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Short communication

Broadening of ^{1}H NMR signals in the spectra of heparin and OSCS by paramagnetic transition metal ions. The use of EDTA to sharpen the signals

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ABSTRACT

Some signals in the ^{1}H NMR spectra of heparin and oversulphated chondroitin sulphate (OSCS) are occasionally broad or very broad owing to the presence of paramagnetic metal ions in those polysaccharides. The addition of very small amounts of EDTA to heparin or to OSCS contaminated heparin solutions was needed to obtain normal looking spectra.

1. Introduction

Heparin and oversulphated chondroitin sulphate (OSCS) are members of the glycosaminoglycan family of carbohydrates and consist of a variably-sulphated repeating disaccharide unit [1,2]. One moiety is uronic acid. The number of sulphate groups per disaccharide units in heparin is one to two; and in OSCS, four.

At the Swedish Medical Product Agency, where this work has been conducted, many different varieties of heparin samples have been analyzed by NMR. Most of the spectra obtained from those heparin samples looked the same concerning the sharpness of the signals, but in some cases some signals in the NMR spectra were much broader than in the average heparin spectrum. When OSCS was identified as a contaminant in those heparin samples with broader ^{1}H NMR signals, the acetate methyl signal at 2.15 ppm was broader than expected. It is of great importance for the identification of heparin as well as for the identification and quantification of OSCS in heparin by NMR spectroscopy to obtain good and reproducible spectra. Broadening of NMR signals is often due to the presence of paramagnetic impurities. Therefore, the effects of transition metal ions and EDTA on the appearance of the heparin and OSCS NMR spectra were studied.

2. Experimental

2.1. Materials and chemicals

OSCS sodium was obtained from NIBSC (UK). Heparin samples came from different (confidential) sources. The following salts, with p.a. quality, come from Merck (Darmstadt, Germany): $FeCl_3{\cdot}6H_2O$, $MnCl_2{\cdot}4H_2O$, $ZnCl_2$, $CoCl_2{\cdot}6H_2O$ and $CuCl_2{\cdot}2H_2O$. Chelex 100 was acquired from Bio-Rad (California, USA). EDTA from Merck (Darmstadt, Germany). Deuterated water (99.8%) from Armar chemicals (Döttingen, Switzerland). Sodium 3-trimethylsilyl tetradeuteriopropionate (TSP) from Dr. Glaser AG (Basel, Switzerland).

2.2. NMR spectroscopy

A 300 MHz Bruker Avance NMR instrument (Bruker Analytik, Rheinstetten, Germany). equipped with a 5 mm broad band probe was used. The heparin concentrations used were ca 50 mg/ml D_2O (higher or lower heparin concentrations can be used). The following parameters were used for the acquisition of the spectra. Time between pulses: 8 s. Number of transients: 64–256. Temperature: 25 or 37 °C. Spectral width: 12 ppm. Number of complex data points: 32K. Reference set at 0.00 ppm for the TSP signal. NMR spectra were obtained at 25 °C. In some cases, spectra were obtained at 37 °C to move the water NMR signal upfield.

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2.3. Sequestering metal ions

To remove metal ions from solution so that they cannot complex with heparin or OSCS, EDTA was added into the NMR tube containing the heparin solution before acquiring the NMR spectrum. The amount of EDTA needed was in the order of 100–1000 μg/g heparin depending on the amount of metal ion present. A second method used for the removal of metal ions was to pass the heparin solution through a bed of cation exchange resin before acquiring the NMR spectrum. Dry resin was used instead of resin suspended in water. The cation exchange resin used was Chelex 100.

3. Results and discussion

3.1. Addition of Mn^{2+} to solutions of heparin spiked with OSCS

The addition of Mn^{2+} (microgram amounts per gram heparin) to a solution of heparin spiked with OSCS causes a broadening of some signals in the heparin NMR spectrum and of the OSCS acetate methyl signal. The NMR signals of the protons H1 and H5 of the iduronic acid moiety in heparin at 5.22 and 4.82 ppm, respectively, are the most affected by the presence of Mn^{2+} (Fig. 1). The more Mn^{2+} added, the broader the NMR signals.

