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Using Pulse Field Gradient NMR-Based Diffusion Experiments To Identify Signals of Low-Molecular-Weight Impurities

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Abstract:

Differences in diffusion rate were utilised to identify signals of low-molecular-weight impurities in NMR spectra of synthetic pentasaccharide samples using NMR pulse field gradients. This method is of general use to discriminate between low-molecular-weight impurities and high-molecular-weight compounds in mixtures.

Synthetic pentasaccharides, currently in development for human therapeutic applications, as shown in Scheme 1, are the product of an elaborate multistep chemical synthesis.[1] NMR spectra of the crude pentasaccharides obtained before purification often contain impurity peaks at a low (<1%) level. These extra peaks can originate either from compounds related to the desired product or from low-molecular-weight unrelated impurities. Unrelated impurities may be introduced by solvents and/or reagents used in the synthesis, solvents used in crystallisation, and leachables released from plastic tubing during processing. These process-related impurities differ in molecular weight and polarity from the pentasaccharide-related compounds, and they are therefore, readily removed by chromatographic techniques. This, and (usually!) the absence of pharmacological action, makes their presence less worrisome than that of related impurities. Hence, it is relevant to discriminate NMR signals from related impurities from those of low-molecular-weight unrelated impurities. Because there is a large difference in molecular weight between the unrelated impurities and the related impurities, we successfully utilised differences in diffusion constant as measured using pulsed field gradients[2−13] to identify signals due to low-molecular-weight impurities.

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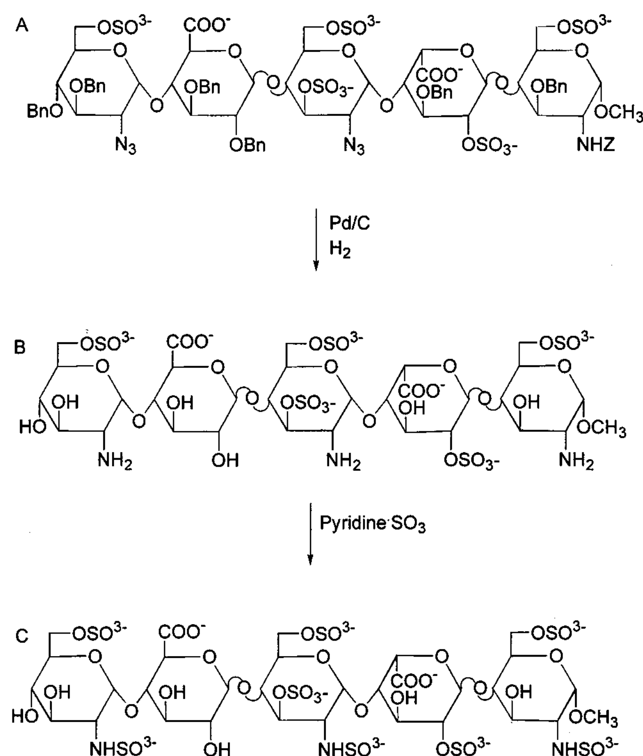
Scheme 1. **Final two steps in the synthesis of the pentasaccharide used in this study[1]**



The synthetic pentasaccharide samples we studied contained only small amounts (<1%) of impurities. In the past, pulse field gradient experiments using differences in diffusion rates have been applied in the analysis of complex, but roughly equimolar, mixtures.[2−13] This was done by 2D diffusion ordered spectroscopy (DOSY).[2,3,6,7] DOSY is a pseudo-two-dimensional NMR technique yielding spectra with the diffusion coefficient along one axis and the spectrum of the component along the other axis.[2] However, neither 2D DOSY nor the related DECODES[9] technique would have been applicable to our samples, since the low level of the impurities (<1%) would have required prohibitively long acquisition times. Trying to establish connectivities between the peaks of the signals in the aromatic region and the aliphatic carbohydrate signals by either homonuclear (TOCSY, 2D NOE) or heteronuclear (HMBC) 2D correlation spectroscopy suffers the same drawback.[12]

