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Assignment of the 750 MHz ^{1}H NMR resonances from a mixture of transacylated ester glucuronic acid conjugates with the aid of oversampling and digital filtering during acquisition

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Abstract

Many drugs containing carboxylic acid functional groups are metabolised in vivo to ester glucuronides (1-O-acyl-β-D-glucopyranuronates) and, of these, a number show a propensity to undergo internal isomerisation via a transacylation process, causing the carboxylic acid moiety to migrate successively to the 2-, 3- and 4-positions of the glucuronic acid. These products may be responsible, through reactions with plasma proteins, for some of the allergenic side effects in a number of non-steroidal anti-inflammatory drugs. It is important to understand those properties of the drug molecules which facilitate this reaction, and to this end we have studied the transacylation product formation and reaction kinetics in a series of aryl carboxylic acid glucuronides using NMR spectroscopy. However, the resulting ^{1}H NMR spectra are very complex with much resonance overlap, and recourse to spectral simplification processes is necessary. Here, improvement in spectral resolution by oversampling and digital filtering to restrict the detection range of the spectrometer, thus yielding improved digital resolution, is demonstrated. The approach has been applied to the assignment of a mixture of transacylated ester glucuronides of 2-trifluoromethylbenzoic acid through the use of a two-dimensional ^{1}H–^{1}H TOCSY experiment.

Keywords: 750 MHz ^{1}H NMR; 2-Dimensional; Glucuronide; Transacylation; Filtering

1. Introduction

One of the major problems in the structural identification of substances in complex mixtures, such as drug metabolites in biofluids, using NMR spectroscopy is the degree of overlap of resonances even at the highest observation frequencies generally available. One approach is to develop extraction methods such as SPEC-NMR [1] or to use directly coupled HPLC-NMR [2]. Alternatively, it may be possible to use a heteronucleus such as ^{19}F present in the drug metabolite species to provide a convenient handle to deduce the ^{1}H NMR spectra via heteronuclear correlation techniques [3].

Such specialised approaches are often not applicable, and then it is necessary to use the full power of multi-dimensional NMR to improve the resonance overlap problem. How-

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ever, this brings its own problems in that large data matrices are required to provide good digital resolution to allow adequate peak definition. This is particularly acute in the second (so called F_1) dimension of a two-dimensional NMR experiment which results from the incremented time period in the two-dimensional pulse sequence [4]. Digital resolution in the F_1 dimension can be improved by the application of suitable data processing methods such as linear prediction [5]. In addition, digital resolution in the acquisition dimension of a two-dimensional NMR experiment (the F_2 axis) can also be less than optimum because of the need to cover a spectral range which includes all peaks, whether their measurement is required or not, in order to avoid peaks outside the spectral window folding back into the spectrum.

The recent introduction of oversampling and digital filtering techniques into NMR data acquisition regimes [6] now means that acquisition spectral windows can be contracted to include only the region of interest in the F_2 dimension, without the problem of signals from outside folding in. Oversampling consists of the collection of digital data points at a rate faster than that required to satisfy the Nyquist criterion of twice the highest desired spectral frequency [7]. In theory, for an oversampling factor of n, a gain in dynamic range of $\log_2(n)$ is obtained, i.e. for eight-fold oversampling an effective gain of three bits in the ADC resolution of the signals results. In practice, the oversampled signal is simply averaged over the n measurements to restore the same number of data points corresponding to the Nyquist criterion, and this prevents folding of noise or artefacts which would have been in the extended spectral region, resulting as a consequence of the oversampling being equivalent to a spectral region n times wider than required to satisfy the Nyquist criterion. Thus, a second consequence of oversampling is an improved signal-to-noise ratio due to the removal of folded noise when the spectral region is truncated. The digital filtering is an improvement on the usual hardware filters applied which are far from ideal and thus also allows the removal of artefacts such as signals which would have been folded in from outside the standard spectral region.