The Mn^{2+} replaces some of the Na^{+} as the counter ion of the carboxylate group in the iduronic acid moiety. Mn^{2+} is a paramagnetic ion and will make the protons close in space (H1 and H5) to relax very effectively. Paramagnetic ions induce paramagnetic relaxation in NMR which in turn will cause a broadening of signals. The shorter the relaxation time, the broader the signals [3]. With all other factors kept equal which contribute to the broadening of NMR signals; the addition of Mn^{2+} shortens the relaxation time of some protons due to the paramagnetic relaxation mechanism. The addition of Mn^{2+} (10 μg/g heparin) to a heparin solution doubles the half-height linewidth of the H1 signal at 5.22 ppm, corresponding to a halving of the relaxation time [4].

The OSCS acetate methyl signal, which is used for the detection and quantification of OSCS in heparin solutions, is very much affected by the presence of Mn^{2+}. In OSCS, there are sulphate groups close to the acetate methyl group. When they complex with the Mn^{2+}, the acetate methyl protons relax more effectively, thus causing broadening of the NMR signal.

The broadening of the NMR signal of the OSCS acetate methyl group affects the identification and levels of detection. In a collaborative NMR study on heparin [5], the limit of detection of OSCS in heparin was set to 0.1% (100 mg/g heparin). To reach such a level, the OSCS acetate methyl signal must be relatively sharp.

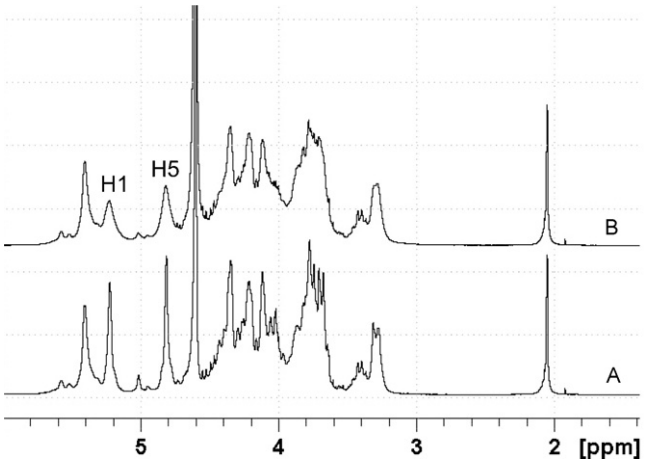

Fig. 1. Spectrum A, heparin sodium. Spectrum B, the same sample after addition of Mn^{2+} (10 μg/g heparin). The spectra were obtained with a 300 MHz NMR instrument at 37 °C.

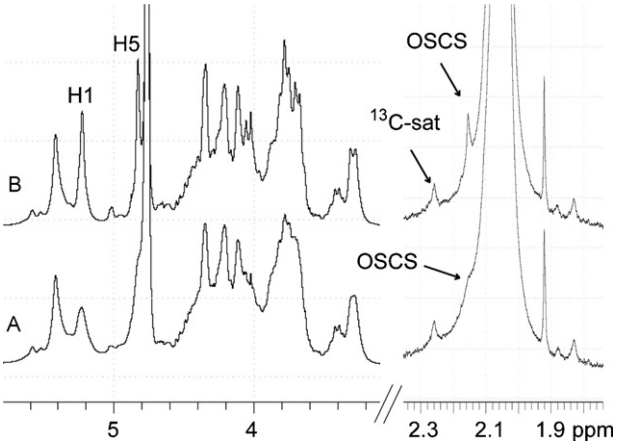

Fig. 2. NMR spectra obtained with a 300 MHz NMR instrument at 25 °C. The right side of the spectra have been expanded in the *x* and *y* axes. Spectrum A: heparin spiked with OSCS (2.5 mg/g heparin) and Mn^{2+} (10 μg/g heparin). Spectrum B: after addition of EDTA (100 μg/g heparin) to the same heparin solution. The ^{13}C-satellites belong to the heparin acetate methyl signal at 2.04 ppm.

The effects of the paramagnetic metal ions: Cu^{2+}, Co^{2+}, Fe^{3+} and Zn^{2+}, on the appearance of the heparin and OSCS NMR spectra were also studied. The addition of Fe^{3+} and Zn^{2+} to a heparin solution spiked with OSCS did not affect the spectrum of heparin or of OSCS. The addition of Cu^{2+} and Co^{2+} to a heparin solution spiked with OSCS did affect the spectrum of heparin but not the OSCS acetate methyl signal.