Because of the large difference in molecular size between the pentasaccharides and pentasaccharide-related impurities on one hand, and low-molecular-weight unrelated impurities on the other hand, a simple gradient on/off experiment

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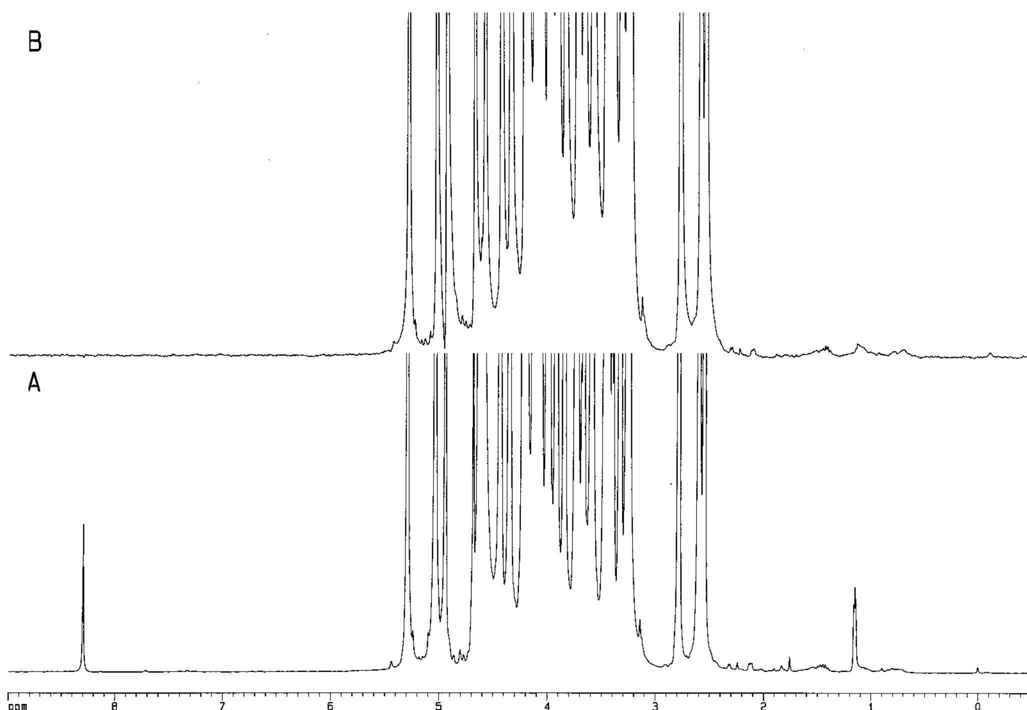


Figure 1. **(A) Reference spectrum of a deprotected synthetic pentasaccharide (Scheme 2B) showing a 20 mol % impurity at 8.3 ppm and additional impurity signals at 1.15 ppm. The signals from 5.4 to 2.3 ppm are from the pentasaccharide, except for the signal at 4.6 ppm, which originates from the residual HDO in D_2O. (B). PFGLED spectrum plotted at the same vertical intensity for the pentasaccharide signals. The peaks at 8.3 and 1.15 ppm are strongly attenuated. This indicates that they originate from low-molecular-weight unrelated impurities. Also, the water signal is attenuated due to its rapid diffusion.**

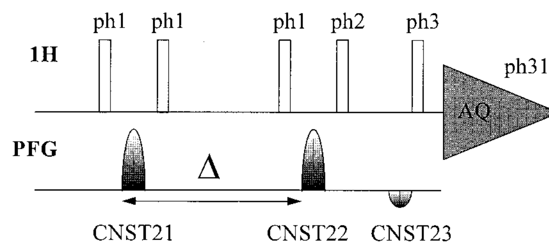
yielding two 1D NMR spectra should be capable of discriminating pentasaccharide-related and low-molecular-weight unrelated molecules.