Many drugs form conjugates with β-D-glucuronic acid in vivo, and in some cases multiple glucuronide species can be formed, for example, from aromatic systems which become ring hydroxylated at various ring positions. We have now tested the oversampling and digital filtering approach using the related problem of ester glucuronide transacylation reaction. This is a chemical process which causes the transfer of the carboxylic acid-containing moiety from the 1-β position of the glucuronic acid to the 2-, 3- and 4-positions successively [8]. Once these other glucuronides are formed, a free hydroxyl group at the 1-β position can mutarotate to include a proportion of the α-anomer, giving a total of seven possible isomeric glucuronides. An additional competing reaction forms the aglycone and free glucuronic acid by hydrolysis. Fig. 1 summarises the reaction scheme. This transacylation process has assumed importance because it has become clear that some of the isomeric glucuronides are particularly reactive towards serum proteins such as albumin and result in modified proteins (haptens) which are recognised by the body as foreign, leading to immunological reactions. In some cases, commercial drugs have had to be withdrawn from the market because of such adverse reactions [9].

This reaction scheme has been modelled using synthetic β-D-glucuronide conjugates of simple aryl carboxylic acids, and we describe here the application of digitally filtered, restricted F_2, two-dimensional NMR to the mixture of the glucuronides formed from 2-trifluoromethylbenzoic acid in buffer solution.

2. Experimental

The synthetic 1-*O*-(2-trifluoromethylbenzoyl)-glucuronide conjugate (see Fig. 1) was synthesised at Zeneca Pharmaceuticals [10] and its transacylation kinetics have been investigated in buffer solution using ^{19}F NMR spectroscopy at pH 7.2 [10]. The assignment of the various species, i.e. the starting material (principally 1-β-O-acyl), the 2-position glucuronides as their α- and β-anomers (2-α-O-acyl and 2-β-O-acyl), the corresponding 3- and 4-position isomers, and the aglycone (2-trifluoromethylbenzoic acid) were made, based only on their ^{19}F NMR spectra by analysis of the time-course of the resonance intensity changes assuming that the $1 \rightarrow 2 \rightarrow 3 \rightarrow 4$ migrations were necessarily sequential.

