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>Dalteparin (n=4)</td>
<td>70 ± 1</td>
<td>13 ± 1</td>
<td>5 ± 0</td>
<td>2 ± 0</td>
<td>2 ± 0</td>
<td>5 ± 0</td>
<td>1 ± 0</td>
<td>2 ± 0</td>
</tr>
<tr>
<td>Tinzaparin (n=3)</td>
<td>60 ± 1</td>
<td>12 ± 0</td>
<td>9 ± 0</td>
<td>6 ± 0</td>
<td>1 ± 0</td>
<td>5 ± 0</td>
<td>1 ± 0</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>Enoxaparin (n=4)</td>
<td>62 ± 1</td>
<td>13 ± 0</td>
<td>8 ± 0</td>
<td>5 ± 0</td>
<td>1 ± 0</td>
<td>4 ± 0</td>
<td>1 ± 0</td>
<td>5 ± 0</td>
</tr>
</tbody>
</table>

- Here we use the same MS instrument and method across a large sample set to establish a set of percent relative composition values.
- Use for specification or surveillance purposes.
Robust?

8-5-2011: After 44 samples
8-23-2011: After 35 samples
8-27-2011: Before 60 samples
8-27-2011: After 60 samples
9-8-2011: After 18 samples
Robust?

One month with a total of 157 injections.
### Manufacturer Signature?

<table>
<thead>
<tr>
<th>Samples</th>
<th>Disaccharides</th>
<th>I-S</th>
<th>II-S</th>
<th>III-S</th>
<th>IV-S</th>
<th>I-A</th>
<th>II-A</th>
<th>III-A</th>
<th>IV-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manufacturer #1 (lots #1-3)</td>
<td></td>
<td>61.0 ± 2.0</td>
<td>14.0 ± 0.4</td>
<td>7.9 ± 0.3</td>
<td>4.5 ± 0.6</td>
<td>1.1 ± 0.0</td>
<td>4.5 ± 0.5</td>
<td>0.8 ± 0.1</td>
<td>6.2 ± 0.8</td>
</tr>
<tr>
<td>Manufacturer #2 (lot #4)</td>
<td></td>
<td>61.7 ± 0.2</td>
<td>17.6 ± 0.1</td>
<td>7.9 ± 0.1</td>
<td>3.8 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td>3.6 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td>Manufacturer #3 (lots #5-7)</td>
<td></td>
<td>56.8 ± 1.2</td>
<td>13.9 ± 0.7</td>
<td>7.8 ± 0.3</td>
<td>5.3 ± 0.2</td>
<td>1.1 ± 0.0</td>
<td>5.3 ± 0.3</td>
<td>0.8 ± 0.1</td>
<td>9.0 ± 0.1</td>
</tr>
<tr>
<td>Manufacturer #3 (lots #8-10)</td>
<td></td>
<td>61.4 ± 1.4</td>
<td>13.8 ± 0.3</td>
<td>7.8 ± 0.3</td>
<td>4.8 ± 0.2</td>
<td>1.0 ± 0.0</td>
<td>3.9 ± 0.2</td>
<td>0.8 ± 0.0</td>
<td>6.4 ± 0.5</td>
</tr>
<tr>
<td>Manufacturer #4 (lots #11-13)</td>
<td></td>
<td>57.8 ± 1.9</td>
<td>16.0 ± 0.9</td>
<td>7.7 ± 0.4</td>
<td>4.8 ± 0.3</td>
<td>1.1 ± 0.1</td>
<td>5.3 ± 0.6</td>
<td>0.7 ± 0.1</td>
<td>6.5 ± 1.0</td>
</tr>
<tr>
<td>Manufacturer #5 (lots #14-16)</td>
<td></td>
<td>61.2 ± 1.0</td>
<td>11.6 ± 0.2</td>
<td>8.4 ± 0.2</td>
<td>5.1 ± 0.3</td>
<td>1.0 ± 0.0</td>
<td>4.4 ± 0.1</td>
<td>1.0 ± 0.0</td>
<td>7.4 ± 0.3</td>
</tr>
<tr>
<td>Manufacturer #6 (lots #17-19)</td>
<td></td>
<td>63.3 ± 1.5</td>
<td>14.8 ± 1.5</td>
<td>7.5 ± 0.2</td>
<td>4.4 ± 0.2</td>
<td>1.0 ± 0.0</td>
<td>3.4 ± 0.0</td>
<td>0.8 ± 0.1</td>
<td>4.8 ± 0.3</td>
</tr>
<tr>
<td>20 (USP Reference Standard)</td>
<td></td>
<td>63.6 ± 0.8</td>
<td>11.2 ± 0.1</td>
<td>7.8 ± 0.2</td>
<td>4.5 ± 0.2</td>
<td>1.0 ± 0.0</td>
<td>4.1 ± 0.2</td>
<td>0.9 ± 0.1</td>
<td>7.0 ± 0.3</td>
</tr>
</tbody>
</table>

