Recovery of Underwater Resonances by Magnetization Transferred NMR Spectroscopy (RECUR-NMR)

Maili Liu,* Huiru Tang,† Jeremy K. Nicholson,† and John C. Lindon†,[1]

Laboratories of Magnetic Resonance and Atomic and Molecular Physics, Wuhan Institute of Physics and Mathematics, Chinese Academy of Sciences, Wuhan 430071, People's Republic of China, and †Biological Chemistry, Biomedical Sciences, Faculty of Medicine, Imperial College of Science, Technology and Medicine, Sir Alexander Fleming Building, South Kensington, London SW7 2AZ, United Kingdom

A method for detecting small molecule NMR resonances under a water peak in biological samples is presented. After high-efficiency solvent suppression using double WATERGATE, either a TOCSY- or ROESY-based coherence transfer sequence is applied to reestablish the resonances close to, or under, water through magnetization transfer using scalar or dipolar coupling, respectively. The use of the TOCSY and ROESY methods ensures an in-phase magnetization transfer, which makes the new approach readily extended for the measurement of transverse relaxation times, internuclear ROEs, and ROE buildup rates. An extension of the new approach for *J*-resolved spectroscopy is also presented and tested using a sample of human blood plasma. © 2001 Academic Press

INTRODUCTION

The suppression of the water resonance has been of fundamental importance for NMR studies of biological systems and there are a number of recent reviews on the topic (*1–4*). The basic criteria for an effective solvent suppression method are (a) high efficiency to suppress the water peak, which is about 10^5–10^6 times as intense as the resonances of the biological molecules of interest, (b) high selectivity to avoid suppression of the resonances close to the water, and (c) short duration to minimize magnetization transfer to the exchangeable protons. Among all of the techniques developed so far, such as WET (*5*) and WATERGATE (*6*), an improved WATERGATE method appears to meet all these criteria (*7*). A large problem of all general methods is that the resonances obscured by the water peak (underwater peaks) are usually also suppressed. Attempts to overcome this have been made in NMR spectroscopic studies of macromolecules by taking advantage of the differences in proton longitudinal relaxation times (T_1) of the water and of the macromolecule. By this means, it is possible to observe the resonances of a macromolecule by the choice of an appropriate

recovery time following an inversion scheme (*8*). In addition, a gradient-enhanced HMQC method has been used for T_1 editing (*9*). This method works at the natural abundance of ^{13}C or ^{15}N and when the experiment is repeated rapidly, the water signal can be partially suppressed because of its long relaxation time compared to the short relaxation times of the spins in large molecules. These gradient-enhanced HMQC and HSQC experiments can give a reasonable result without further water suppression, but if a longer prescan delay is used in the case of small molecules, additional water suppression would be necessary and also ^{12}CH suppression using a BIRD sequence. Alternatively, a diffusion filter can be used to attenuate the resonance from the fast diffusing water and leave resonances of macromolecules to be observed such as in the DRYCLEAN method (*10*). However, these approaches are also not generally suitable for small metabolites in biological fluids because of the similar T_1 and diffusion coefficients of the protons of the water and metabolites. Methods based on differences in T_2 relaxation times between the resonances of water and those of obscured peaks have also been demonstrated. This is especially efficient if the water resonance T_2 can be artificially shortened, albeit by contaminating the sample (*11*). In this paper, we present a method termed RECUR NMR, which stands for recovery of the underwater resonances by magnetization transfer NMR and which is based on the modified WATERGATE peak suppression scheme followed by a magnetization transfer step. This is similar in concept to other approaches (*12, 13*) but, as will be seen, shows significant advantages. The method has been tested using a mixture of D-glucose and D-galactose, and also by using human blood plasma as an example of biofluid. Extensions to the method for measurement of ROE buildup curves and transverse-relaxation times (T_2) of the underwater resonances, as well as *J*-resolved spectroscopy, are also demonstrated.

