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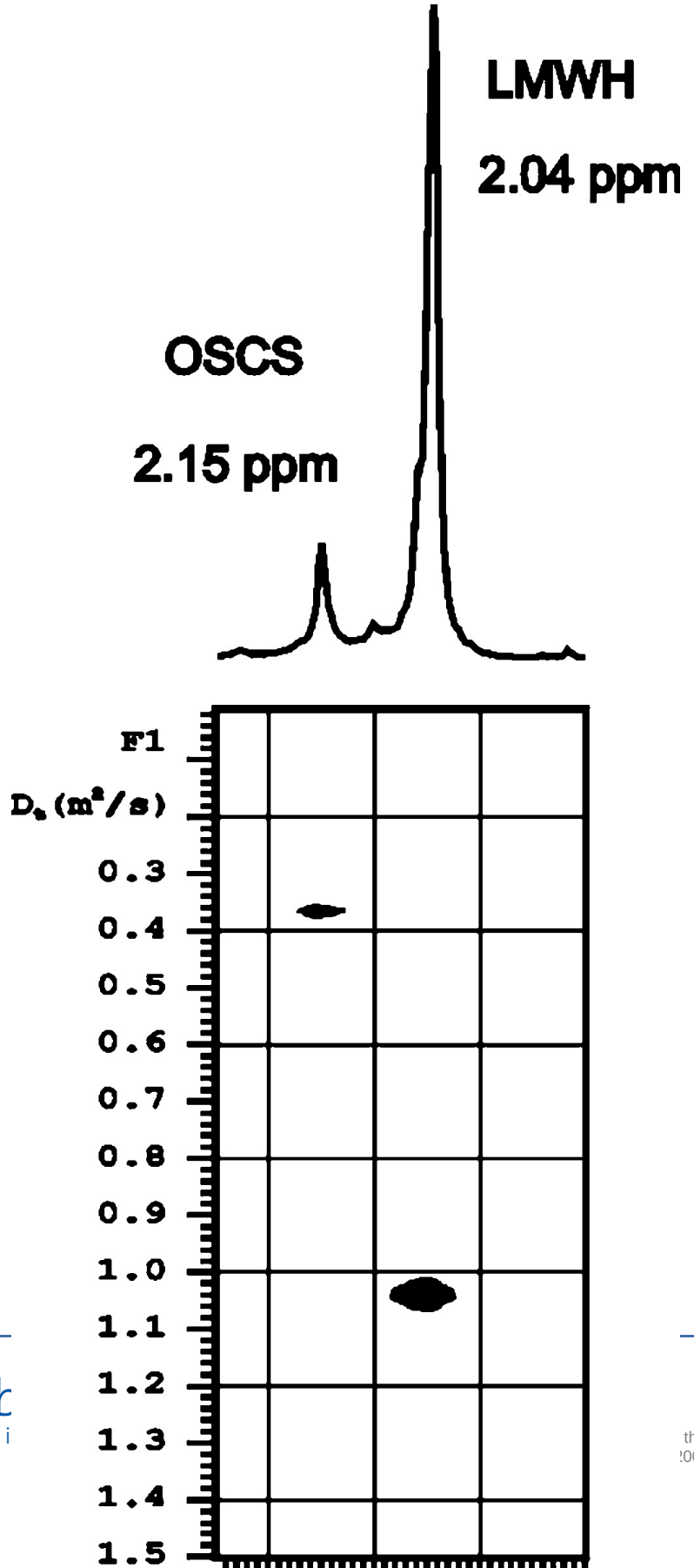

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Letters

Assessment of Oversulfated Chondroitin Sulfate in Low Molecular Weight and Unfractioned Heparins Diffusion Ordered Nuclear Magnetic Resonance Spectroscopy Method

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Abstract: Heparins can be contaminated with oversulfated chondroitin sulfate, OSCS, the impurity being linked to adverse clinical events that certain lots of heparins have had on humans. Here, we propose labeling of the *N*-acetyl peaks in ^{1}H NMR spectra of heparins with the parameter D_t, describing the translational diffusion coefficient available from DOSY NMR. We show how DOSY can be applied as a routine method for screening the lots of heparins for obtaining the impurity profile when using ^{1}H NMR.

