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Short communication

Effect of Ca^{2+} on the ^1H NMR chemical shift of the methyl signal of oversulphated chondroitin sulphate, a contaminant in heparin

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ABSTRACT

The chemical shift of the methyl signal of oversulphated chondroitin sulphate (OSCS) is dependent on the type and concentration of the counterion. When OSCS is present as a contaminant in heparin sodium, the reported methyl ^1H chemical shift is 2.15 ± 0.02 ppm. In this report, a value of 2.18 ± 0.01 ppm is reported for the OSCS in the presence of Ca^{2+}. The chemical shift of the methyl signal of pure OSCS varies linearly from 2.13 ppm to 2.18 ppm with increasing amounts of Ca^{2+}, until reaching the saturation point of four Ca^{2+} ions per OSCS disaccharide unit, which contains four sulphate groups (a 1:1 ratio between sulphate groups and Ca^{2+}). This Ca^{2+} effect can be used for OSCS identification as well as to facilitate quantification.

1. Introduction

Heparin is widely used as an anticoagulant [1]. At the end of 2007, some patients died after its administration [2]. Until then, analyses done following the pharmacopeia heparin analytical methods [1] did not detect any impurities. Other advanced analytical techniques were then used to find out if there was something wrong with the heparins. With NMR, other signals apart from the heparin signals were observed. After intense studies, the contaminant was identified as oversulphated chondroitin sulphate (OSCS) [3,4], which, like heparin, has an anticoagulant effect [5]. It has been shown that OSCS has a hypotension effect, which has probably been the cause of the death of some patients [6]. Other analytical methods are being developed to detect and identify OSCS, among others, capillary electrophoresis and SAX-HPLC (strong anion exchange-HPLC).

OSCS is a synthetically modified oligosaccharide (Fig. 1), which cannot be formed in any of the steps in the production of heparin. So far, the main belief is that OSCS has been intentionally added to heparin [7].

Dermatan sulphate is another naturally occurring polysaccharide, which is a process related impurity found in levels up to a few percent in heparin products. Dermatan sulphate, at these concentrations, is not considered to be harmful [8].

Heparin has a basic repeating disaccharide unit of uronic acid and glucosamine. OSCS and dermatan sulphate have a basic repeating disaccharide unit of uronic acid and galactosamine. In the case of OSCS and dermatan sulphate, each amino group is acetylated; but in heparin, roughly every fifth amino group is acetylated.

The NMR signals of the acetyl methyl groups are well separated from the other NMR signals and are found at 2.04 ± 0.01 ppm for heparin, 2.08 ± 0.02 ppm for dermatan sulphate, and 2.15 ± 0.02 ppm for OSCS in heparin sodium [3]. In our experience the OSCS methyl signal is found between 2.15 ppm and 2.16 ppm. The mentioned OSCS methyl chemical shifts have been obtained when OSCS is in heparin sodium. But there are also calcium heparin products. Ca^{2+}could also be added intentionally or unintentionally to heparin sodium. In this paper, it will be shown that the OSCS methyl chemical shift is dependent on the type and concentration of the counterion. This has implications in the correct identification of OSCS in heparin, as well as in the practical NMR work.

2. Experimental

2.1. Materials and chemicals

OSCS sodium, Nadroparin, Calciparin and OSCS contaminated heparin were obtained from our counterpart (medicines control authority) AFSSAPS in France. Purity of the OSCS sodium was more than 90%. The identification of the OSCS was conducted with 2D-NMR HSQC and other NMR experiments and confirmed published data [5].

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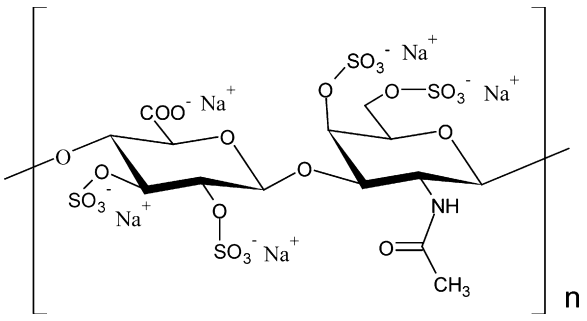

Fig. 1. Oversulphated chondroitin sulphate (OSCS) repeating disaccharide unit.

Calcium acetate monohydrate (99%) was obtained from Sigma–Aldrich; $CaCl_2{\cdot}2H_2O$ p.a. quality from Merck; deuterated water (99.8%) from Armar Chemicals and sodium 3-trimethylsilyl tetradeuteriopropionate (TSP) from Dr. Glaser AG.