This experiment is essentially the stimulated echo experiment originally proposed by Tanner,[13] applied to achieve "diffusion weighing" of the intensities of the NMR signals. Conversely, this application could be thought of as a DOSY experiment in which resolution in the diffusion direction has been traded for sensitivity to an extreme: only two points are obtained in the diffusion direction. The consequence is that no quantitative information is obtained on the value of the diffusion coefficient. However, for our application this is not severe, since our goal is qualitatively identifying low-molecular-weight impurities

This is illustrated by the analyses of a number of synthetic pentasaccharide samples.

Scheme 1 shows the final two steps of the synthesis of the active pentasaccharide fragment of the drug heparin.[1] During catalytic hydrogenation to remove the benzylic protecting group (Scheme 1A → B), a small portion of the reaction mixture was worked-up and analysed by NMR to check the progress of the reaction. The ^{1}H NMR spectrum still showed only a small amount of signal in the aromatic region of the spectrum (Figure 1A). However, a peak was observed at about ~8.3 ppm corresponding to an impurity of 20 mol %. To determine whether this signal originated from a related impurity, or from an unrelated impurity, an experiment was done using pulse field gradients. The pulse sequence used for the experiment is the pulse field gradient longitudinal encode−decode (PFGLED)[5] sequence, shown in Scheme 2, with and without applying gradients. Since the

Scheme 2. **Pulse sequence of a pulse field gradient longitudinal encode−decode (PFGLED) 1D ^{1}H NMR experiment[5]**



magnetisation is flipped back along the Z axis after the first gradient and before the delay Δ, no T_2 relaxation effects are active, while T_1 effects will be minor if Δ is kept <100 ms.[5] The strength and length of the gradient pulses and the delay separating them should be tuned in such a way as to allow a clear distinction between low- and high-molecular-weight species (see also Experimental Section). This will result in two 1D NMR spectra, in which the spectrum obtained with gradients will show strongly attenuated signals from rapidly diffusing, smaller species. Also, more slowly diffusing, larger molecules will show attenuated signals, but the intensity of their signals will be less affected. Figure 1A and B shows the spectra obtained without and with applying gradients, respectively. The spectra have been vertically expanded to show the impurity, while the intensity of the carbohydrate signals has been plotted at the same intensity level in both spectra to allow comparison. In Figure 1B, the signal at 8.30 ppm is clearly almost completely attenuated as compared to the carbohydrate signals. This indicates that this signal originates from a species with a different diffusion coef-