EXPERIMENTAL

The water suppression is based on the double WATERGATE method with the 5-element pulse train (W5) as shown in Fig. 1a.
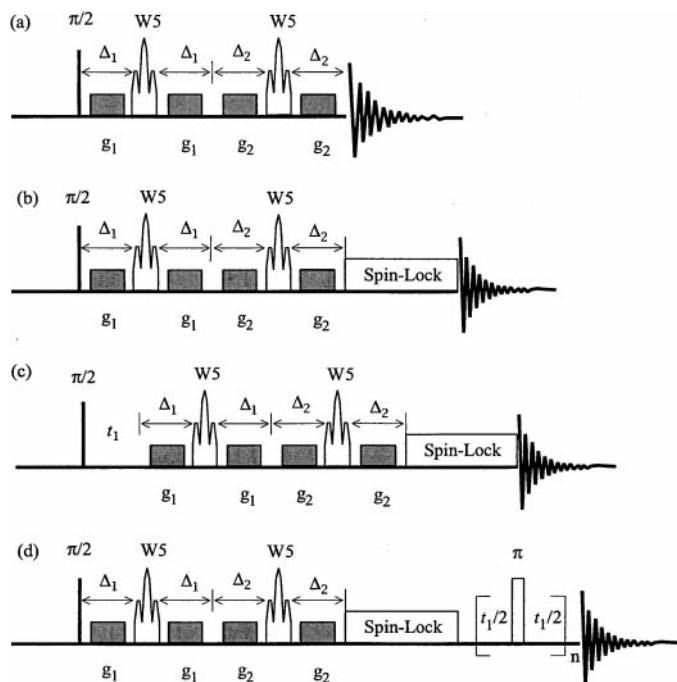

FIG. 1. (a) Standard double WATERGATE-W5 pulse sequence. (b) One-dimensional and (c) two-dimensional RECUR-TOCSY and RECUR-ROESY pulse sequences containing a spin-lock pulse sequence of either a MLEV-17 or continuous pulse to achieve TOCSY- or ROESY-type magnetization transfer for reestablishing the resonances under the water peak. (d) RECUR-JRES ($n = 1$) and RECUR-CPMG pulse sequences.

The underwater resonances can be recovered by either a TOCSY or a ROESY sequence, which provide magnetization transfer back to the underwater resonances using scalar or dipole interactions, resulting in one-dimensional (1D) RECUR-TOCSY and RECUR-ROESY experiments, respectively, and this pulse sequence is given in Fig. 1b. It should be noted that both of the coherence transfer schemes give rise to in-phase magnetization. Therefore, it is possible to measure the ROE buildup rate using 1D experiments by employing the pulse sequence of Fig. 1b, or using two-dimensional (2D) experiments with the pulse sequence of Fig. 1c. It is also possible to measure the transverse relaxation times of any underwater resonances by introducing a CPMG scheme before data acquisition as shown in Fig. 1d (RECUR-CPMG). If the CPMG scheme is replaced by a single spin echo, the sequence can be used for the homonuclear ^{1}H–^{1}H J-resolved experiment (RECUR-JRES). The pulse sequence in Fig. 1c is different from the previously proposed WATERGATE-TOCSY and WATERGATE-ROESY (*12, 13*), where the WATERGATE sequence was applied after the coherence transfer scheme. In those cases, the underwater resonances were suppressed, and the ^{1}H NMR spectra were used for studies of macromolecules in a 2D mode. Those methods cannot be used in 1D experiments and would not allow the measurement of the ROE buildup curves or the determination of T_2 of resonances under the water peak.

All of the experiments were carried out on a Bruker DRX600 spectrometer operating at 600.13 MHz for ^{1}H observation with a 5-mm broadband probe. The spectrometer was equipped with an actively shielded gradient unit with a maximum gradient-strength output of 49 G/cm. An interpulse delay of 250 μs, corresponding to a spectral width of 4000 Hz between the null points, was used for the double WATERGATE suppression sequence with gradient strengths of 15 and 12% of the maximum output for the first and second WATERGATE components, respectively. An MLEV-17 sequence with a trim pulse length of 3 ms was used for TOCSY transfer and a continuous spin-lock pulse was used for the ROESY transfer.

