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

21st OCTOBER, 2016 Pavia

Modern analytical techniques in Pharmaceutical Industry

Donata Bensi

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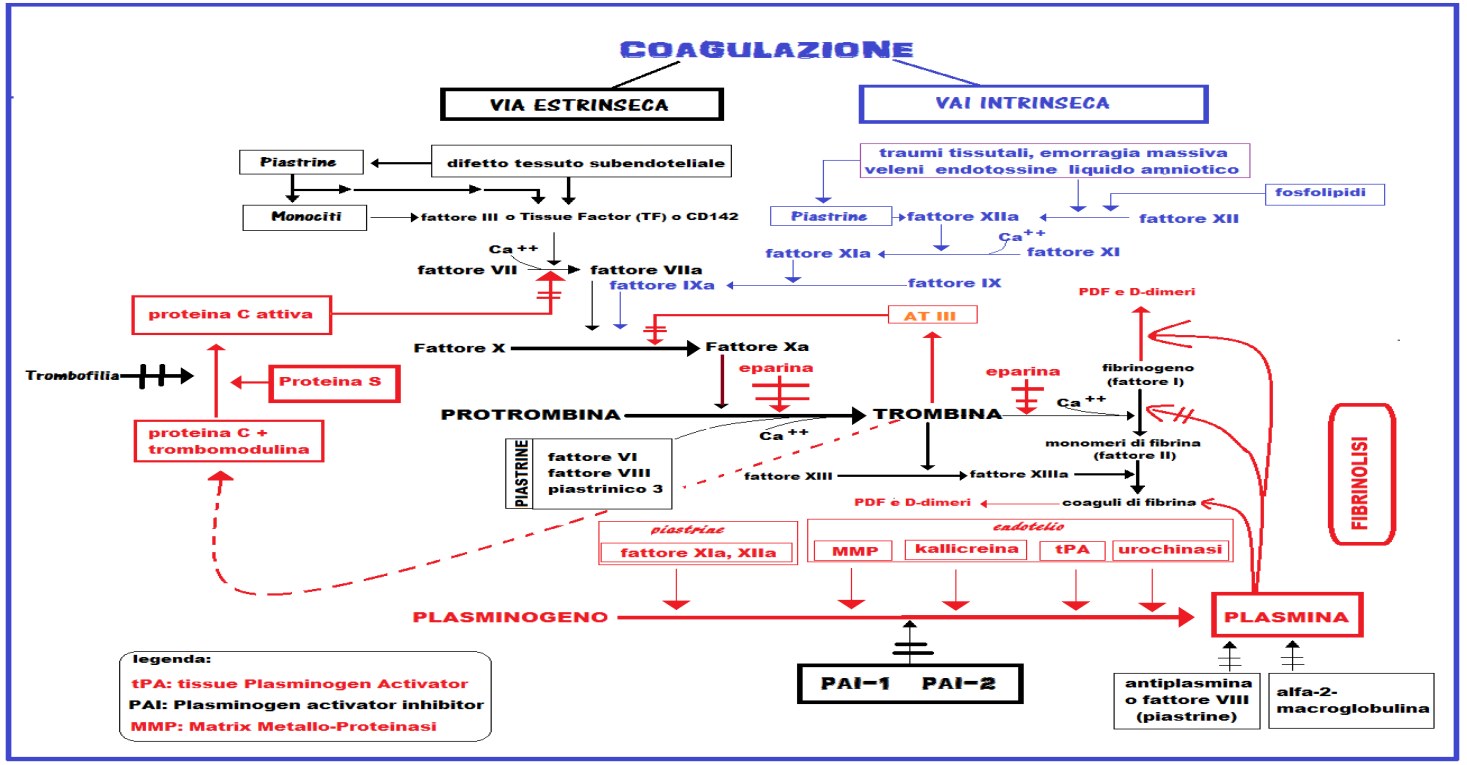
HEPARIN

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.



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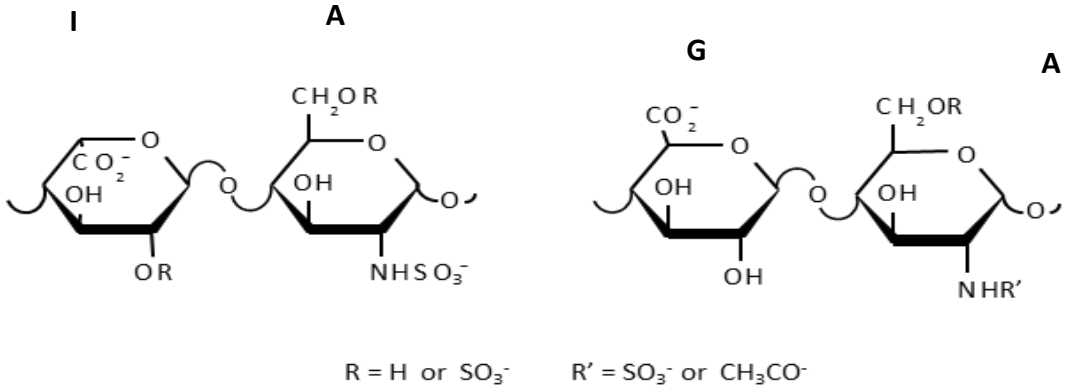
Heparin history

- 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- In 1990s bovine Heparin was withdrawn from the market because of the potential risk of infection with bovine spongiform encephalopathy (BSE) prion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 Structure



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

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Heparin Crisis

In 2008, after an extensive investigation, a contaminant was identified in HP coming from Cina.

Chondroitin sufated, a sulfomucopolysaccharide of the same family of HP, with natural origin and subsequently sulfated, was intentionally added in order to reduce costs.

AM2 PAT, Inc. Issues Nationwide Recall of All Lots and All Sizes of Pre-Filled Heparin and Normal Saline Flushes

HealthDay

THE WALL STREET JOURNAL.

