

NMR Test Protocol for OSCS determination in API Heparin Sodium and Calcium

Introductory notes

The present protocol has been elaborated between September and December 2008 by an ad-hoc expert group composed of five representatives from Official Medicines Control Laboratories (OMCL) and one member of a university laboratory under the coordination of EDQM. The purpose of this document, which was established following several discussions and a one-month testing phase, is to provide to all users of the European Pharmacopoeia (Ph.Eur.) a reference method for the quantitative determination of the contaminant oversulphated chondroitin sulphate (OSCS) in unfractionated heparins by ¹H-Nuclear Magnetic Resonance (NMR) spectrometry. Since August 2008 the two official unfractionated heparin monographs of the Ph.Eur., namely Heparin Calcium 08/2008:0332 and Heparin Sodium 08/2008:0333, include in the production section additional requirements for the determination of contaminants by NMR spectrometry and Capillary electrophoresis (CE) without further specification. The work of the expert group contributes to the reliability and comparability of results/data by using a standardised reference test method.

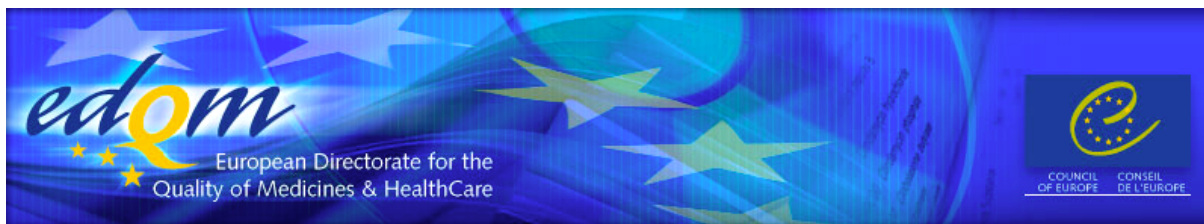
1 Instrumentation

From 300 MHz and upwards; depending on the instrument used, the left ¹³C satellite of the heparin methyl signal may interfere with the signal of the oversulphated chondroitin sulphate (OSCS) methyl group.

NMR instrument	Chemical shift of left ¹³ C satellite in ppm
600 MHz	2.15 – 2.16
500 MHz	2.17 – 2.18
400 MHz	2.21 – 2.22
300 MHz	2.26 – 2.27

2 Reference material/spectrum

Spiked material with defined level of OSCS (1%) for system suitability test.
OSCS sample with defined quality for quantification/spiking experiments, if applicable.



3 Reagents

Deuterated water (D₂O) with 0.002% sodium trimethylsilyl tetradeuteriopropionate (TSP-d₄) as an internal standard (reference peak set at 0.00 ppm).

4 Preparation of samples

No pre-treatment of the sample is needed. About 40 mg is dissolved in ca 0.7 ml deuterated water.¹

5 NMR acquisition parameters

- 5.1 The number of transients (16 upwards) should be adjusted until the signal to noise ratio (S/N) is at least 2000:1 for the heparin methyl signal. The noise level is defined in a signal free 200 Hz window in a region 0.1 – 0.9 ppm.
- 5.2 Temperature: 25 °C ± 5 °C (at a defined stable temperature) – reference spectra have to be recorded at the same temperature.
- 5.3 Transmitter offset: at the water resonance (ca 4.7 ppm depending on temperature).
- 5.4 Acquisition time: 2 s
- 5.5 Repetition time (acquisition time plus delay): at least 8 s.
- 5.6 Spectral width: 10-12 ppm.
- 5.7 Pulse width: to give a flip angle of 90° or less.

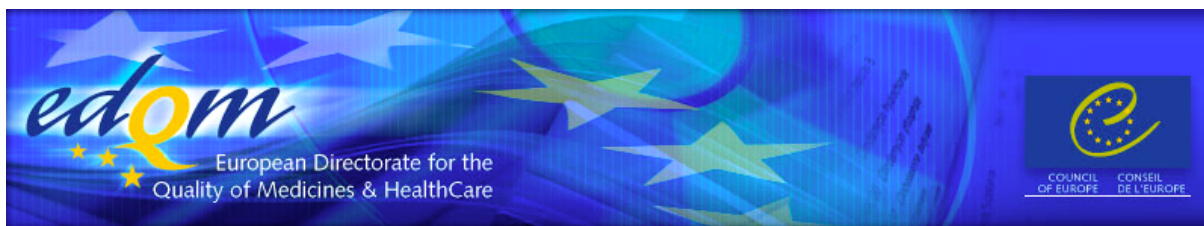
6 NMR processing parameters

- 6.1 Exponential line broadening window function (LB): 0.3 Hz.
- 6.2 Phasing may have to be done manually.
- 6.3 Baseline correction.
- 6.4 Zero filling: equal to data points used in acquisition for a given spectrometer frequency.

7 Criteria for identification

- 7.1 Heparin methyl group: 2.04 ± 0.01 ppm for heparin sodium; 2.05 ± 0.01 ppm for heparin calcium
- 7.2 Dermatan Sulphate (DS) methyl group: 2.08 ± 0.02 ppm
- 7.3 OSCS methyl group: 2.15 ± 0.02 ppm in heparin sodium; 2.18 ± 0.01 ppm in heparin calcium.

