Heparin and Heparin-like substances: NMR as release analytical control

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\textit{Modern analytical techniques in Pharmaceutical Industry}

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Heparin is a biological product, mainly used as anticoagulant drug, due to its ability to link proteases, and in particular Antithrombin (AT).

The complex Hp AT reacts with factor IIa and with factor Xa, acting on the coagulation cascade by increasing the inhibitory activity of Antithrombin. Recently other possible activities and applications have been studied. Due to presence of High molecular chains (Mw 15000-20000Da), the prolonged use of heparin is linked to adverse effects of bleeding and thrombocytopenia.
Heparin history

- Heparin was discovered in 1916 and commercialized in the early 1920s
- First pharmaceutical formulation was made in 1939 in USA named Liquemin from Organon, extracted from bovine lung
- In the 1950s bovine lung was replaced by porcine mucosa and partially by bovine mucosa
- In the 1970s fractioning and depolymerization studies started, that gain to produce low molecular weight heparins
- In 1990s bovine Heparin was withdrawn from the market because of the potential risk of infection with bovine spongiform encephalopathy (BSE) prion
- In the late 2007 and early 2008, Heparin was involved in a second crisis mainly in the USA, where adverse effects, including fatalities, were reported.
Heparin is the sodium salt of a highly sulfated and polydispersed linear polysaccharide chain, with an heterogeneous structure.

HP consists of disaccharide units of uronic acid and α-D-glucosamine linked with a 1 to 4 bond. Uronic acid can be α-L-iduronic or β-D-glucuronic, which can be 2-O sulfated. Glucosamine can be N-acetylated or N-sulfated, and 3-O or 6-O sulfated.

- Complexity and variability
- Differences due to the production processes
- Biological origin with a very low API/starting material ratio, equal to 1/20000
Heparin Crisis

In 2008, after an extensive investigation, a contaminant was identified in HP coming from Cina. Chondroitin sulfated, a sulfomucopolysaccharide of the same family of HP, with natural origin and subsequently sulfated, was intentionally added in order to reduce costs.
Heparin Crisis

Published on FDA web site

Impurity Evaluation of Heparin Sodium by $^1$H-NMR Spectroscopy
Over Sulfated Chondroitin Sulfated

Chondroitin sulfate

Dermatan Sulfate

Heparin
Over Sulfated Chondroitin Sulfated

No peak should be visible at 2.15 ±0.02 ppm.

The use of this technique for OSCS detection has been further developed with new specifications with the aim to identify new impurities

Criteria for identification
- Heparin methyl group: 2.04 ± 0.01 ppm for heparin sodium; 2.05 ± 0.01 ppm for heparin calcium
- Dermatan Sulpate (DS) methyl group: 2.08 ± 0.02 ppm
- OSCS methyl group: 2.15 ± 0.02 ppm in heparin sodium; 2.18 ± 0.01 ppm in heparin calcium.

<table>
<thead>
<tr>
<th>Characters</th>
<th>USP 30</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IDENTIFICATION</strong></td>
<td></td>
</tr>
<tr>
<td>a) Coagulation</td>
<td>It delays the clotting of recalcified citrated sheep plasma</td>
</tr>
<tr>
<td>b) Optical rotation (sol. 4%)</td>
<td>≥ + 35°</td>
</tr>
<tr>
<td>c) Electrophoretic mobility</td>
<td>0,9 - 1,1</td>
</tr>
<tr>
<td>d) Sodium reaction</td>
<td>positive</td>
</tr>
<tr>
<td><strong>COLOR OF THE SOLUTION</strong></td>
<td>≤ 5</td>
</tr>
<tr>
<td>pH (sol. 1%)</td>
<td>5,5 - 8,0</td>
</tr>
<tr>
<td>Reading at UV 260 nm</td>
<td>≤ 0,200 OD</td>
</tr>
<tr>
<td>Reading at UV 280 nm</td>
<td>≤ 0,150 OD</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>≤ 2,5 % o.d.b.</td>
</tr>
<tr>
<td>Sodium</td>
<td>9,5 - 12,5 % o.d.b.</td>
</tr>
<tr>
<td>HEAVY METALS</td>
<td>≤ 30 ppm</td>
</tr>
<tr>
<td>LOSS ON DRYING</td>
<td>≤ 8,0 %</td>
</tr>
<tr>
<td>SULPHATED ASH</td>
<td>30 % - 43 % o.d.b.</td>
</tr>
<tr>
<td>ANTICOAGULANT ACTIVITY Ph.Eur.</td>
<td>≥ 150 IU/mg o.d.b.</td>
</tr>
<tr>
<td>Endotoxins</td>
<td>&lt; 0.01 EU/ IU of heparin</td>
</tr>
<tr>
<td>Sterility</td>
<td>X</td>
</tr>
<tr>
<td>Anti-Xa Activity</td>
<td>X</td>
</tr>
<tr>
<td>Residual solvent</td>
<td>X</td>
</tr>
</tbody>
</table>

Criteria for heparin release
Before OSCS crisis
Due to heparin crisis, a deeper characterization of this complex macromolecule became necessary.