News | Today's Newspaper | My Online Journal | Multimedia & Online Extras

HEALTH BLOG

WSJ's blog on health and the business of health. Blog Search:

< Youth 'Choking Game' Can Be [...] -- Previous | SEE ALL POSTS FROM THIS BL

February 15, 2008, 8:27 am

Heparin Trail: Pig Intestines From China Via Wisconsin

WSJ.com THE WALL STREET JOURNAL. ONLINE

March 7, 2008

German Firm Recalls Heparin With China Link

FDA U.S. Food and Drug Administration

FDA Home Page | Search FDA Site | FDA A-Z Index | Contact FDA

FDA News

FOR IMMEDIATE RELEASE
February 11, 2008

Media Inquiries:
Karen Riley, 301-827-6244
Consumer Inquiries:
888-INFO-FDA

Baxter's Multiple-dose Vial Heparin Linked to Severe Allergic Reactions

FDA advises health care practitioners to switch suppliers and limit use of drug until problem identified

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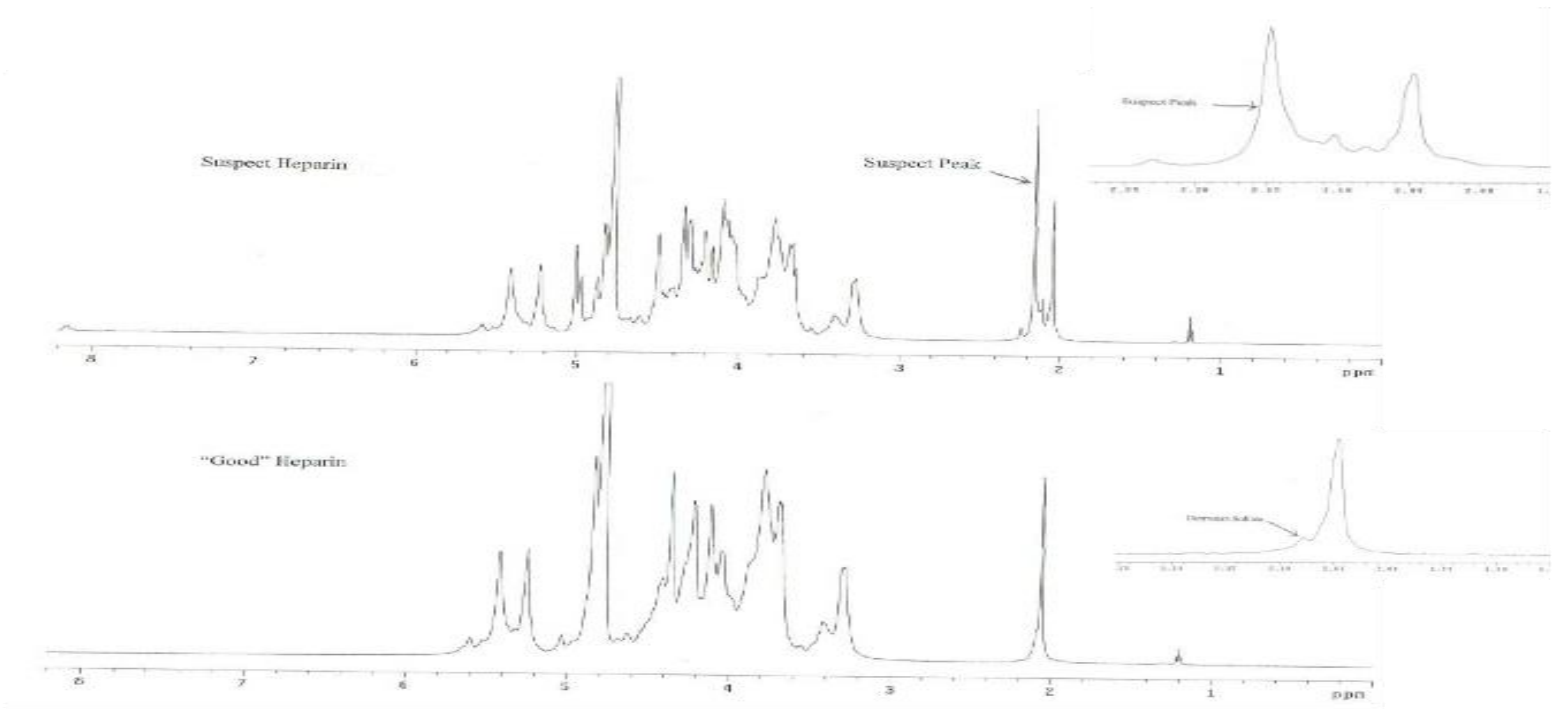
Heparin Crisis

Published on FDA web site

Impurity Evaluation of Heparin Sodium by ^{1}H-NMR Spectroscopy



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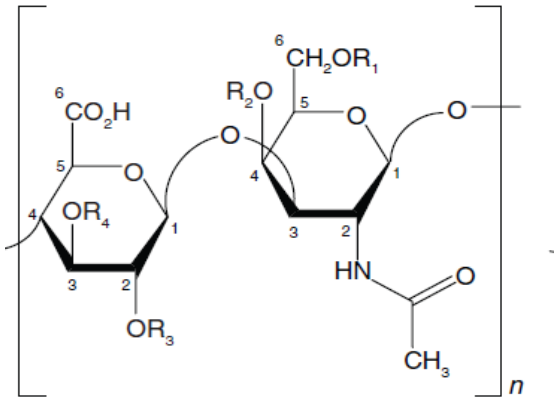
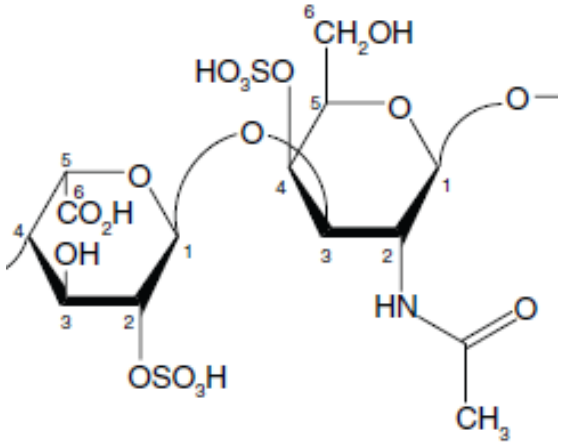
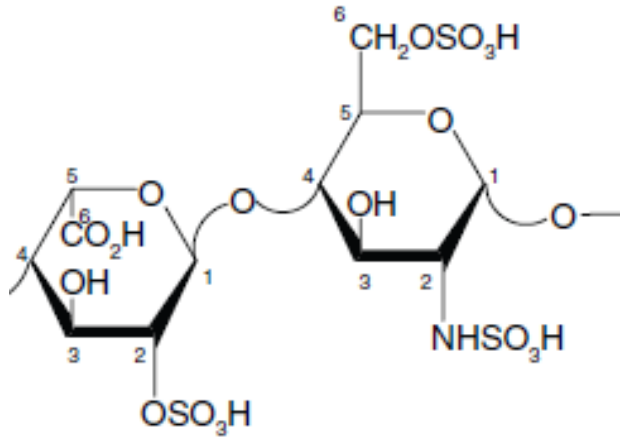
Over Sulfated Chondroitin Sulfated