The new pulse sequences were tested initially using a sample consisting of a 4:1 mixture of D-glucose (20 mg.ml^{-1}) and D-galactose (5 mg.ml^{-1}) in H$_2$O/D$_2$O solution (1/1). Coherence transfer times of 80 ms and 1.5 s were used for 1D RECUR-TOCSY and RECUR-ROESY experiments, respectively. The ROE buildup rate was measured using four 2D experiments of the type shown in Fig. 1c with spin-lock times of 200, 300, 400, and 600 ms. Typically, 2048 (F_2) complex data points were acquired for 16 transients in each of the 512 (F_1) increments, covering a spectral width of 4800 Hz in both dimensions. A $\pi/2$ phase-shifted sine-bell window function was applied to both dimensions before Fourier transformation with zero-filling in the F_1 dimension to 1024 points. The transverse relaxation times of the underwater resonances were determined using the pulse sequence in Fig. 1d in a standard manner with 12 spin-echo times ranging from 0.1 ms to 4 s. A human blood plasma taken from a healthy volunteer was used as an example of biofluid in which approximately 10% of D$_2$O by volume was added for the spectrometer magnetic field lock. One-dimensional RECUR-TOCSY and RECUR-ROESY experiments with mixing times of 70 ms and 1 s respectively were obtained on the plasma sample. A 2D RECUR-JRES spectrum was also acquired with 64 increments in F_1, covering a spectral width of 40 Hz. In this study, only 32 transients were acquired for the glucose/galactose solution, 128 scans for the blood plasma, and 32 scans per increment for the 2D spectra.

RESULTS AND DISCUSSION

The 1D ^{1}H NMR spectra of the glucose/galactose mixture solution without and with water suppression using the double WATERGATE technique at 310 K are shown in Figs. 2a and 2b, respectively. It can be seen from the figure that the resonances of the H1 protons of both β-galactose and β-glucose located at both sides of the water peak have been eliminated by the solvent suppression pulse. After the magnetization transfer by either a TOCSY (Fig. 2c) or a ROESY (Fig. 2d) approach with a mixing time of 80 ms and 1.5 s, respectively, the underwater resonances have been recovered while the water resonance remains suppressed. Due to the large J-coupling constants, there are small phase distortions observable in the
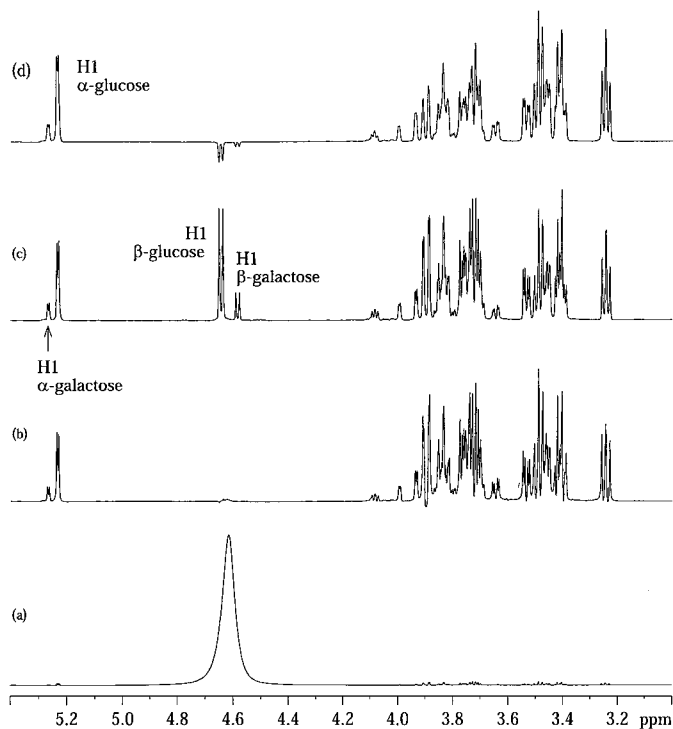
FIG. 2. One-dimensional ^{1}H NMR spectra of D-glucose and D-galactose (a) without and (b) with water suppression using the double WATERGATE technique. The underwater resonances from the H1 protons were fully recovered using (c) the RECUR-TOCSY and (d) RECUR-ROESY approaches with magnetization transfer times of 80 ms and 1.5 s, respectively.