The biopolymeric carbohydrate drug heparin is the oldest and widely used anticoagulant[1,2] and is a member of the glycosaminoglycan family extracted from animal tissues. It has been used clinically for over 75 years. More recently, low molecular weight heparins, LMWHs,[a] obtained by partial depolymerization of heparin in controlled chemical or enzymatic processes, have been introduced as anticoagulant and antithrombotic agents.[3] Capillary/gel electrophoresis, CE/GE, has been used to map the oligosaccharide composition,[4,5] and NMR spectroscopy aided successfully in the structure characterization and elucidation of its conformation in solution.[6−8]

Earlier this year, authorities in the U.S. and Europe were informed of adverse effects that certain lots of heparin have had on humans. Oversulfated chondroitin sulfate, OSCS,[9] has been identified as a contaminant associated with these adverse clinical effects.[10] Following this study a thorough account of the quality assessment of more than 100 unfractionated heparin samples from international markets appeared.[11] ^{1}H-1D NMR allowed reliable quantification of the major contaminants OSCS and DS (dermatan sulfate). Furthermore, the chemical stability of OSCS in five depolymerization reactions, similar to those used in the preparation of LMWHs, has been investigated.[12] It was shown that base-catalyzed β-elimination partially degrades OSCS and hydrogen peroxide treatment results in its complete degradation, whereas nitrous acid, heparin lyase, and periodate oxidation treatments leave the OSCS essentially intact. Therefore, depolymerized OSCS could be a possible contaminant of pharmaceuticals with enoxaparin or ardeparin as the API. It is thus clear that the complex mixtures produced in the depolymerization of contaminated heparin via base-catalyzed β-elimination or oxidative depolymerization with hydrogen peroxide would make analysis of the impurity profile of LMWHs difficult using either capillary electrophoresis or ^{1}H-1D NMR. The polydispersity of the degraded OSCS prevents characterization of the contamination by CE using known standards. Furthermore, it was not clear whether the chemical shifts of *N*-acetyl signals in degraded OSCS coincide with the chemical shift of the OSCS standard at 2.15 ppm.

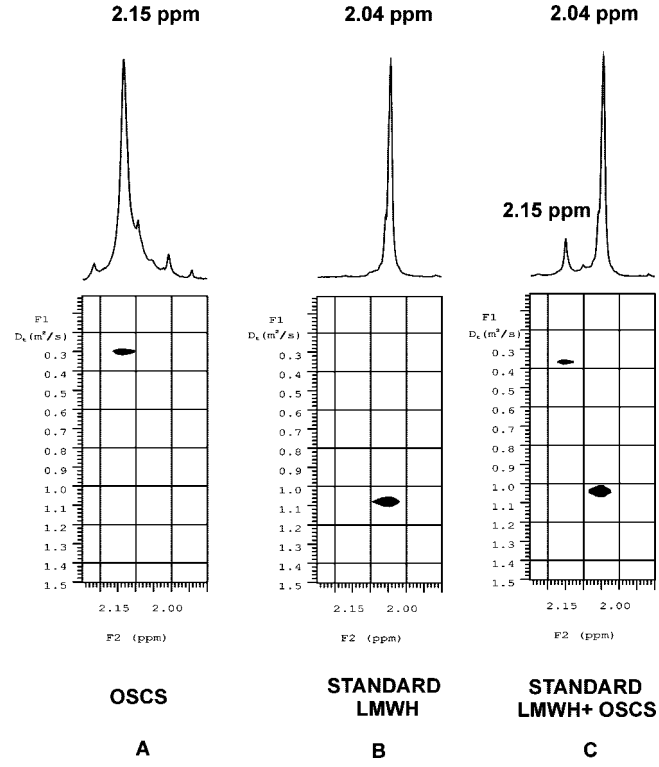

Figure 1. ^{1}H NMR DOSY traces of the *N*-acetyl region of (A) OSCS, (B) low-molecular-weight heparin standard, and (C) LMWH spiked with OSCS. Each cross-peak relates the translational diffusion coefficient, $D_t \times 10^{-10}$ m^2/s, on the F1 axis to its chemical shift on the F2 axis.

Neither the present USP nor the Ph Eu protocol specifies disclosure of the OSCS as a contaminant. Therefore, the responsible authorities have suggested that LMWHs and unfractioned heparins are examined by ^{1}H-1D NMR, utilizing the established differences in the *N*-acetyl chemical shifts of the three major components of the finished pharmaceutical product, API (2.04 ppm), DS (2.08 ppm), and OSCS (2.15 ppm).

From the considerations discussed above it seemed obvious that separately neither CE nor ^{1}H-1D NMR alone can guarantee reliable evaluation and therefore the safety of LMWH pharmaceuticals. The complex situation requires a method that will label each chemical shift in the ^{1}H-1D NMR spectrum with the molecular weight of the species it represents. Here, we propose that this parameter can be the translational diffusion coefficient,

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[a] Abbreviations: LMWHs, low molecular weight heparins; HMWHs, high molecular weight heparins; DOSY, diffusion ordered spectroscopy; OSCS, oversulfated chondroitin sulfate; CE, capillary electrophoresis; GE, gel electrophoresis; NMR, nuclear magnetic resonance; DS, dermatan sulfate; MW, molecular weight; API, active pharmaceutical ingredient.