Chondroitin sulfate



Dermatan Sulfate



Heparin

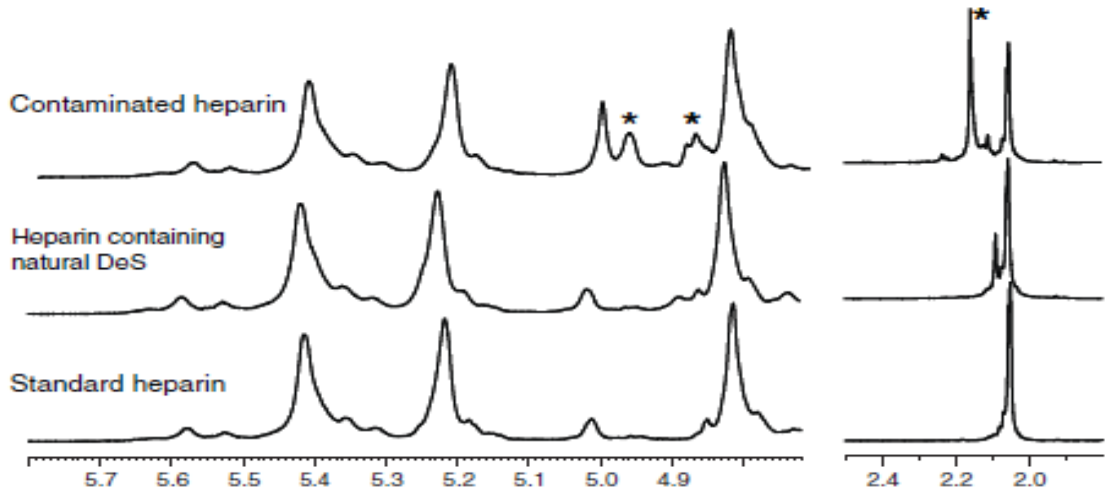
Over Sulfated Chondroitin Sulfated

No peak should be visible at 2.15 ±0.02 ppm.

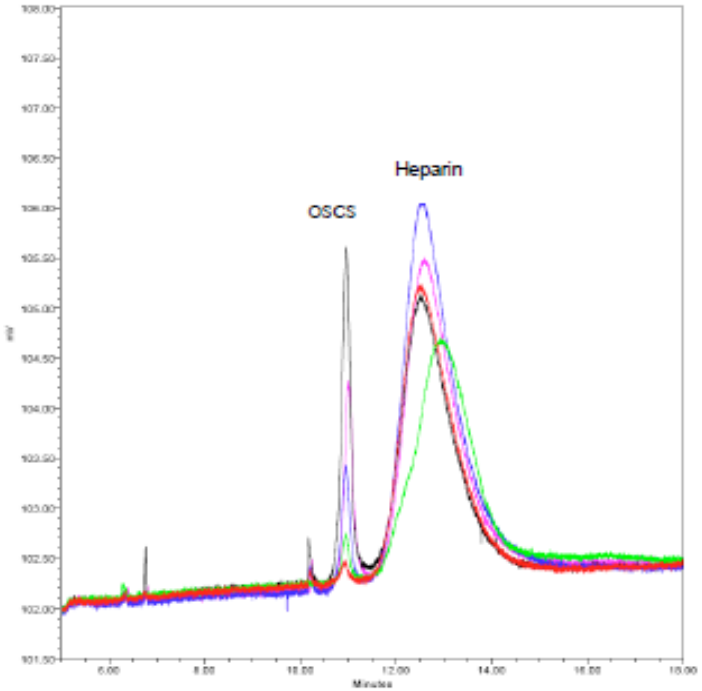
The use of this technique for OSCS detection has been further developed with new specifications with the aim to identify new impurities

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.



Guerrini, Nature Biotechnology,2008 .



[#]Somsen Govert, Department of Biomedical Analysis, Utrecht University, P.O. Box 80082, 3508 TB, Utrecht, NL

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Heparin Sodium Monograph 2007

Criteria for heparin release
Before OSCS crisis

	Ph. Eur. 01/2005 :0333	USP 30
Characters	*White or almost white powder, hygroscopic, freely soluble in water*	
IDENTIFICATION		
a) Coagulation	*It delays the clotting of recalcified citrated sheep plasma*	✓
b) Optical rotation (sol. 4%)	*≥ + 35°*	*X*
c) Electrophoretic mobility	*0,9 - 1,1*	*X*
d) Sodium reaction	*positive*	*X*
COLOR OF THE SOLUTION	*≤ 5*	*X*
pH (sol. 1%)	*5,5 - 8,0*	*5.2 – 7.5*
Reading at UV 260 nm	*≤ 0,200 OD*	*X*
Reading at UV 280 nm	*≤ 0,150 OD*	*1 ml of a 1 in 100 solution, 5 drops of TCA; not ppt or turbidity*
Nitrogen	*≤ 2,5 % o.d.b.*	*1.3 – 2.5 %*
Sodium	*9,5 - 12,5 % o.d.b.*	*Flame test for sodium*
HEAVY METALS	*≤ 30 ppm*	*≤ 0.003 %*
LOSS ON DRYING	*≤ 8,0 %*	*≤ 5.0 %*
SULPHATED ASH	*30 % - 43 % o.d.b..*	*28.0 – 41.0%*
ANTICOAGULANT ACTIVITY Ph.Eur.	*≥ 150 IU/mg o.d.b.*	✓
Endotoxins	*< 0.01 EU/IU of heparin*	*< 0.03 EU/U USP units*
Sterility	*X*	✓
Anti-Xa Activity	*X*	✓
Residual solvent	*X*	✓