WATERGATE spectrum (Fig. 2b), but the distortion has been removed in the spectra obtained using the RECUR-TOCSY (Fig. 2c) and RECUR-ROESY (Fig. 2d) sequences because of the effective spin-lock scheme. The ratio of the peak areas of the H1 protons of the β-isomer and the α-isomer are 1.5 for D-galactose and 1.4 for D-glucose as calculated from the spectrum in Fig. 2c and these values are close to the values expected. The intensity of the H1 resonance of α-glucose at $\delta 5.23$, a peak not affected by the water suppression, is similar in both Fig. 2b (normal suppression) and Fig. 2c (RECUR-TOCSY), and the intensity in Fig. 2d (RECUR-ROESY) is about 45% of that of Fig. 2b as a consequence of the longer spin-lock time. Thus it can be seen that the TOCSY sequence has a magnetization transfer efficiency much higher than that of the ROESY sequence because of the different transfer mechanism. However, when the underwater resonances have no scalar coupling or very small scalar coupling constants, the ROESY sequence may be the method of choice. Also it might be possible if the samples were concentrated enough to use the maximum quantum filtering method, MAXY, (*14, 15*) to recover the resonances of singlets via the use of attached ^{13}C nuclei at natural abundance. Using this sample, ^{1}H transverse relaxation times were obtained using the RECUR-CPMG pulse sequence as follows: 1.1 s, H1, β-

glucose; 1.5 s, H1, β-galactose; 1.5 s, H1, α-glucose; 1.3 s, H1, α-galactose.

The measurement of the ROE buildup rate of the peaks under the water in the sample of the mixture of glucose and galactose using the 2D RECUR-ROESY method is demonstrated in Fig. 3. The spectra inserted in the figure are the slices extracted from a 2D RECUR-ROESY experiment along the F_1 dimension at the chemical shifts of the H1 protons of β-galactose (right) and β-glucose (left), with mixing times of 200, 300, 400, and 600 ms, as shown. Concomitant with the increment of the mixing time, the NMR peak intensities increased at different rates, reflecting the difference in the distances to the correlated protons. Assuming that there is no secondary dipolar contribution, the ratio of the peak areas is inversely related to the sixth power of the distances between the two protons. It is interesting to note that the ROE cross peaks from the other protons to H1 of the β-isomer are much larger than those to H1 of the α-isomer, and this is a consequence of the configurational difference of the isomers with the other ring protons being closer to H1 in the β-isomer than to H1 in the α-isomer.

The 1D RECUR-TOCSY and RECUR-ROESY NMR spectra of a sample of human blood plasma, with water suppression using the double WATERGATE pulse sequence, are given in Figs. 4a and 4b, respectively. High efficiencies of both recovery of the underwater resonance of H1 of the β-glucose (glucose is
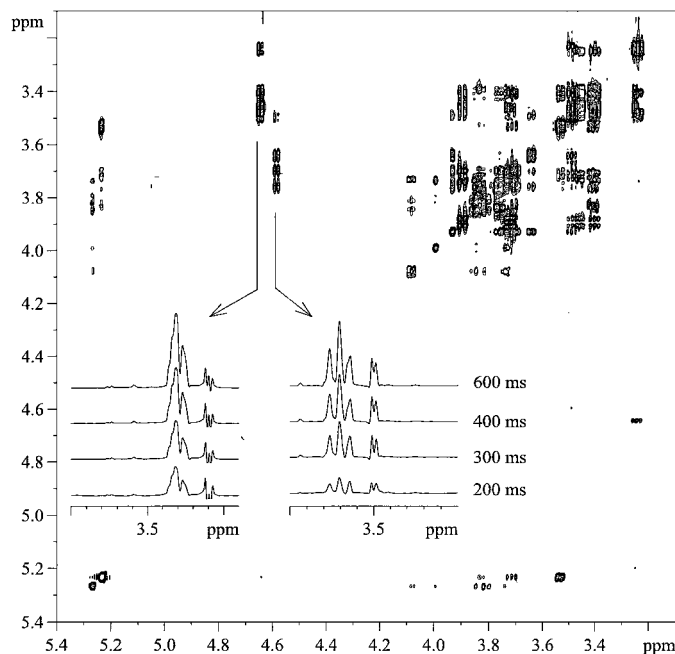