¹ Ca²⁺ should be added to the heparin sodium solution (5-7 mg CaCl₂·2H₂O to 40 mg heparin) if a 600 MHz NMR instrument is used (to avoid having the OSCS signal on top of the ¹³C satellite signal). The addition of Ca²⁺ can also be used to sharpen the OSCS signal, or as a way of OSCS identification (the OSCS signal moves progressively to 2.18 ppm with increasing amounts of Ca²⁺).



8 Detection limit (DL or LoD)

LoD = 0.1 weight%. This corresponds to an OSCS methyl peak height equivalent to that of the ¹³C satellite of the heparin methyl signal.

9 Quantification limit (QL or LoQ)

LoQ = 0.5 weight%.

10 Quantification

Measuring of the height of the OSCS methyl peak and of the heparin methyl peak, both with a common baseline, and by utilising the formula given below:

Amount(%) = (H_{imp} / H_{hep}) / RF, where the Response Factor (RF) is calculated by a standard curve to 0.047.

Relative Standard Deviation (RSD) - Interlaboratory reproducibility: 20%.

11 Literature Reference

The work of the ad-hoc expert group is published in PHARMEUROPA BIO 2008-1, December 2008 under the following title:

Determination of Oversulphated Chondroitin Sulphate and Dermatan Sulphate in unfractionated Heparin by ¹H-NMR

Collaborative study for quantification and analytical determination of LoD

I. McEwen, B. Mulloy, E. Hellwig, L. Kozerski, T. Beyer, U. Holzgrabe, A. Rodomonte, R. Wanko, J-M. Spieser.

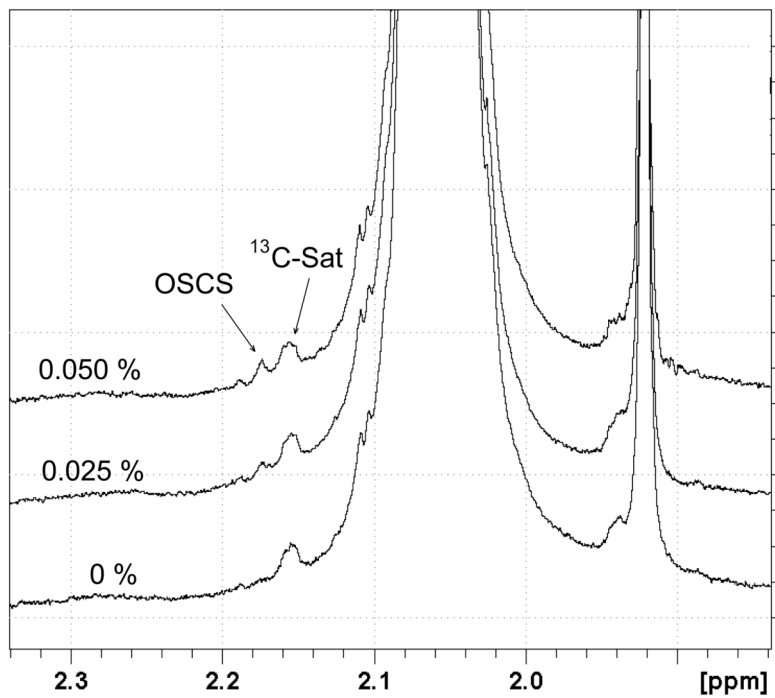


Figure 1 - Titration of OSCS to heparin sample. The spectra were obtained with a 600 MHz NMR instrument at an S/N of 5000:1

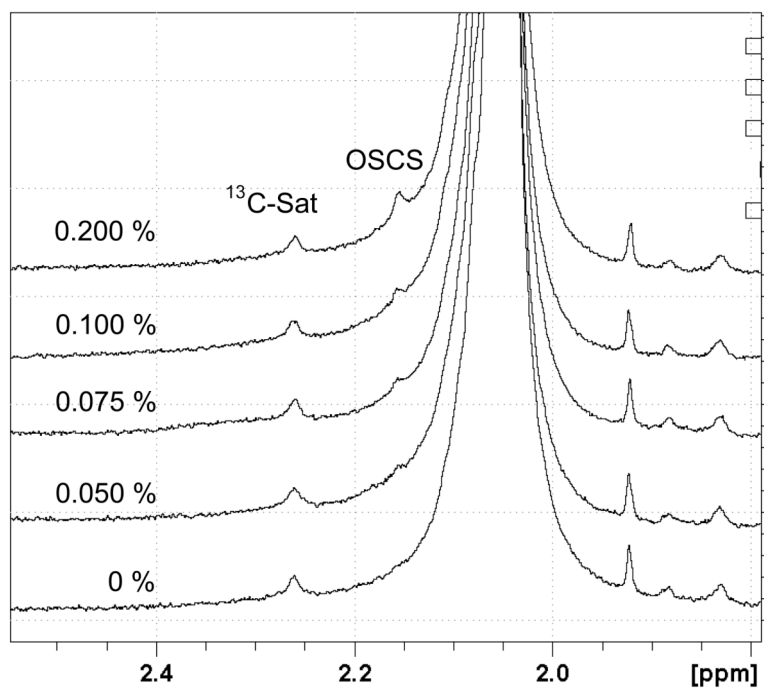


Figure 2 - Titration of OSCS to heparin sample. The spectra were obtained with a 300 MHz NMR instrument at an S/N of 1800:1