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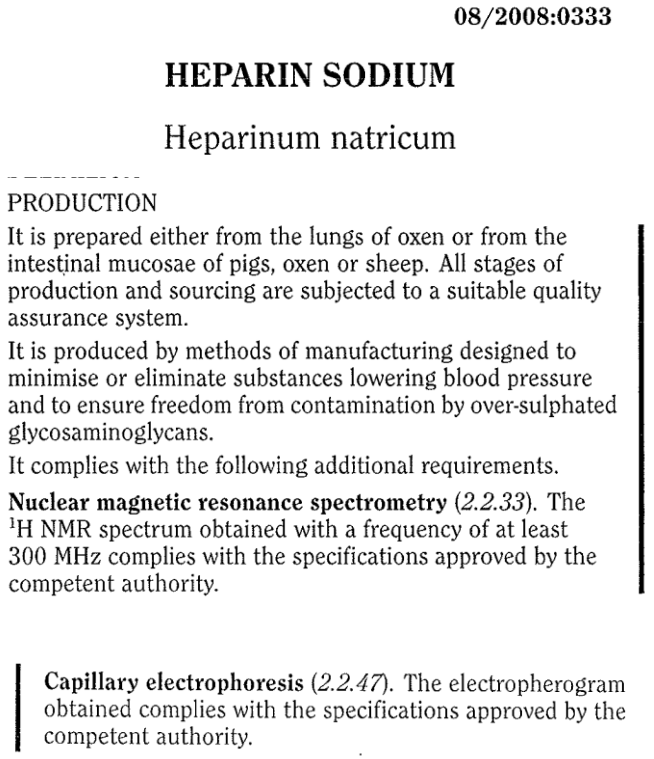
Monograph development

- ✓ 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.



08/2008:0333

HEPARIN SODIUM

Heparinum natricum

PRODUCTION

It is prepared either from the lungs of oxen or from the intestinal mucosae of pigs, oxen or sheep. All stages of production and sourcing are subjected to a suitable quality assurance system.

It is produced by methods of manufacturing designed to minimise or eliminate substances lowering blood pressure and to ensure freedom from contamination by over-sulphated glycosaminoglycans.

It complies with the following additional requirements.

Nuclear magnetic resonance spectrometry (*2.2.33*). The ^{1}H NMR spectrum obtained with a frequency of at least 300 MHz complies with the specifications approved by the competent authority.

Capillary electrophoresis (*2.2.47*). The electropherogram obtained complies with the specifications approved by the competent authority.

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Monograph hystory after 2007 crisis

AUG 2008
Ph. Eur. 6.4 08/2008:0333

CE e NMR tests added in section "production"

AUG 2010
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

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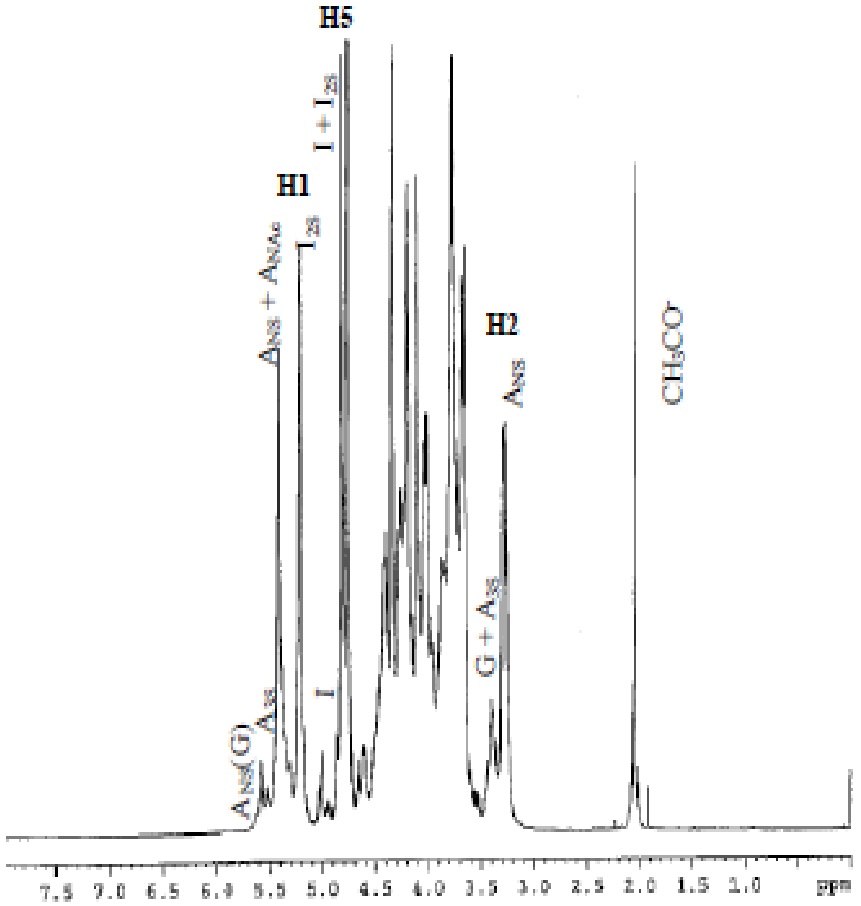
EP NMR release specification

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.