Revision of EP, USP and JP monographs occurred in more steps, in order to guarantee a higher and safer quality standard for patients.
Monograph history after 2007 crisis

**AUG 2008**
Ph. Eur. 6.4 08/2008:0333
CE e NMR tests added in section “production”

**AUG 2008**
Ph. Eur. 7.0 08/2010:0333
HPLC and NMR as Identification Test
Impurities: CE deleted
HPLC: 2,0 % DS+ CS,
Other impurities: absent
Potency Anticoag ≥ 180 IU/mg o.d.b.

**JAN 2015**
Ph.Eur. 8.3 01/2015:0333
Potency: changed with Anti II Act

**MARCH 2008**
FDA Alert on contaminated Heparins

**JUNE 2008**
additional tests required by FDA for HP: NMR and CE

**MAY 2009**
USP 32 stage 1
New
b) 1H NMR <761>
c) Capillary electrophoresis <1053>

**OCT 2010**
USP 33 Suppl 1 Stage 2
b) 1H NMR, reviewed
b) HPLC
c) Potency Anti IIa
Impurities:
HPLC Gal NMT 1%
HPLC Other impurities
Added test protein

**AUG 2016**
USP 39 - Suppl 1
Identification d)
Mw, 15000 Da - 19000 Da,
Ratio:
M8000-16000/M16000-24000 ≥ 1,0
M24000 ≤ 20 %
Lower Limit for Proteins and Nucleic acid
The $^1$H-NMR spectrum obtained with the test sample and that obtained with heparin sodium for NMR identification CRS are compared qualitatively after the two spectra have been normalized so as to have a similar intensity.

The spectrum shows the typical heparin signals at: 2.04 ppm, 3.27 ppm (doublet), 4.34 ppm, 5.22 ppm and 5.42 ppm, all within ± 0.03 ppm.

No unidentified signals larger than 4 per cent compared to the height of the heparin signal at 5.42 ppm are present in the ranges 0.10-2.00 ppm, 2.10-3.10 ppm and 5.70-8.00 ppm.
HPLC release test: Identification and Related Substances
Current criteria for heparin evaluation

After BSE and OSCS crisis, criteria for heparin evaluation changed

- Monographs were reviewed
- Heparin was added to the list of substances of biological origin and Regulatory registration procedures changed consequently
  - «Heparins and Changing Regulatory» PHARMEUROPA Vol. 23, January 2011
  - Heparin for Drug and Medical Device Use: Monitoring Crude Heparin for Quality, Guidance for Industry June 2013
- Complete traceability of materials in the Supply chain by planning the detailed supervision of: healthy status of animals and of slaughterhouses, check of the materials used in the production chain, Technical agreements and periodical audits
  - EMA/CHMP/BWP/429241/2013 “Guideline on the use of starting materials and intermediates collected from different sources in the manufacturing of non-recombinant biological medicinal products”
NMR applied to heparin analysis

NMR analysis routinely applied allows to identify:

• Impurities coming from reagents of the production process
• Typical characteristics of the process for the production of LMM
• Structures produced during particular steps of the process
• Variability range
• Control tests for import
Process Flow sheet

mucosa → Alkaline enzymatic digestion → Heparin binding

LMWH ↔ Heparin API

Solvent Precipitation → Oxidation → Elution
Process impurities

Acetone
2.2360 ppm
4.5%

Acetates
1.9202 ppm
5.6%
NMR for heparin derivatives: LMM

- NMR analysis becomes critical for the identification of Low Molecular Mass Heparin
- Different LMM HP are available on the market depending on the different depolymerization processes
- Obtained molecules are specific for the applied process

**Dalteparinum natricum**

**Nitrous acid depolymerisation**

The majority of the components have a 2-0-sulfo-a-L-idopyranosuronic acid structure at the non-reducing end and a 6-0-sulfo-2,5-anhydro-D-mannitol structure at the reducing end of their chain.
**H₂O₂ cupric oxidative depolymerisation**

The majority of the components have a 2-0-sulfo-a-L-idopyranosuronic acid structure at the non-reducing end and a 2-N,6-0-disulfo-D-glucosamine structure at the reducing end of their chain.

**alkaline depolymerisation of the benzyl ester**

Enoxaparin consists of a complex set of oligosaccharides that have not yet been completely characterized. Based on current knowledge, the majority of the components have a 4-enopyranose uronate structure at the non-reducing end of their chain. 15 % to 25 % of the components have a 1,6-anhydro structure at the reducing end of their chain.
Nitrous acid depolymerisation
The majority of the components have a 2-0-sulfo-a-L-idopyranosuronic acid structure at the non-reducing end and a 6-0-sulfo-2,5-anhydro-o-mannitol structure at the reducing end of their chain.