- Lots of heparin sodium made by manufacturer #3 have more total N-acetyl content than lots from other manufacturers: mean total N-Acetyl 12.5 ± 2.3% vs. #3 16.2%
- Heparin begins as $G_{20H}$-$A_{NAC}$
<table>
<thead>
<tr>
<th>Sample</th>
<th>I-S</th>
<th>II-S/III-S</th>
<th>IV-S</th>
<th>I-A</th>
<th>II-A/III-A</th>
<th>IV-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP#1</td>
<td>65.6 ± 0.2</td>
<td>25.6 ± 0.2</td>
<td>2.7 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>2.3 ± 0.1</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
<td>MP#1+0.1% OSCS</td>
<td>67.1 ± 0.3</td>
<td>27.5 ± 0.4</td>
<td>3.0 ± 0.2</td>
<td>1.4 ± 0.0</td>
<td>0.8 ± 0.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>MP#1+0.5% OSCS</td>
<td>73.3 ± 0.4</td>
<td>22.7 ± 0.6</td>
<td>2.1 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td>0.6 ± 0.0</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td>MP#1+1.0% OSCS</td>
<td>82.0 ± 0.9</td>
<td>15.2 ± 1.1</td>
<td>2.0 ± 0.2</td>
<td>0.4 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>MP#1+2.0% OSCS</td>
<td>82.8 ± 0.1</td>
<td>9.9 ± 0.1</td>
<td>2.0 ± 0.2</td>
<td>0.3 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>MP#1+5.0% OSCS</td>
<td>94.6 ± 0.4</td>
<td>3.3 ± 0.6</td>
<td>2.0 ± 0.3</td>
<td>n.o.</td>
<td>n.o.</td>
<td>n.o.</td>
</tr>
<tr>
<td>MP#1+10.0% OSCS</td>
<td>100 ± 0</td>
<td>n.o.</td>
<td>n.o.</td>
<td>n.o.</td>
<td>n.o.</td>
<td>n.o.</td>
</tr>
</tbody>
</table>

• How accurate are these values?
  – 2D-NMR as an orthogonal assay.
Orthogonal assay

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>$A_{NX,6S}$</th>
<th>$A_{NX,6OH}$</th>
<th>$A_{NaC}$</th>
<th>$I_{2S}$</th>
<th>$I_{2OH}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMR-Heparin Sodium$^a$</td>
<td>81 ± 2%</td>
<td>19 ± 2%</td>
<td>15 ± 2%</td>
<td>78 ± 2%</td>
<td>24</td>
</tr>
<tr>
<td>MS-Heparin Sodium</td>
<td>81</td>
<td>20</td>
<td>13</td>
<td>74</td>
<td>30</td>
</tr>
</tbody>
</table>

$^a$Keire et al. unpublished results on the analysis of 2D-NMR data of intact heparins.
Conclusions

1. NMR and MS are information-rich assays for surveillance of complex drug structure and composition:
   - Extra peaks or intensity changes are indications of impurities, contaminants or structure alterations.
   - Important to establish the normal range of variability for each drug with a marketplace survey.
   - Information-rich data sets from a robust method are amenable to pattern recognition analysis that can make non-subjective pass/fail judgments for complex drugs.
People that did the work!

• Bo Wang (ORISE)
• Adam Brustkern (ORISE)
• Supported by:
  – Michael Boyne
  – Lucinda Buhse
  – Ali Al-Hakim
Thank You

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