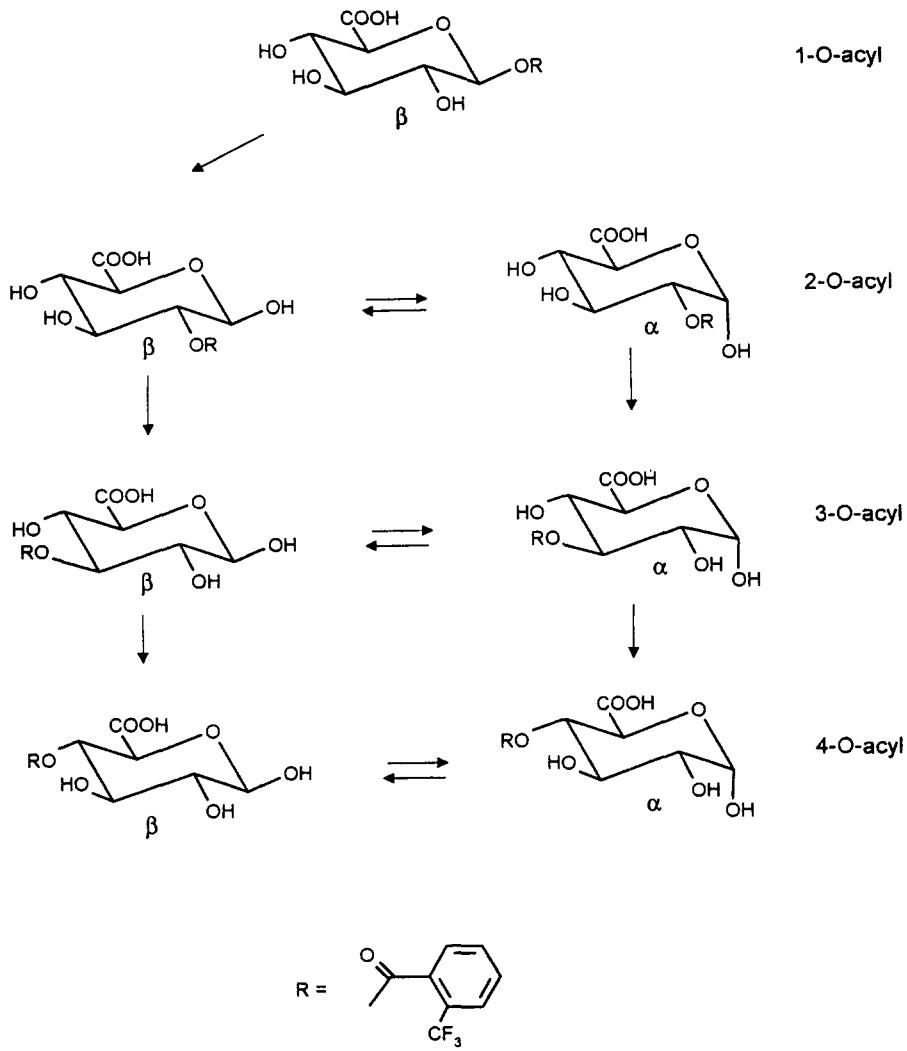

Fig. 1. Reaction scheme showing the transacylation products formed from the 1-β-O-acyl glucuronide of 2-trifluoromethylbenzoic acid.

To confirm these assignments, we have now measured 750 MHz ^{1}H two-dimensional TOCSY spectra using a restricted F_2 spectral window with oversampling and digital filtering to eliminate any folded resonances from outside the spectral window. The sample was a mixture of the 1-O-acylglucuronides of 2-trifluoromethylbenzoic acid comprising a mixture of the α- and β-forms in the proportions of 1:7 (as deduced from a freshly made solution) dissolved in 0.1 M disodium phosphate buffer and allowed to undergo transacylation reactions at 310 K for 24 h. The NMR spectra were acquired at 750.13 MHz using a Bruker DMX-750 instrument. Conventional one-dimensional spectra were acquired using eight scans and a spectral width of 4006 Hz into 32 K data points. The data were zero-filled by a factor of two before Fourier transformation and a line-broadening factor of 0.7 Hz was applied. Cubic spline baseline correction was used before plotting. Oversampled one-dimensional spectroscopy and a two-dimensional TOCSY experiment used eight scans per increment for 512 increments, with a spectral width of 861 Hz in F_2 and 4006 Hz in F_1, to give 1 K data points. This corresponds to an oversampling factor of 128. In addition, the large water resonance was suppressed using a shaped-pulse with off-resonance excitation comprising a square pulse of 100 ms duration repeated 15 times. Before Fourier transformation, the data were multiplied by a shifted sine-bell function and zero-filled by a factor of two in F_2, and forward linear prediction was used to double the number of data points in F_1 using the values of 128 previous points to predict the next.