A Glucosamine

I Iduronic acid

G Glucuronic Acid

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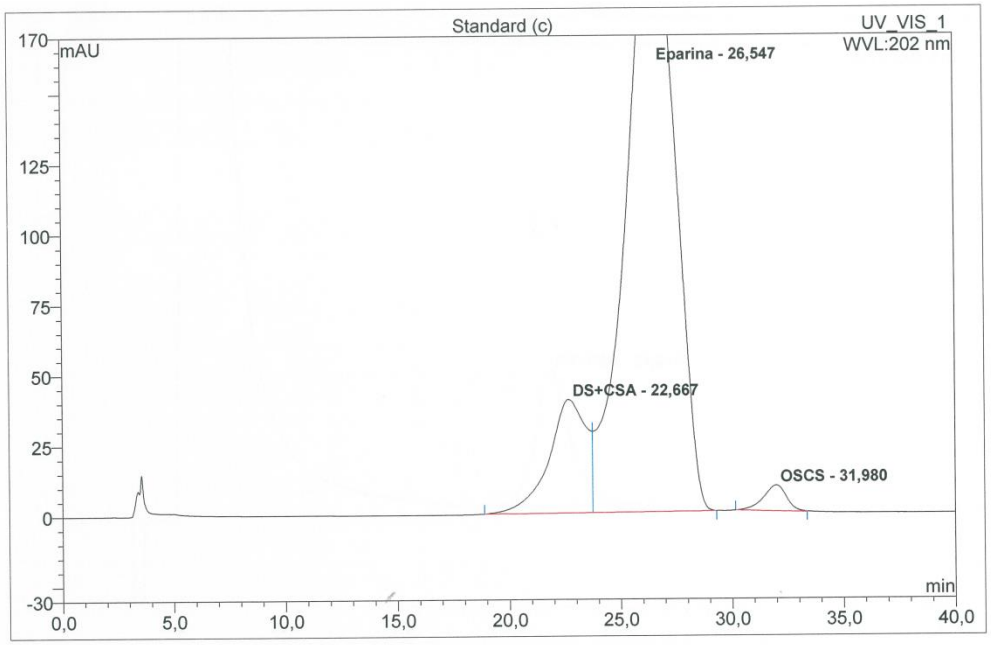
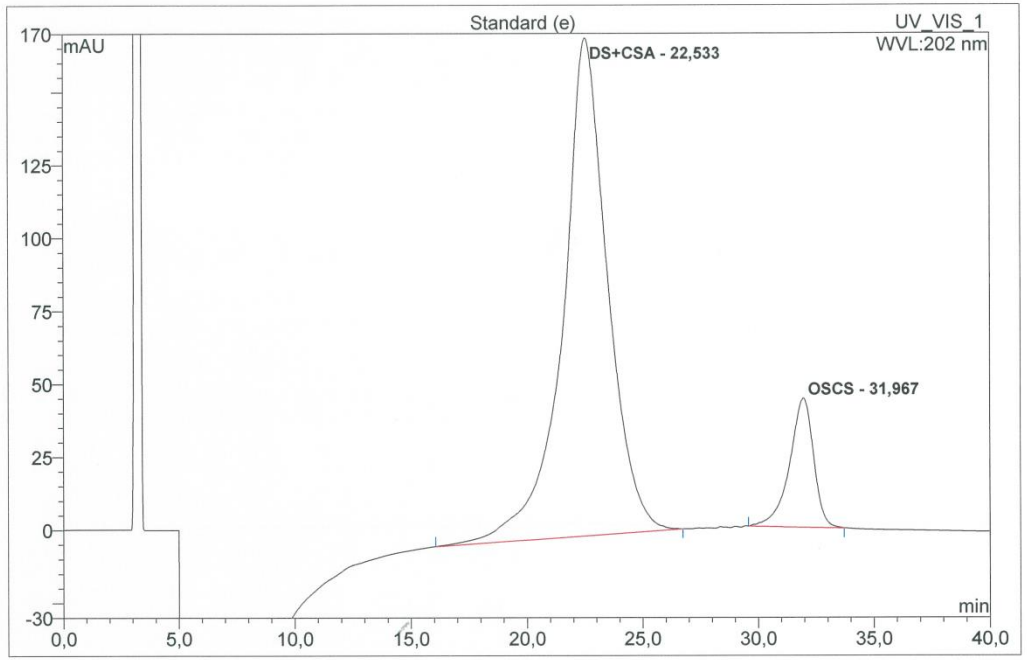
HPLC release test: Identification and Related Substances





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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"

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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

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Process Flow sheet

mucosa → Alkaline enzymatic digestion → Heparin binding → Elution → Oxidation → Solvent Precipitation → Heparin API → LMWH