2.2. NMR spectroscopy

A 600 MHz Bruker Avance NMR instrument equipped with a CryoProbe was used. The following parameters were used for the acquisition of the spectra. Time between pulses: 8 s, number of transients: 64, temperature: 25 °C, spectral width: 12 ppm and number of complex data points: 32 K. The following parameters were used for the processing of the raw data. Exponential line broadening function: 0.3 Hz. Reference set at 0.000 ppm for the TSP signal.

3. Results and discussion

3.1. Effect of Ca^{2+} on the ^1H NMR chemical shift of OSCS methyl signal

OSCS is a glycosaminoglycan with a repeating disaccharide unit containing a glucuronic acid and an *N*-acetylgalactosamine (Fig. 1). OSCS is chemically produced from chodroitin sulphate by the sulphonation of the hydroxy groups.

Fig. 2 shows the NMR chemical shift of the OSCS methyl signal versus the Ca^{2+} added to a pure OSCS solution. The *x*-axis is given as the concentration ratio of [Ca^{2+}]:[OSCS disaccharide unit]. The chemical shift of the OSCS methyl signal moves downfield (higher ppm values) linearly until the value four is reached on the *x*-axis. At this "saturation point", there are four Ca^{2+} present to complex one to one with the four sulphate groups in the OSCS disaccharide unit. The fact that the curve is linear to the saturation point means that there is a strong affinity of the sulphate groups for Ca^{2+}. After the saturation point, there is still a progressive but slight increment of the chemical shift of the OSCS methyl signal. This could be attributed to other types of Ca^{2+} complexes.

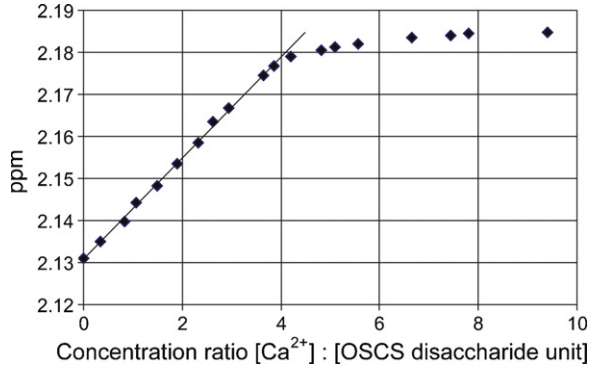

Fig. 2. Titration of OSCS (0.4 mg/ml) in deuterated water with calcium acetate. The *y*-axis is the NMR chemical shift of the OSCS methyl signal; and the *x*-axis, the concentration ratio of [Ca^{2+}]:[OSCS disaccharide unit].

The addition of Ca^{2+} to pure dermatan sulphate or heparin sodium affects only very slightly the chemical shift of their methyl signal.

OSCS has two sulphate groups per monosaccharide unit and they lie close in space to each other. At a first glance, one could suggest that each Ca^{2+} could bind to two contiguous sulphate groups and in that way only two Ca^{2+} per OSCS disaccharide unit would suffice to reach the saturation point. The graph in Fig. 2 clearly shows that the saturation point is only reached when each sulphate group is coordinated to a single Ca^{2+} ion.

The downfield effect (higher ppm values) of the OSCS methyl signal in the presence of Ca^{2+} is probably due to polarization of the OSCS acetamide group when it complexes with the Ca^{2+}. Charge is withdrawn from the carbonyl group and inductively also from the methyl group. Charge withdrawing causes a downfield effect of NMR signals. Alternatively, the downfield effect could be due to conformational changes of the polysaccharide.

Before the saturation point is reached (4 Ca^{2+} on the *x*-axis in Fig. 2), there are two states: one where the acetamide group is coordinated to or affected by the Ca^{2+} and the other when it is not. One could perhaps expect to observe two separate OSCS methyl signals. In fact, only one is observed because there is a rapid exchange between the two states; and with NMR, only the weighed mean value of the two states can be observed.

Below, three examples are shown of the interaction of Ca^{2+} with OSCS in heparin.

3.2. Titration of OSCS with Nadroparin

In Fig. 3, a pure OSCS solution is titrated with Nadroparin, which is a low molecular mass calcium heparin. At a concentration ratio [OSCS]: [Nadroparin] of 1:2 (spectrum B), the chemical shift of OSCS is 2.156 ppm. OSCS has four sulphate groups per disaccharide unit and Nadroparin about 2.3. At the concentration ratio of 1:2, the total number of available sulphate groups is $4+(2.3\times2)$, namely: 8.6; and the number of added calcium is (2×2.3), namely: 4.6. Assuming that the affinity of the sulphate groups for Ca^{2+} is the same in OSCS as in Nadroparin, the calcium ions are then shared proportionally between OSCS and Nadroparin. OSCS will then complex with 2.1 Ca^{2+} ($(4\times4.6)/8.6$), which corresponds to a chemical shift