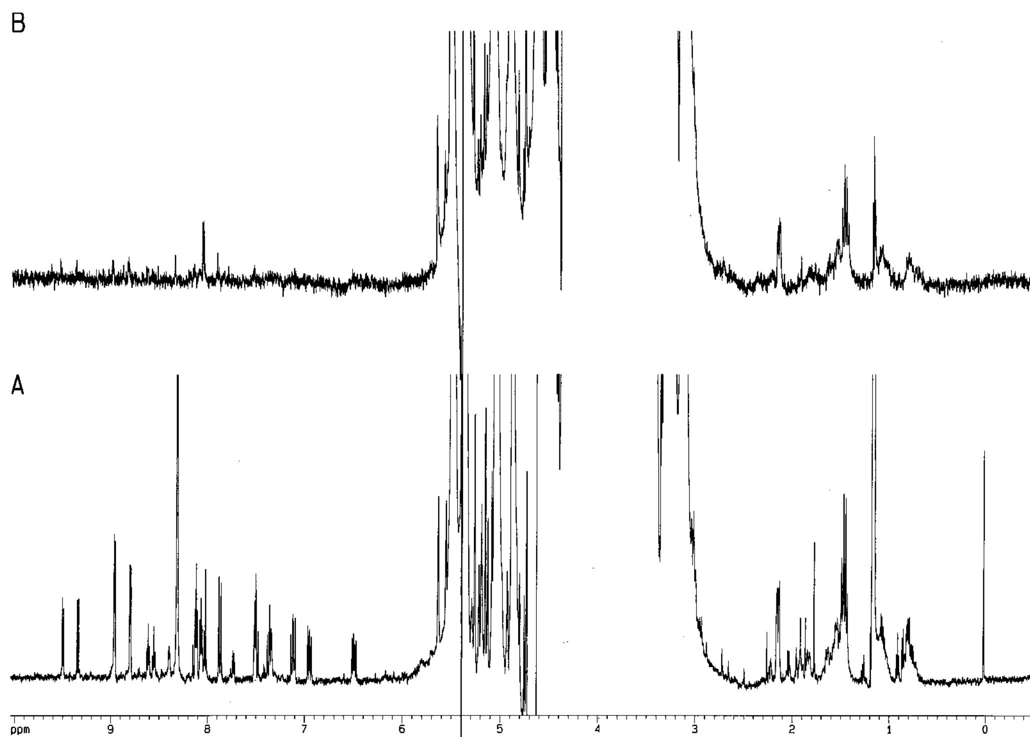


Figure 2. **(A) Reference spectrum. Vertically expanded proton NMR spectrum of a synthetic pentasaccharide. The peaks >6 and <2.5 ppm originate from impurities, while the signal at 0 ppm is from the reference compound TSP. The remaining signals are from the pentasaccharide. (B) PFGLED spectrum plotted at the same vertical intensity for the pentasaccharide signals. The impurity signals are strongly attenuated relative to the saccharide peaks. This indicates that they arise from low-molecular-weight, rapidly diffusing species, unrelated to the pentasaccharide. The TSP signal is undetectable, also due to its fast diffusion.**

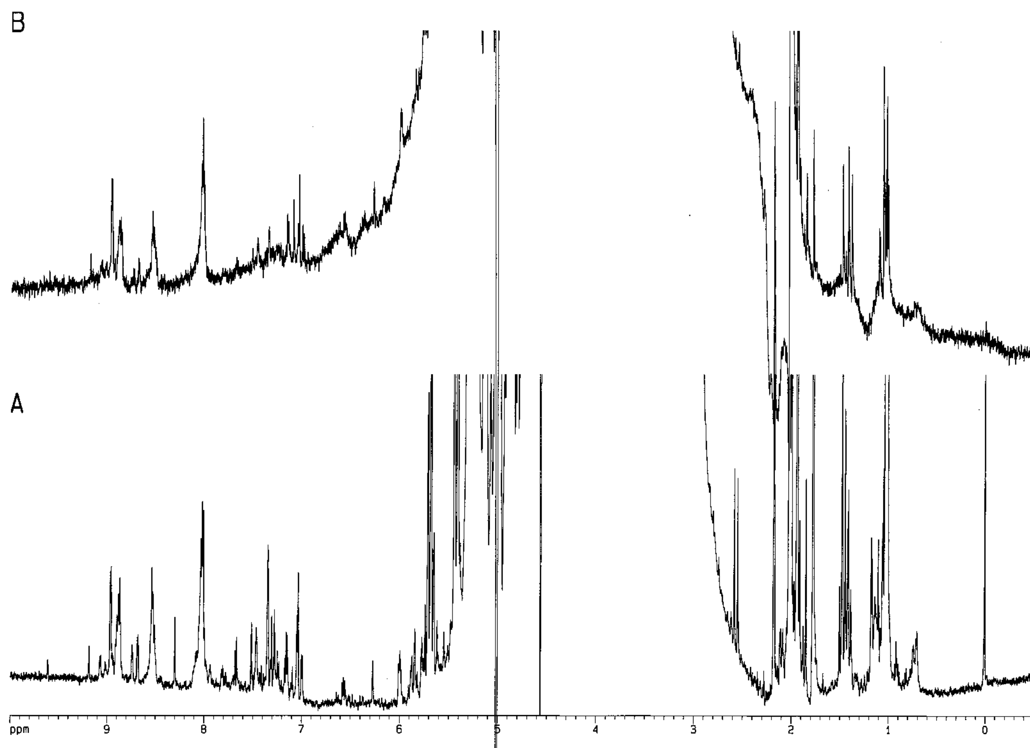


Figure 3. **(A) Reference spectrum of a synthetic pentasaccharide, showing numerous impurity signals in the aromatic region. (B) PFGFLED spectrum plotted at the same vertical intensity for the pentasaccharide signals. The singlet signals at about 8.3 and 0 ppm, attributable to formic acid and TSP, respectively, are strongly attenuated, while other signals are virtually unaffected. This may indicate that they originate from related impurities bearing aromatic substituents.**