3.2. Addition of EDTA to heparin solutions which show broad NMR signals

EDTA is a very effective chelating agent that will sequester Mn^{2+} and other metal ions. Fig. 2 shows a much improved spectrum of heparin and a much improved OSCS acetate methyl signal after the addition of EDTA (100 μg/g heparin) to a solution of heparin with OSCS (2.5 mg/g heparin) that had previously been spiked with Mn^{2+} (10 μg/g heparin). The metal ions are sequestered by the chelating agent so that they will not interact any more with the polysaccharides. The protons in heparin and OSCS can relax in the usual more slowly way thus giving sharper signals.

Out of those heparin samples (not previously spiked with metal ions) that showed different degrees of broadening of some NMR signals compared to those in the average heparin spectrum, a heparin sample with a very similar broadening pattern as that obtained with a pure heparin sample spiked with Mn^{2+} (10 μg/g heparin) was chosen in this discussion (Spectra A in Figs. 2 and 3). Fig. 3 shows a much improved spectrum of heparin and a much improved OSCS acetate methyl signal after the addition of EDTA (100 μg/g heparin) to a solution of the chosen heparin spiked with OSCS (5 mg/g heparin).

The amounts of EDTA (100 μg/g heparin) and Mn^{2+} (10 μg/g heparin) used in these experiments correspond to roughly 1.2 to 1 molar equivalent. No NMR signals from EDTA were visible at such low concentrations.

The same amount of EDTA (100 μg/g heparin) was needed for cancelling the broadening of the OSCS and heparin signals of a solution of heparin with OSCS spiked with Mn^{2+} (10 μg/g heparin) (Fig. 2B) as to cancel the broadening of the OSCS and heparin signals of the chosen heparin (not spiked with metal ions) (Fig. 3B).

Instead of adding EDTA into the NMR tube containing the heparin sample, which is the simplest way, a heparin solution spiked with OSCS was passed through a bed of cation exchange resin (for

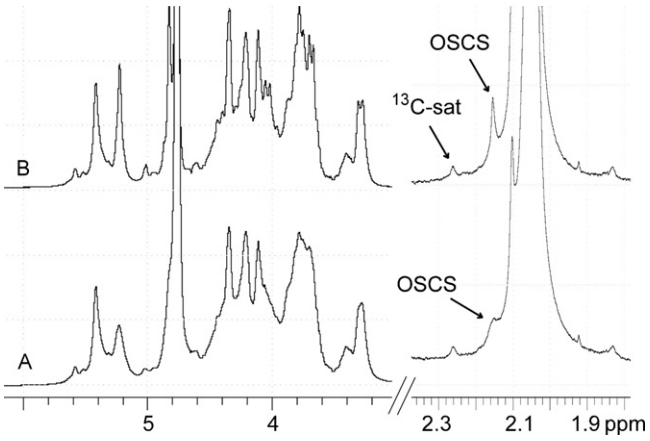

Fig. 3. NMR spectra obtained with a 300 MHz NMR instrument at 25 °C. The right side of the spectra have been expanded in the *x* and *y* axes. Spectrum A: example of a heparin spiked with OSCS (5 mg/g heparin) but not previously spiked with metal ions. Spectrum B: after addition of EDTA (100 μg/g heparin) to the same heparin solution. The ^{13}C-satellites belong to the heparin acetate methyl signal at 2.04 ppm.

instance Chelex 100). The same results were obtained as when EDTA was used.

4. Conclusions

The presence of heavy metals (up to 30 μg/g heparin) is allowed in heparin according to the European Pharmacopoeia [6]. Depending on the source of the heparin, the production method (for instance permanganate bleaching), the way of storing it etc, metals ions (within the range of the allowed quantities) with high affinity for heparin and good paramagnetic relaxation enhancement, may end up in the heparin and cause broadening of the signals in the ^{1}H NMR spectra. This may make the identification of heparin problematic. Metal ions also affect the OSCS acetate methyl signal. This could make the detection and quantification of OSCS in heparin difficult.

If there is any broadening of the heparin NMR signals, the metal ions that cause this broadening should be taken away with EDTA or by filtering the heparin solution through a cation exchange resin. If the height of the NMR heparin signal at 5.22 ppm is less than 70–80% of the height of the signal at 5.42 ppm, treatment of the heparin solution is highly recommended. If EDTA is used, 300 μg EDTA/g heparin is the recommended amount.

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