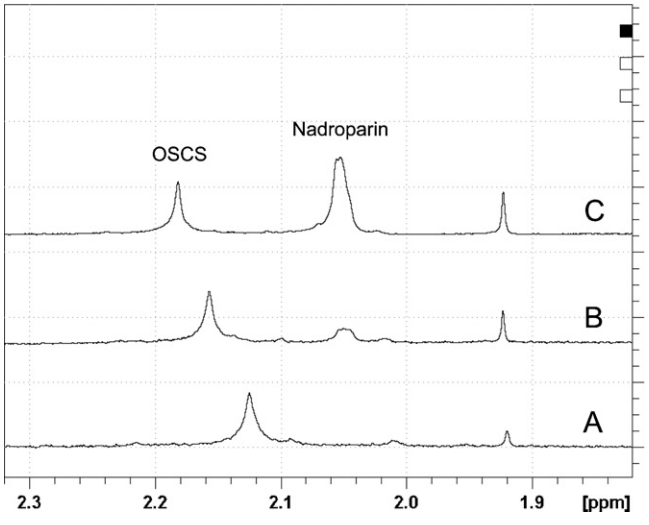

Fig. 3. Titration of OSCS (0.3 mg/ml) in deuterated water with Nadroparin (δ_H = 2.05 ppm). Some sodium acetate (δ_H = 1.92 ppm) is present in all spectra. Spectrum A, 100% OSCS. Spectrum B, 1:2 concentration ratio of [OSCS]: [Nadroparin]. Spectrum C, 1:15 concentration ratio of [OSCS]: [Nadroparin].

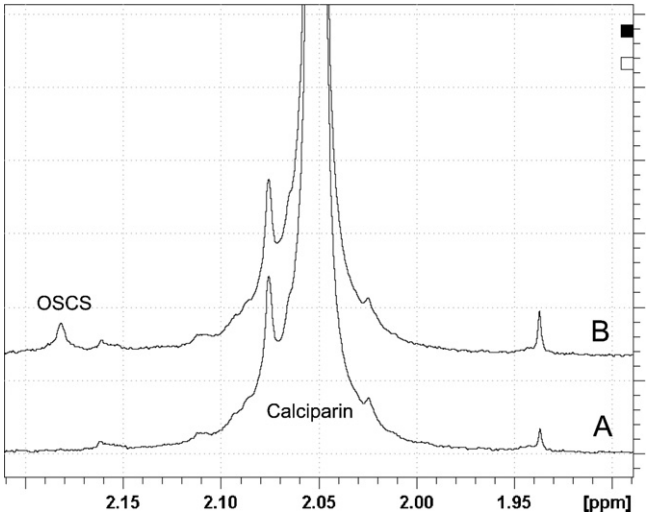

Fig. 4. Spectrum A, a Calciparin solution (20 mg/ml). Spectrum B, same Calciparin spiked with OSCS.

of 2.155 ppm (see plot in Fig. 2). Well in accordance with the OSCS methyl chemical shift in spectrum B in Fig. 3.

3.3. Addition of OSCS to Calciparin

Fig. 4 shows Calciparin, which is a calcium heparin (spectrum A); and Calciparin spiked with a low amount of OSCS (spectrum B). The methyl signal of the OSCS is at 2.182 ppm. Because of the small amount of OSCS compared with the calcium heparin, practically all sulphate groups in OSCS are complexed with Ca^{2+}.

Any OSCS contamination of a heparin calcium will then show a signal at about 2.18 ppm, which is outside the 2.15 ± 0.02 ppm for the identification of OSCS in heparin sodium [3].

3.4. Addition of Ca^{2+} to an OSCS contaminated heparin sodium solution

Spectrum A in Fig. 5 shows an OSCS contaminated heparin sodium sample. The OSCS methyl signal coincides with the ^{13}C satellite of the heparin methyl signal when a 600 MHz NMR instrument is used. The addition of $CaCl_2{\cdot}2H_2O$ to the heparin solution

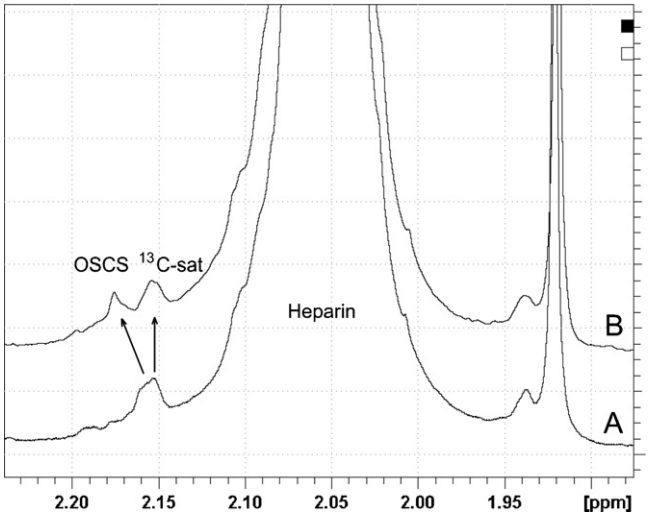

Fig. 5. Spectrum A, an OSCS contaminated heparin sodium solution (20 mg/ml). The OSCS methyl signal coincides with the chemical shift of the ^{13}C satellite of the heparin methyl signal. Spectrum B, same heparin solution with added $CaCl_2{\cdot}2H_2O$.