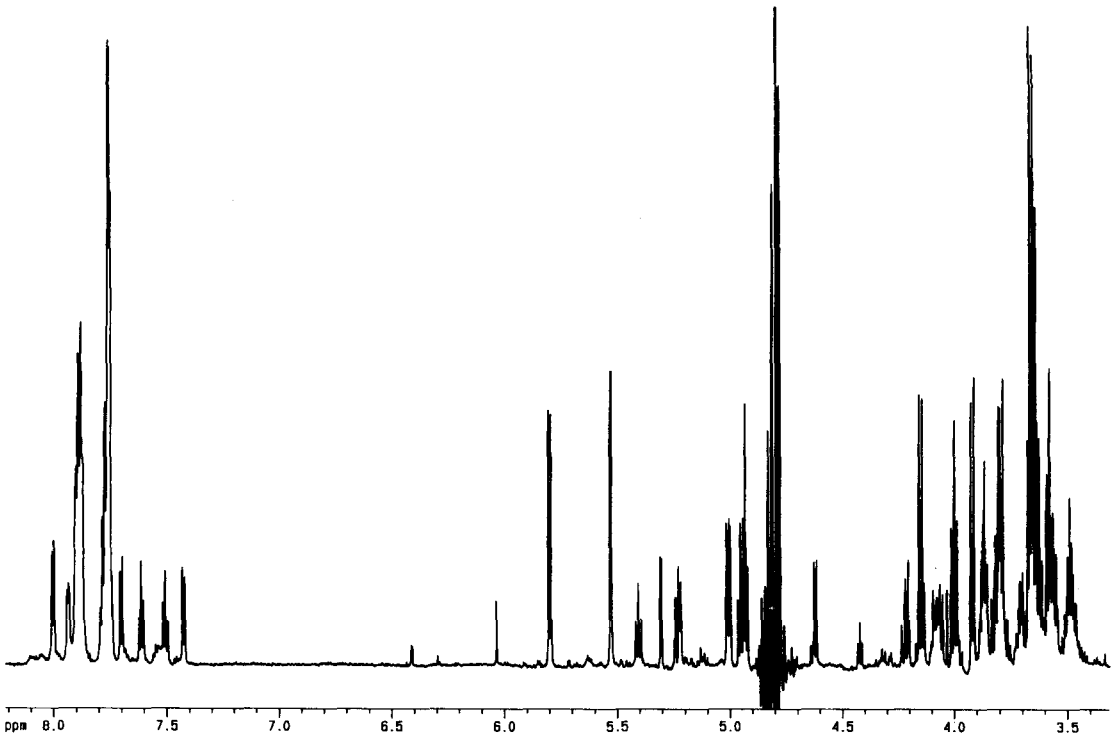

Fig. 2. 750 MHz ^{1}H NMR spectrum (δ3.3–8.2) of a partially equilibrated mixture of transacylated glucuronide conjugates of 2-trifluoromethylbenzoic acid in 0.1 M disodium phosphate buffer at pH 7.4.

3. Results and discussion

The standard single pulse 750 MHz ^{1}H NMR spectrum (δ3.3–8.2) of the partially equilibrated mixture of 2-trifluoromethylbenzoic acid glucuronide isomers in buffer obtained from presaturation of the large water signal using the NOESYPRESAT method [11] is shown in Fig. 2. The signals from the aromatic protons of the glucuronides are clearly visible as well as the complex bands seen between about δ3.5 and the suppressed water resonance at about δ4.8. In addition, a number of resonances can be seen to high frequency of the water resonance, and these arise from some of the anomeric protons of the various glucuronide isomers and from other deshielded protons caused by esterification of the different hydroxyl groups on the glucuronic acid moiety. The H1 proton resonances of the various isomers appear as doublets with characteristic coupling constants to the H2 protons for α- and β-isomers of about 3.5–4.0 Hz and 7.5–8.5 Hz respectively. Other signals which appear in this region and which have higher multiplicity arise from the protons at other positions on the glucuronic acid ring which have been esterified at the hydroxyl group attached to the same carbon. However, there is considerable overlap in the spectrum and this results in difficulty of assigning most of the resonances, particularly those to low frequency of the HDO signal.

Fig. 3 depicts the two-dimensional 750 MHz ^{1}H–^{1}H TOCSY NMR spectrum obtained with the F_2 observation window limited to the region of the spectrum between δ4.9 and δ5.9 using the oversampling technique. The F_1 axis covers the same region as the ^{1}H NMR spectrum shown in Fig. 2. This particular sample had not reached equilibrium and so the major component remains the 1-β-O-acyl isomer with lower proportions of the other isomers. Assignments can be made based on the chemical shifts, spin–spin coupling patterns and integrals in the one-dimensional spectrum shown in Fig. 2, and on the connectivity information from the two-dimensional TOCSY experiment, as shown in Fig. 3. Thus, for example, the resonance at δ5.800 is a doublet of 7.7 Hz and therefore arises from an anomeric H1 proton in a β-anomer. From its intensity and chemical shift, this is the starting material, the 1-β-O-acyl compound. From this point it is possible to map out the chemical shifts for the other protons around the glucuronide ring, assuming that the H5 proton resonance is more deshielded than the others, as is usually seen in