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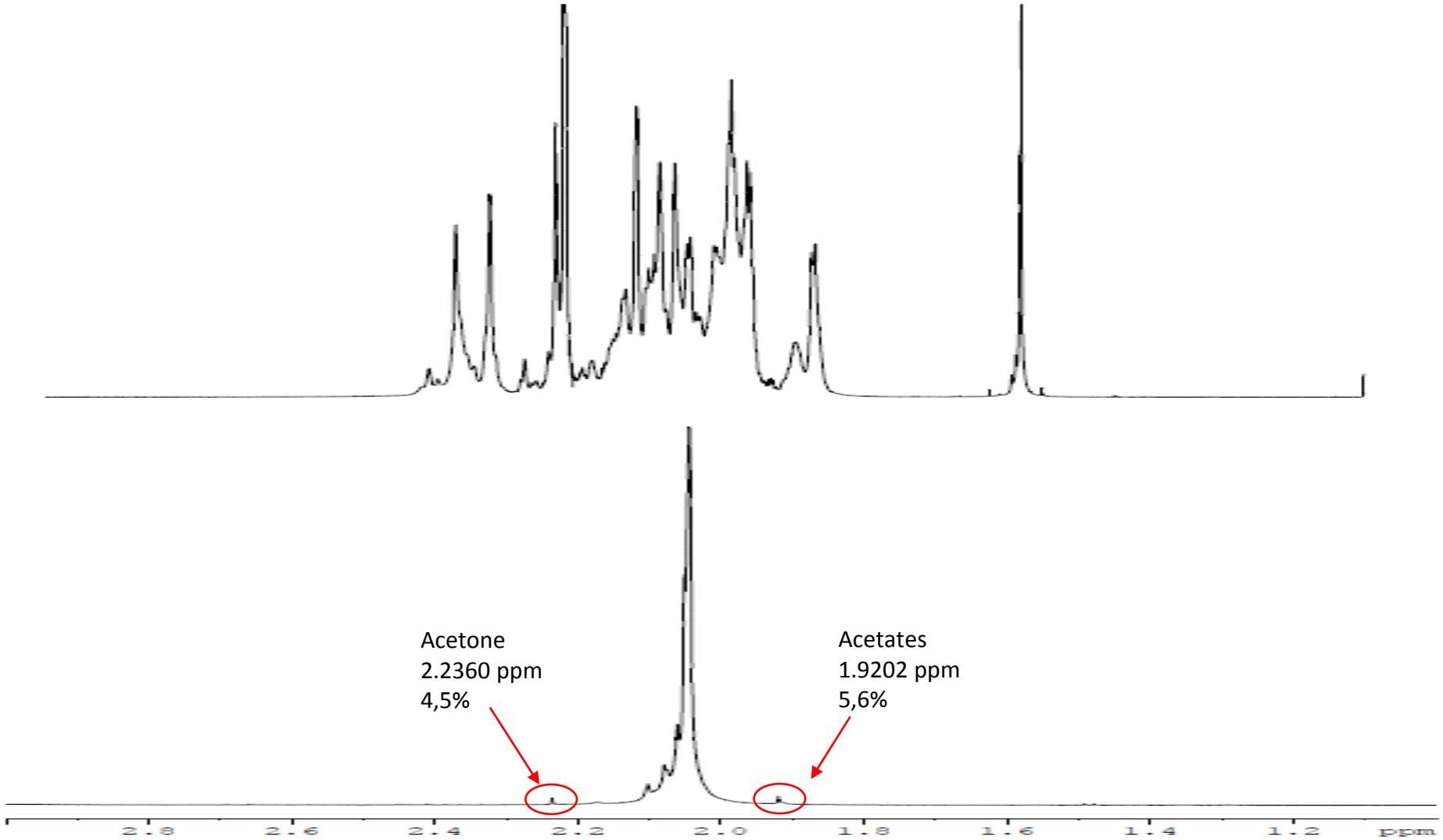
Process impurities



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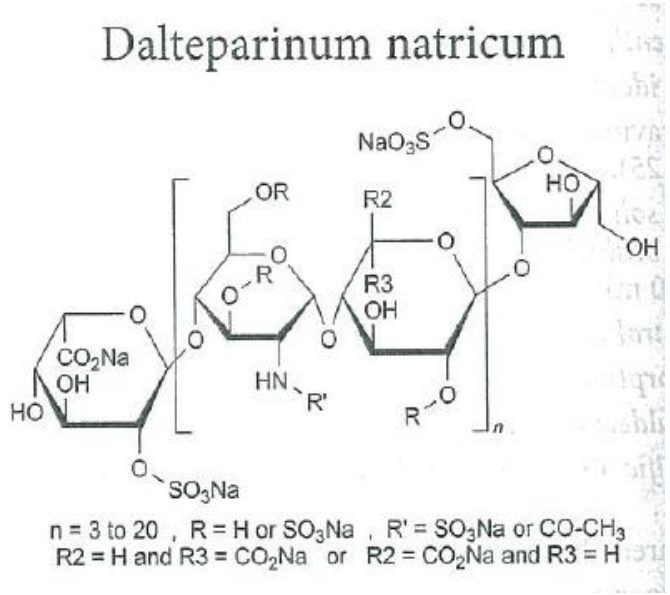
NMR for heparin derivatives: LMM

- 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



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.

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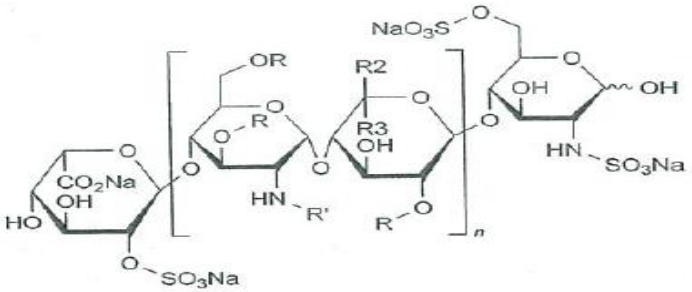
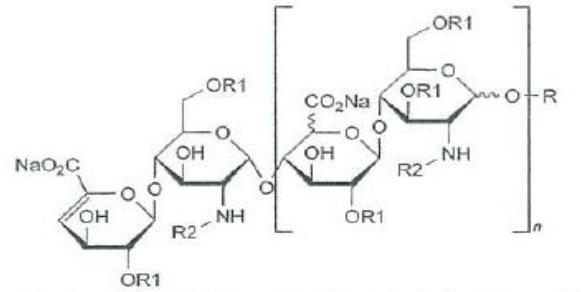
NMR for heparin derivatives: LMM

Parnaparinum natricum

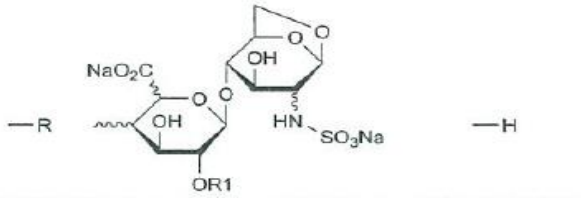


n = 1 to 21 , R = H or SO_3Na , R' = SO_3Na or $CO\text{-}CH_3$
R2 = H and R3 = CO_2Na or R2 = CO_2Na and R3 = H

Enoxaparinum natricum



	Structure at the 'reducing end'	
	1,6-anhydro	non 1,6-anhydro
n	0 to 20	1 to 21



R1 = H or SO_3Na R2 = SO_3Na or $CO\text{-}CH_3$

H_2O_2 cupric oxidative depolymerisation

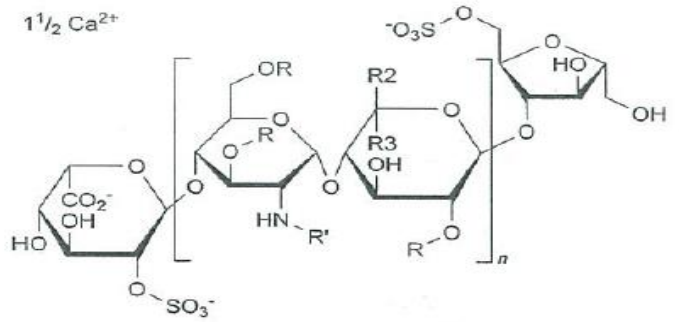
The majority of the components have a 2-0-sulfo-a-L-idopyranosuronic acid structure at the non-reducing end and a 2-N,6-0-disulfo-D-glucosamine structure at the reducing end of their chain.