ficient, probably indicating a low-molecular-weight unrelated impurity. Also, as expected, the water resonance at ~4.6 ppm is strongly attenuated by the applied pulse sequence. The aliphatic impurities at 0.7−2.1 ppm also seem to be of lower

molecular weight. Although the 20 mol % impurity could also have been analysed using a regular DOSY experiment, this example nicely illustrates the potential of the method. The peak at ∼8.30 ppm was later shown to be due to formic acid.

Scheme 1B → C shows the final step in the synthesis of the pentasaccharide: the N-sulphation using a pyridine trioxide complex. The spectrum of the crude reaction product again shows extra peaks in the aromatic region (Figure 2A): the formic acid peak of about 20 mol % at ∼8.30 ppm noticed earlier, and several other peaks of compounds probably related to pyridine residues at a lower level. Again, using pulsed field gradients, it could be shown that the extra signals in the aromatic region originate from rapidly diffusing, probably unrelated, low-molecular-weight impurities (Figure 2B). This was confirmed by a purification based on molecular size using gel filtration, which efficiently removed these impurities (results not shown).

Figure 3A and B shows the spectra of another crude pentasaccharide sample obtained after sulphation with pyridine trioxide.[1] In this case, some signals in the aromatic region are not attenuated after applying the gradients, while the intensities of other aromatic signals are reduced. This probably indicates that both related and unrelated impurities give rise to signals in the aromatic area in this sample. However, it should be pointed out that, although the attenuation of impurity signals points to a low-molecular-weight, and therefore unrelated, impurity, the reverse is *not* necessarily true: since no quantitative information is obtained on the diffusion rate, unattenuated signals cannot definitely be said to arise from related impurities. Stated otherwise; this method is suited for identifying unrelated impurities but not for identifying related impurities.

Conclusion

Low-molecular-weight impurities in synthetic pentasaccharides samples could readily be identified on the basis of their (relative) diffusion rates, determined using a PFGLED experiment. The experiment requires ca. 30 min of experimental time. This technique is expected to be of general applicability to identify signals of low-molecular-weight impurities which differ significantly in molecular size and, therefore, in diffusion coefficient. Because only two 1D NMR spectra are required, in contrast to the 2D DOSY experiment and more conventional 2D NMR correlation experiments, low-level impurities can also be analysed using this technique.

Experimental Section

Spectra were obtained using a Bruker DRX 600 spectrometer and a Bruker 5-mm inverse probe equipped with shielded pulsed field Z gradients. Approximately 20 mg of pentasaccharide was dissolved in 0.7 mL of D_2O. A modified PFGLED sequence was used, as shown in Scheme 1.[5] Δ was set at 50 ms. Between 128 and 512 scans were obtained, acquiring 32*K* data points for the spectra displayed in Figures 1 and 2. The strength of the first two gradient pulses (CNST 21 and 22) was first set at 0 to record the reference spectrum, and then to 60 to obtain the diffusion spectrum. These values are appropriate for our probe and these particular samples and need not be the same for other samples and/or probes. The probe used in this study has a maximum gradient strength of 50 G/cm^2 and can be adjusted linearly from 0 to 95 (maximum strength). The length of the gradient pulses was 3 ms, while the duration Δ between the gradients was 50 ms. Total acquisition time was ca. 30 min. For the spectrum displayed in Figure 3, 8*K* scans were collected, resulting in a measuring time of ca. 8 h. Spectra were referenced either relative to the remaining HDO resonance set at 4.60 ppm or relative to trimethylsilanepropionic acid (TSP) at 0.0 ppm.

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Supporting Information Available

Pulse sequence used and a list of acquisition parameters. This material is available free of charge via the Internet at http://pubs.acs.org.

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