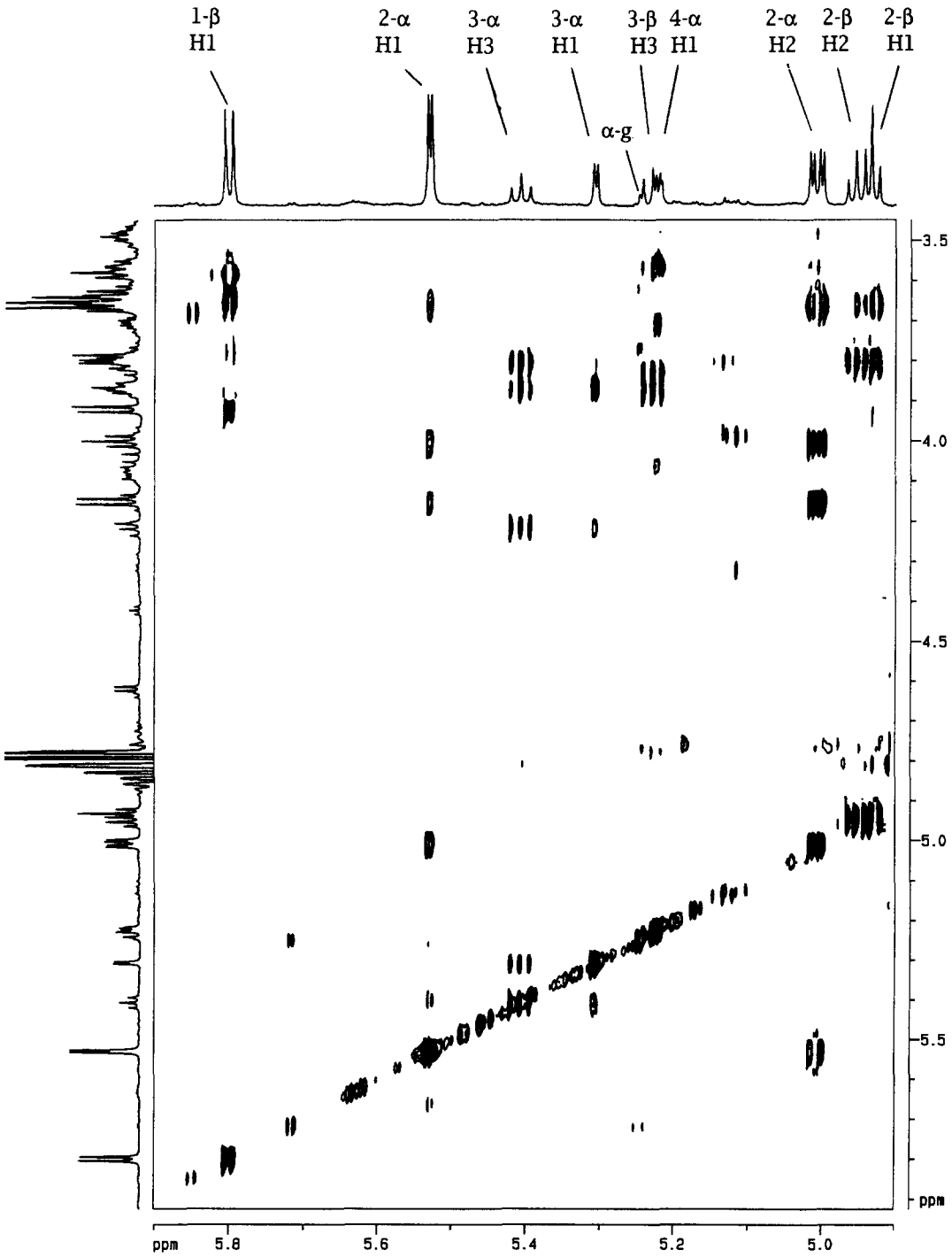

Fig. 3. Restricted F_2 ^1H–^1H 750 MHz TOCSY spectrum (δ4.9–5.9) of a partially equilibrated mixture of acyl glucuronides from 2-trifluoromethylbenzoic acid in 0.1 M disodium phosphate buffer at pH 7.4. α-g denotes the H1 proton chemical shift of α-glucuronic acid. The diagonal is observed as the band of resonances appearing at an angle from the lower left corner.

other drug metabolites [11,12], and will be a simple doublet owing to coupling to H4. The specific assignment of the H2, H3 and H4 resonances cannot be made because of their closeness and the degree of overlap in this region of the spectrum even at 750 MHz. Evidence that a low level of the 1-α-O-acyl compound is present is confirmed by the observation of a doublet at δ6.41 corresponding to the H1 proton chemical shift, but its low

Table 1
^{1}H NMR chemical shifts (δ) and coupling constants (Hz) [a] for the O-acylglucuronides of 2-trifluoromethylbenzoic acid