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.

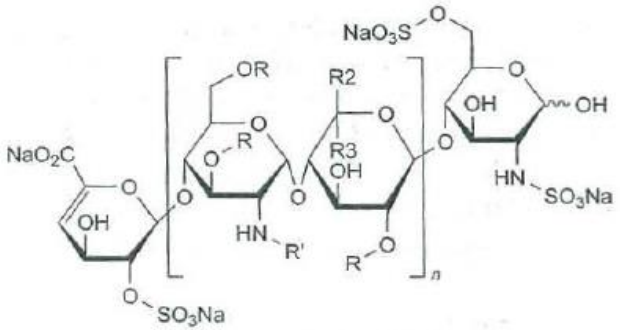
NMR for heparin derivatives: LMM

Nadroparinum calcicum



R = H or $SO_3(^1/_2\ Ca)$, R' = H or $SO_3(^1/_2\ Ca)$ or $CO\text{-}CH_3$
R2 = H and R3 = $CO_2(^1/_2\ Ca)$ or R2 = $CO_2(^1/_2\ Ca)$ and R3 = H

Tinzaparinum natricum



n = 1 to 25 , R = H or SO_3Na , R' = H or SO_3Na or $CO\text{-}CH_3$
R2 = H and R3 = CO_2Na or R2 = CO_2Na and R3 = H

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.

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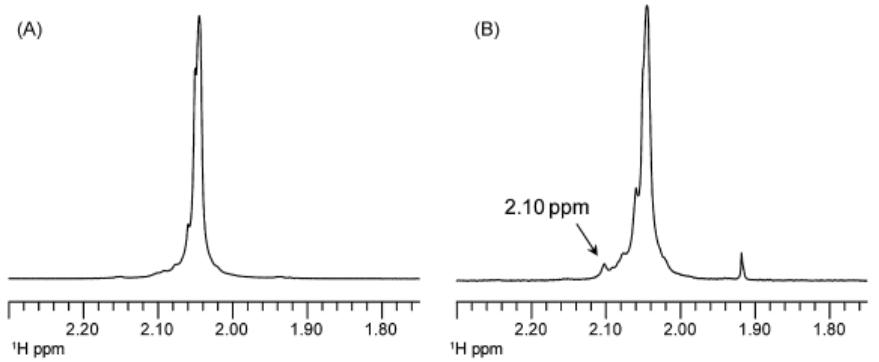
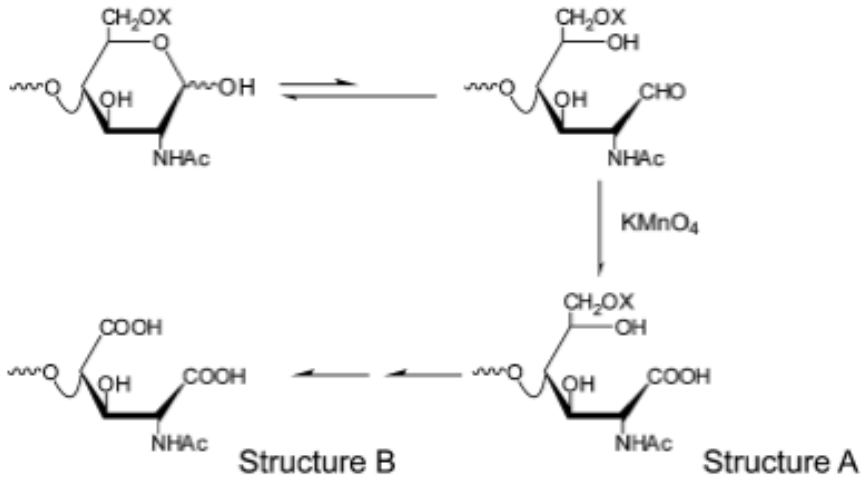
Modified heparin structures

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 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.