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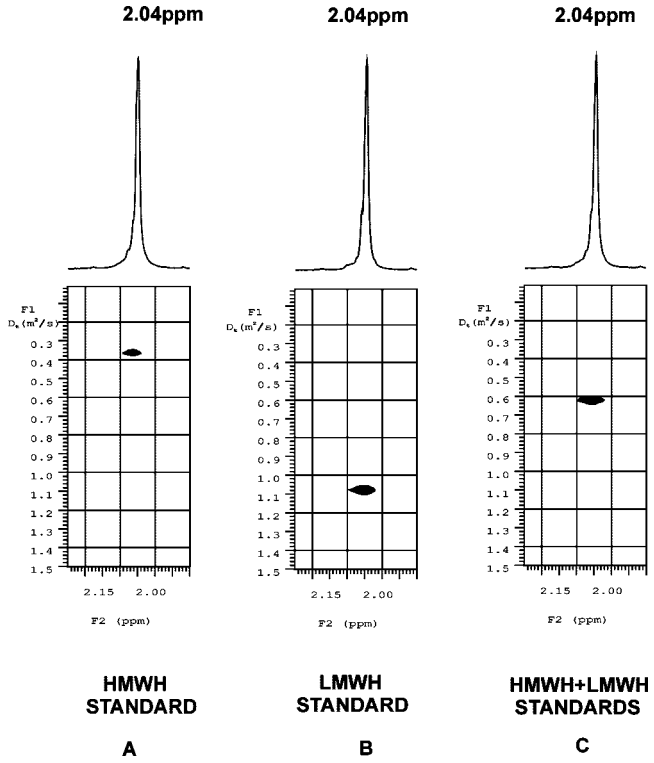

Figure 2. ^{1}H NMR DOSY traces of the *N*-acetyl region of (A) high molecular weight heparin standard, (B) low molecular weight heparin standard, and (C) a 1:1 mixture of both.

D_t (m^2/s), which is readily available from DOSY NMR. DOSY combines the information that is specific to CE and ^{1}H-1D NMR.

DOSY NMR is a powerful method for analyzing mixtures of chemical species in solution;[13] it measures the translational diffusion coefficient D_t. Larger molecules diffuse more slowly than smaller ones. Each spectral line in the ^{1}H -1D NMR spectrum of a given chemical entity should be characterized by the same D_t, providing the line is separated from spectral lines of other species in solution, which are characterized by their own D_t. Separation of the lines is better achieved with higher magnetic fields. The larger is the difference in MW of the two species, the larger is the difference in D_t between them. This method can therefore be applied to differentiation between unfractionated heparins, the OSCS impurity of about 18 kDa MW[12] and the LMWHs of about 4−8 kDa.

Here, we show how DOSY can be applied as a routine method for screening different lots of heparins for obtaining their impurity profile. Figure 1 demonstrates how the ^{1}H DOSY trace of the *N*-acetyl region can be used to identify OSCS in the LMWHs. It is clearly seen that the diffusion coefficient of OSCS has a much lower value, $D_t \approx 0.3 \times 10^{-10}$ m^2/s, than the LMWH, $D_t \approx 1.05 \times 10^{-10}$ m^2/s. In the absence of other species in solution these values may be treated as characterizing each type of polysaccharide. Therefore, each chemical shift was labeled with its specific diffusion coefficient reflecting its molecular weight (Figure 1C).

It is of primary importance to note that pure low and high molecular weight heparins cannot be distinguished on the basis of their *N*-acetyl chemical shifts, which are the same. Figure 2 demonstrates how the DOSY experiment gives a clear distinction between the two because the diffusion coefficients differ dramatically, 1.05×10^{-10} vs 0.38×10^{-10} m^2/s. Therefore, the purity of the sample can be easily judged from the value of D_t. A mixture of both heparins will be characterized by a diffusion coefficient that is the weighted average of the diffusion coefficients of both components of the mixture (Figure 2C).