Glucuronide	Relative amount	δ(H1)	δ(H2)	δ(H3)	δ(H4)	δ(H5)
β-1-O-acyl	100	5.80	3.58 [b]	3.65 [b]	3.65 [b]	3.92
α-1-O-acyl	7	6.41	[c]	[c]	[c]	[c]
β-2-O-acyl	72	4.93	4.95	3.80	3.65	3.80
α-2-O-acyl	114	5.53	5.01	4.00	3.66	4.15
β-3-O-acyl	38	4.78	3.85 [b]	5.23	3.57 [b]	3.88
α-3-O-acyl	43	5.31	3.87	5.41	3.80	4.21
β-4-O-acyl	?	$\approx$4.8	[c]	[c]	[c]	[c]
α-4-O-acyl	38	5.22	3.56	3.70	?	4.06
β-Glucuronic acid	?	$\approx$4.8	[c]	[c]	[c]	[c]
α-Glucuronic acid	10	5.24	3.76 [b]	3.61 [b]	3.76 [b]	4.17

[a] Coupling constants fall into characteristic ranges of 3.6–3.7 Hz for J(H1–H2) in α isomers, 7.7–8.1 Hz for J(H1–H2) in β isomers, and 9.5–10.3 Hz for couplings between H2–H3, H3–H4 and H4–H5. Some impurity peaks which are not related to the glucuronide spin system can be observed including a triplet of 7.3 Hz at δ4.42, a triplet of 12.0 Hz at δ4.22, and doublets of 8.1 Hz at δ4.62 and δ.64.
[b] Assignments may be reversed.
[c] Resonance from the β-4-O-acyl isomer and free β-glucuronic acid cannot be assigned because the H1 chemical shift is expected to be close to the HDO resonance, and no TOCSY cross peaks will appear in the spectral window acquired.

level precludes detection of TOCSY cross-peaks. The doublet signal at δ5.53 can be assigned to the H1 proton of the 2-α-O-acyl compound because the smaller coupling constant of 3.6 Hz indicates an axial–equatorial orientation of the two coupled protons. The assignment is confirmed because the H2 proton chemical shift is easily assigned from the TOCSY connectivity to H1 and comprises a doublet of doublets; the remaining protons of this molecule can also be mapped out from their TOCSY spectra. The 2-β-O-acyl compound gives more complex resonances because the H1 and H2 proton chemical shifts are close together at δ4.93 and δ4.95 respectively. The H2 proton is more deshielded than the H1 proton, but the TOCSY spectrum allows straightforward assignment of the other chemical shifts for this molecule. The 3-α-O-acyl isomer gives rise to the two resonances at δ5.31 and δ5.41, the former being due to H1 because it is a doublet of 3.6 Hz indicating an α-configuration, and the latter arising from H3 in this molecule which is expected to be deshielded owing to the esterification shift effect and has the expected triplet structure with couplings typical of an axial proton vicinal to two other axial protons. The 3-β-O-acyl compound gives rise to one resonance in the spectral region at higher frequency than the HDO resonance due to H3 which appears as a triplet at δ5.23. The TOCSY spectrum then allows assignment of the H1 resonance which can be seen at δ4.78 which, being so close to the HDO resonance, would not be visible in the one-dimensional spectrum. The other ring proton chemical shifts for this molecule can also be mapped out using the TOCSY spectrum. The 4-α-O-acyl isomer can be detected in the spectrum with its H1 resonance almost coincident with that from H3 of the 3-β-O-acyl compound. The 4-β-O-acyl isomer is not detected because it is expected that all of the chemical shifts will be outside the range of the TOCSY experiment, including both H1 and H4 resonances which will be close to the HDO resonance [12]. No evidence from free β-glucuronic acid can be found because the chemical shift for the H1 proton is expected at about δ4.8 underneath the HDO signal. A low level of α-glucuronic acid is detected via the TOCSY connectivities from the H1 proton chemical shift observed at δ5.24. The chemical shifts and coupling constants for all species are summarised in Table 1, together with the relative concentrations derived by integration of well resolved ^{1}H NMR resonances. These are in agreement with those derived previously using ^{19}F NMR spectroscopy.

These results show that through the combination of a very high NMR observation frequency of 750 MHz, the use of methods to improve digital resolution and the application of two-dimensional spectroscopy, it is possible to achieve assignments of the ^{1}H NMR resonances of this mixture of glucuronide conjugates. Even for this relatively simple

carbohydrate mixture, analysis is not possible by inspection. The application of digital filtering and oversampling provides a restricted F_2 spectral window and allows high digital resolution along this axis. In addition, the technique of linear prediction gives good resolution along the F_1 axis by extrapolating the experimental data effectively.

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