Enzymatic depolymerisation
The majority of the components have a 2-0-sulfo-4-enepyranosuronic acid structure at the non-reducing end and a 2-N,6-0-disulfo-D-glucosamine structure at the reducing end of their chain.
Some heparins were not compliant to specifications due to an unidentified peak at 2.1 ppm.

The signal was attributed to a structure produced during the oxidative treatment with Potassium Permanganate performed by some producers.

As a consequence of the NMR release test, the attention and the interest in defining the modified heparin structures derived from particular purification steps grew.
Some heparins have an unidentified peak at 3.13/66.4 ppm.

The signal was attributed to a structure produced during the oxidative treatment with hydrogen peroxide and subsequent reduction with sulfite.

Modified heparin structures

**Analytical Chemistry, 2012 Daniela Beccati et al.**
«Identification of a Novel Structure in Heparin Generated by sequential oxidative-reductive treatment»
NMR analysis was introduced as quality evaluation parameter

FDA Documentation required for import

- **crude Heparin**
  
  SAX-HPLC chromatograms
  
  The 500 MHz 1D-1H-NMR spectrum, which includes an expansion of the 1.8 to 2.3 ppm plot region along with the full spectrum

- **Heparin Sodium API and finished dosage forms**
  
  (1)H-NMR spectrum (testing conducted according to current USP method), with expanded 1.8 to 3.0 ppm region
  
  HPLC-SAX chromatogram (testing conducted according to USP method)

- **Documentation Required for importing low molecular weight heparin (LMW) products**
  
  (1)H-NMR spectrum (testing conducted according to current USP method), with expanded 1.8 to 3.0 ppm region
  
  HPLC-SAX chromatogram (testing conducted according to USP method)
Plots of anomeric region A and aliphatic region B of the 2D $^1$H-$^{13}$C HSCQ spectrum, obtained on a sample of HP Na.

Selected signals are labeled based on the assignements.

NMR for determining composition

Percentage of monosaccharide composition, derived from H-C HSQC data collected on intact Heparin products compared to values derived from LC-MS studies on Heparin digested APIs

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>$A_{NX,6S}$</th>
<th>$A_{NX,6O\text{H}}$</th>
<th>$A_{NAc}$</th>
<th>$I_{2S}$</th>
<th>$I_{2O\text{H}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMR-heparin sodium</td>
<td>81 ± 2%</td>
<td>19 ± 2%</td>
<td>9 ± 1%</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>
<tr>
<td>NMR-dalteparin</td>
<td>91 ± 0%</td>
<td>8 ± 1%</td>
<td>6 ± 1%</td>
<td>77 ± 1%</td>
<td>23</td>
</tr>
<tr>
<td>MS-dalteparin</td>
<td>90</td>
<td>10</td>
<td>9</td>
<td>78</td>
<td>22</td>
</tr>
<tr>
<td>NMR-tinzaparin</td>
<td>81 ± 0%</td>
<td>19 ± 0%</td>
<td>7 ± 0%</td>
<td>78(^c)</td>
<td>22</td>
</tr>
<tr>
<td>MS-tinzaparin</td>
<td>77</td>
<td>23</td>
<td>14</td>
<td>72</td>
<td>29</td>
</tr>
<tr>
<td>NMR-enoxaparin</td>
<td>77 ± 1%</td>
<td>23(^d)</td>
<td>6 ± 0%</td>
<td>73(^c)</td>
<td>27(^e)</td>
</tr>
<tr>
<td>MS-enoxaparin</td>
<td>81</td>
<td>19</td>
<td>12</td>
<td>77</td>
<td>23</td>
</tr>
</tbody>
</table>

Keire Analytical method 2013
NMR analysis was initially introduced for characterization studies.

Enlargement of NMR applications allowed to increase investigation possibilities.

2D (bidimensional analysis of H and C spectra) allows for the definition of the composition through the sulfation pattern.  
Marco Guerrini, Seminars in thrombosis and Hemostasis 2001, 2005

An extensive study would allow to the definition of the variability range of UFH and of LMM available on the market.  
Keire Analytical method 2013; Guerrini Analytical Chemistry 2015
CONCLUSIONS

- Currently in EU and in USP, NMR technique is used for 17 substances only of the 10000 monographs; however, regarding heparin, this test is critical.

- Even if it is a complex technique, it is extremely useful for the definition of the specifications, for the quality evaluation and for the composition analysis.

- Processes used to produce heparin generate typical and quantifiable structures that can permit to define an allowed range for such products.

- Standardization of Heparin parameter allows qualification for highlighting differences both on raw material and on final product.