FIG. 3. Two-dimensional RECUR-ROESY spectrum with a mixing time of 600 ms. The slices extracted from the 2D RECUR-ROESY spectra with mixing time of 200, 300, 400, and 600 ms at the chemical shifts of the H1 protons of β-glucose and β-galactose are plotted as insets to show the dependence of ROE upon the mixing time. The vertical axis is F_1 and the horizontal axis is F_2.
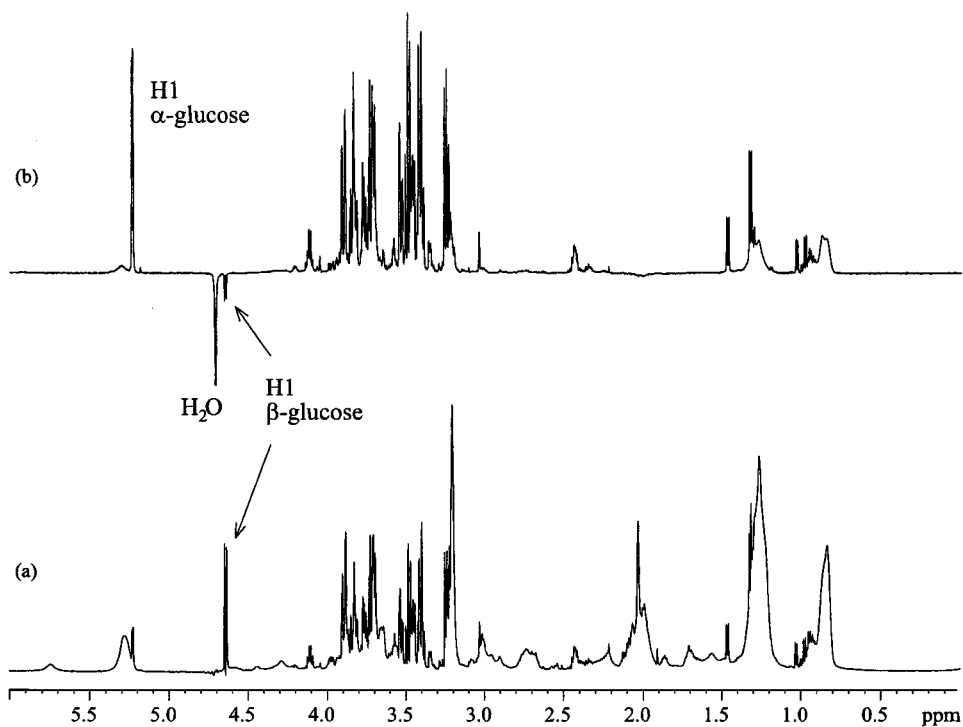
FIG. 4. One-dimensional ^{1}H NMR spectra of human blood plasma using (a) RECUR-TOCSY with a 70-ms magnetization transfer time and (b) RECUR-ROESY with a 700-ms magnetization transfer time to recover the underwater peaks.
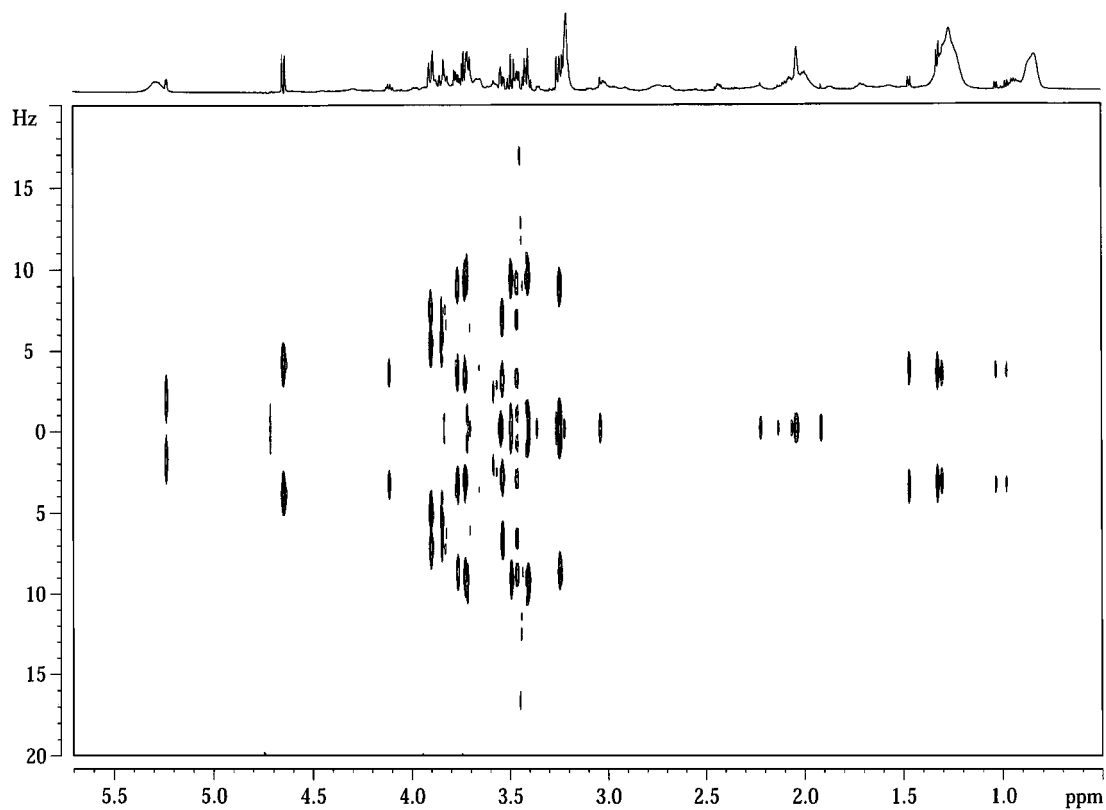