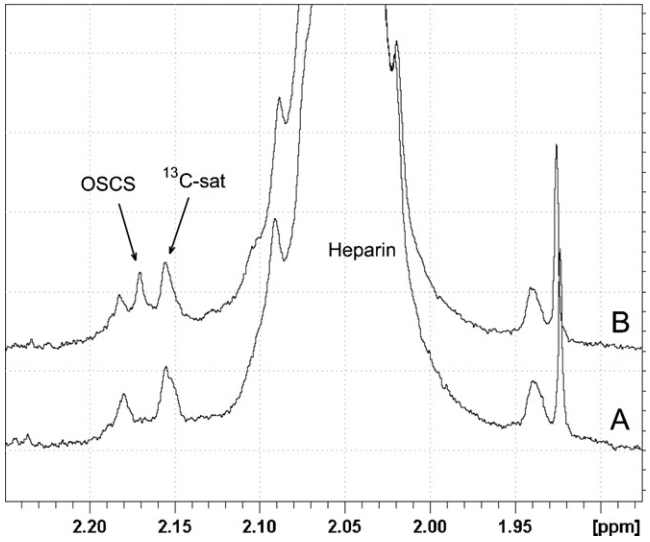

Fig. 6. Spectrum A, a Fragmin solution. Spectrum B, same solution spiked with OSCS and $CaCl_2{\cdot}2H_2O$.

(spectrum B) makes the OSCS methyl signal to move downfield to 2.18 ppm. Enough $CaCl_2{\cdot}2H_2O$ has to be added as to saturate all the sulphate groups in heparin and OSCS. By choosing to add less $CaCl_2{\cdot}2H_2O$ to the heparin solution, the OSCS chemical shift can be placed where it is wanted.

When a 600 MHz NMR instrument is used, it is recommended that $CaCl_2{\cdot}2H_2O$ is added on a routine basis before obtaining a spectrum (1 mg $CaCl_2{\cdot}2H_2O$ per 10 mg heparin giving a chemical shift of about 2.17 ppm, or 2 mg $CaCl_2{\cdot}2H_2O$ per 10 mg heparin giving a chemical shift of about 2.18 ppm). With a 500 MHz instrument, the OSCS methyl signal, when Ca^{2+} is added, will coincide with the ^{13}C satellite of methyl heparin signal.

The Ca^{2+} effect on the chemical shift of the OSCS methyl signal can be conveniently used to demonstrate the presence of OSCS in heparin sodium samples if there is any doubt of the identity of the OSCS signal.

It has been observed that that the OSCS methyl signal becomes a bit sharper with the addition of Ca^{2+}. This means that the intensity of the signal becomes higher and therefore the limit of detection (LoD) improves somewhat.

The presence of a signal at 2.18 ppm in a heparin solution does not mean by itself that it is OSCS. In the low molecular mass sodium heparin Fragmin and in some unfractionated heparins, there is a signal at 2.18 ppm (Fig. 6). But this is not OSCS. If Fragmin is spiked with OSCS, a new signal appears corresponding to the signal of the OSCS methyl group.

4. Conclusions

The chemical shift of the OSCS methyl signal varies from 2.131 ppm to 2.185 depending on the concentration of Ca^{2+}.

When studying contamination of OSCS in heparin, the presence, or possible presence of Ca^{2+} should be taken into account. In calcium heparin, the OSCS methyl signal appears at 2.18 ± 0.01 ppm, well outside the region 2.15 ± 0.02 ppm of the OSCS methyl signal in heparin sodium.

Addition of $CaCl_2$ to contaminated heparin sodium can be used so that the OSCS methyl signal moves away from the heparin and dermatan sulphate methyl signals so that OSCS can be more easily quantified. Also, addition of Ca^{2+} makes the OSCS methyl signal sharper, which improves the LoD.

Ca^{2+} can be used for spiking a heparin sodium solution if a confirmation of the presence of OSCS is needed instead of spiking with OSCS.

$CaCl_2$ can be added to a contaminated heparin sodium solution if there is an interference with the ^{13}C satellite of the heparin methyl signal.

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