Figure 3C shows the DOSY trace of the *N*-acetyl region in a contaminated LMWH market product. The three major components of current interest, LMWH, DS, and OSCS, are readily identified by their *N*-acetyl chemical shifts, 2.04, 2.08, and 2.15 ppm, respectively.[11]

The D_t of the LMWH sample shown in Figure 3C is found be close to its reference value of 1.05×10^{-10} m^2/s (see Figure 1B), with its diffusion coefficient being negligibly dependent on the closely lying signals of DS and OSCS present in small

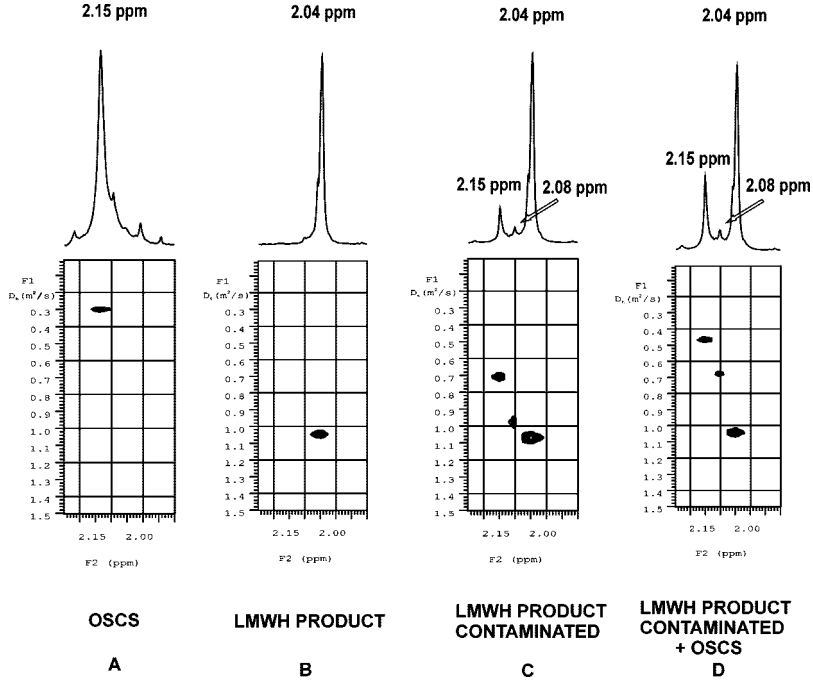

Figure 3. ^{1}H NMR DOSY traces of the *N*-acetyl region in the spectra of (A) OSCS, (B) low molecular weight market product, (C) low molecular weight market product contaminated with DS and OSCS, and (D) sample in (C) spiked with OSCS.

amounts. The diffusion coefficients of each of the three species are dependent mainly on their molecular weight. To a lesser extent they are also dependent on the overlap of closely lying spectral lines. This is more severe in the case of the DS *N*-acetyl signal, which lies very close, 2.08 ppm compared to the LMWH signal at 2.04 ppm. By comparison to the spiking of LMWH with OSCS (an amount similar to that added to the sample in Figure 3C) in Figure 1C, the D_t value for the signal at 2.15 ppm in Figure 3C is unexpected. If it was high molecular weight OSCS that was spiked in, then the D_t value should be about 0.35×10^{-10} m^2/s, not 0.7×10^{-10} m^2/s as seen in Figure 3C. This discrepancy can tentatively be interpreted as indicating a contaminated pharmaceutical sample with a peak at 2.15 ppm that has a much larger diffusion coefficient D_t associated with it (Figure 3C). This explanation seems plausible in view of the results published by Linhardt[12] who discloses the fact that OSCS, present in unfractioned heparin which undergoes alkaline treatment to obtain LMWH, can degrade. It can therefore be expected that the degradation products of OSCS may be present in the finished product at the same chemical shift, 2.15 ppm. To confirm this hypothesis, spiking the sample of Figure 3C with OSCS was performed. Spiking the sample with pure OSCS resulted in a rise of the intensity of a signal at 2.15 ppm and a change of its D_t to lower value, from 0.7×10^{-10} to 0.5×10^{-10} m^2/s (Figure 3D) as expected for a species having the same *N*-acetyl chemical shift. It is well-known that the proton NMR chemical scale has low dispersion, and coincidental overlap of chemical shift values of different species can occur.

We strongly believe that these results demonstrate that ^{1}H NMR can be used for screening market samples for contamination with OSCS. It is noted that the interpretation of the D_t values, likewise the interpretation of ^{1}H NMR chemical shifts, requires consideration of the overlap of closely lying spectral lines, which changes their true D_t value, and more importantly, one needs to be aware that ^{1}H chemical shift can be identical for two species differing markedly in molecular weight. Finally, noncovalent binding of the two species in solution can influence the true D_t and chemical shift values. While we have addressed some problems that should be clarified before eventually implementing the method into pharmaceutical practice, i.e., defining the D_t values of reference materials in standard conditions or the effect on its D_t value of spiking pure heparin with OSCS or DS, additional answers are required. A full investigation is ongoing.

Supporting Information Available: Experimental details, sample preparation, pulsed field gradient spin echo experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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