FIG. 5. Two-dimensional RECUR-JRES spectrum showing coupling properties of the resonances of the metabolite molecules, including the H1 of β-glucose, in blood plasma.

present naturally at about 5 mM) and the water resonance suppression are clearly demonstrated. Clearly from the signal–noise ratio in the plasma spectra the method will give good detection, if not quantitation, at approximately 10-fold dilution, i.e., 500 μM even with the limited acquisition times used and the detection limits could be reduced by acquiring more transients. A small water peak is seen in the 1D RECUR-ROESY spectrum, and this may arise from macromolecule-bound water. Finally, it can be mentioned that the RECUR-TOCSY and RECUR-ROESY sequences also have a function of spectral editing. This can be seen from Figs. 4a and 4b, in which the broad peaks, mainly from the lipoproteins, in the blood plasma have been attenuated. Using a spin-lock time of 70 ms, the resonances of the most flexible structural moieties of the lipoproteins are observable (Fig. 4a) but when the spin-lock time is increased to 700 ms (Fig. 4b), there is considerable further attenuation of these peaks. On the other hand, the resonances from the small metabolite molecules are less suppressed because of their relative long relaxation times.

The approach can also be applied to 2D JRES spectra, and Fig. 5 shows the 2D RECUR-JRES result obtained using the pulse sequence in Fig. 1d with a 70-ms TOCSY transfer scheme to detect the underwater resonances. It can be seen from the figure that all of the peaks from small molecules appear, including those obscured by the water peaks.

CONCLUSION

An effective method for detecting small molecule NMR resonances under a water peak in biological samples (RECUR-NMR) has been developed. After high-efficiency solvent suppression using double WATERGATE, either a TOCSY- or ROESY-based coherence transfer sequence is applied to reestablish the resonances close to, or under, water through magnetization transfer using scalar or dipolar coupling, respectively. The use of the TOCSY and ROESY methods ensures an in-phase magnetization transfer, which makes the new approach readily extended for the measurement of the transverse relaxation time, internuclear ROEs, and ROE buildup rates, which are all important for investigating molecular structure and dynamics. An extension of the new approach for J-resolved spectroscopy is also presented and tested using a sample of control human blood plasma.

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