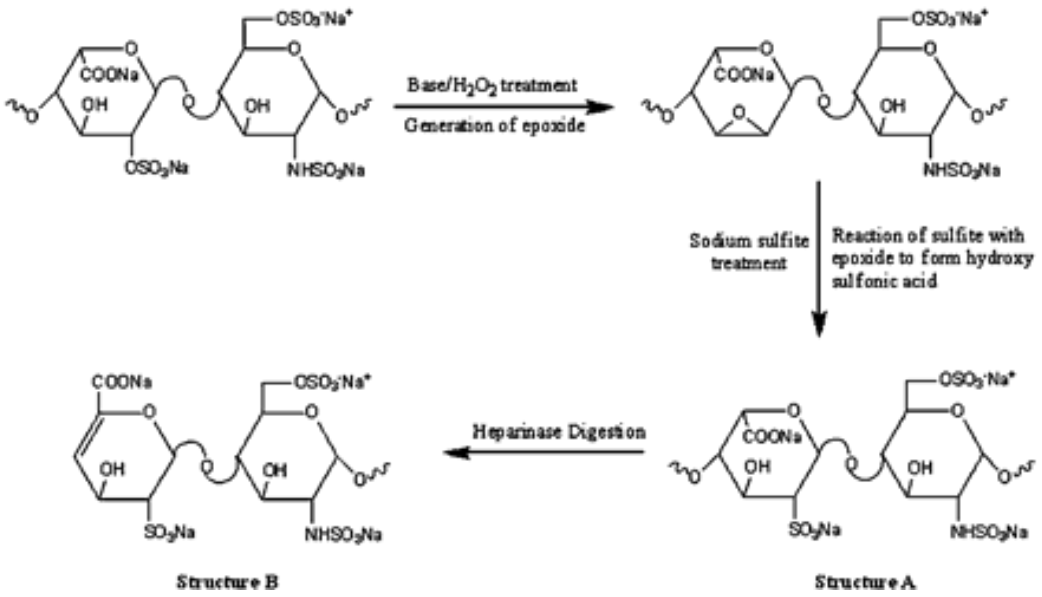


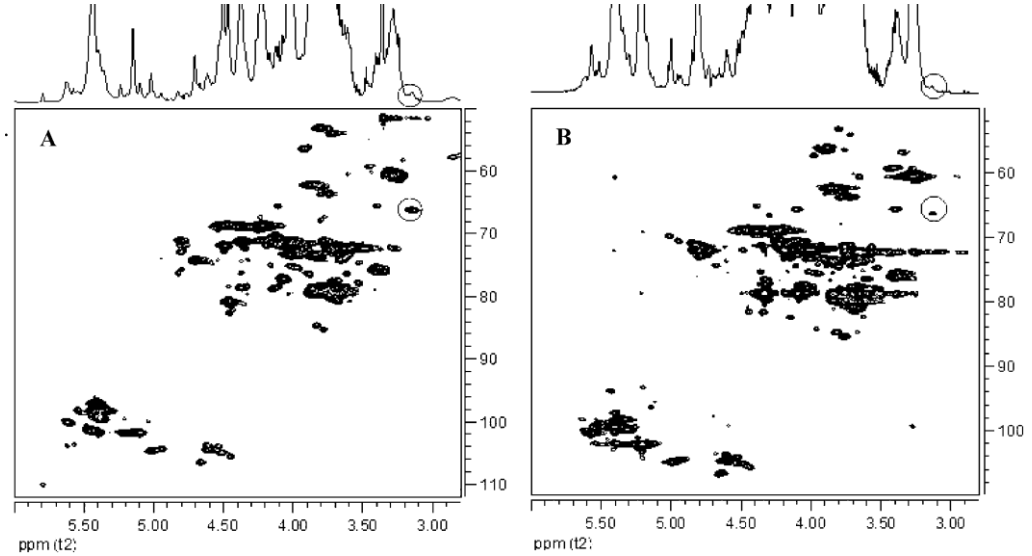
Daniela Beccati et al. Carbohydrate Polymers 2010

Modified heparin structures

- 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





Analytical Chemistry, 2012 Daniela Beccati et al.
« Identification of a Novel Structure in Heparin Generated by sequential oxidative-reductive treatment»

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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)

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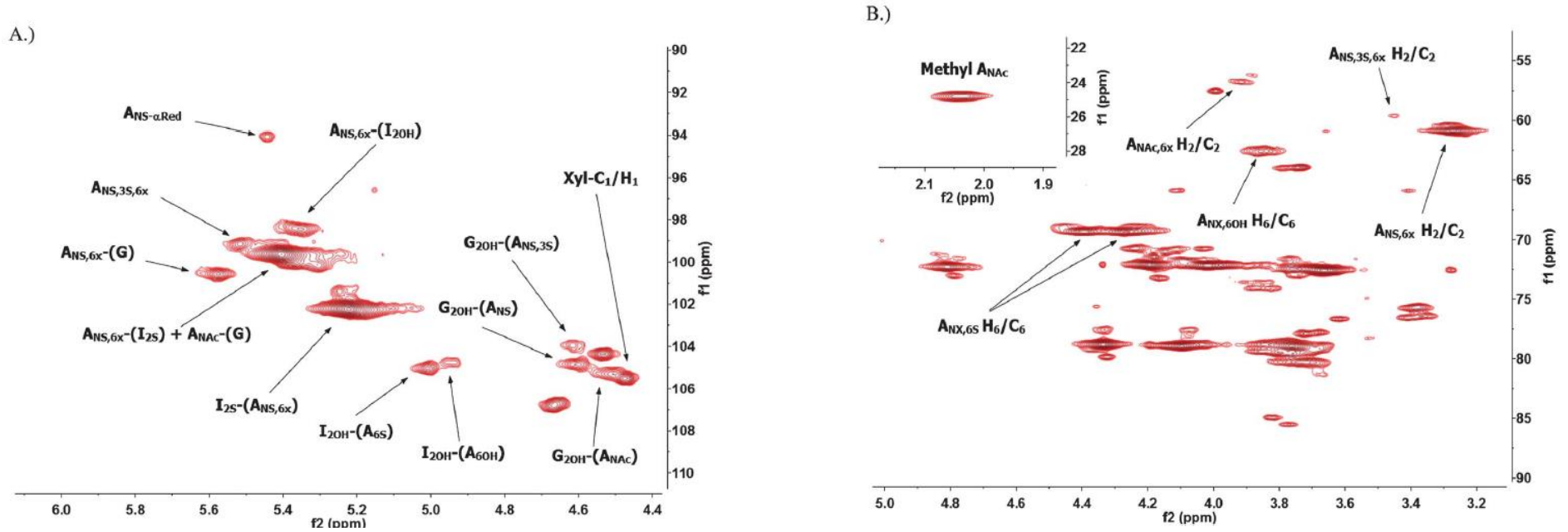
NMR for determining composition



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

M. Guerrini, Semin. Throb.Hemostasis,2007

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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

Monosaccharide	$A_{NX,6S}$	$A_{NX,6OH}$	A_{NAc}	I_{2S}	I_{2OH}[a]
NMR-heparin sodium	81 ± 2%	19 ± 2%	9 ± 1%	78 ± 2%	24
MS-heparin sodium[b]	81	20	13	74	30
NMR-dalteparin	91 ± 0%	8 ± 1%	6 ± 1%	77 ± 1%	23
MS-dalteparin	90	10	9	78	22
NMR-tinzaparin	81 ± 0%	19 ± 0%	7 ± 0%	78[c]	22
MS-tinzaparin	77	23	14	72	29
NMR-enoxaparin	77 ± 1%	23[d]	6 ± 0%	73[c]	27[e]
MS-enoxaparin	81	19	12	77	23

Keire Analytical method 2013

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Summary

- NMR analysis was initially introduced for characterization studies.

- Enlargement of NMR applications allowed to increase investigation possibilities.

- 2D (bidimensional analysys 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

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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.