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*Short Communication*

# Proton NMR analysis of plasma from renal failure patients: evaluation of sample preparation and spectral-editing methods*

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## Introduction

High resolution proton nuclear magnetic resonance spectroscopy ($^1$H-NMR) is a powerful, exploratory technique for investigating perturbed metabolic patterns in biofluids such as plasma, urine and bile [1–5]. $^1$H-NMR spectroscopy provides a unique probe of body fluid composition and dynamic interactions between molecules in solution [2, 3, 6]. The signal linewidths (and hence proton $T_2$ relaxation times), are highly sensitive to molecular motions and molecular mobility in solution. It is therefore possible to use $T_2$ experiments to edit the spectra of complex biofluids such as plasma according to the molecular mobility of the biochemical components [2]. Exact solution conditions, for example pH and ionic strength, also influence dynamic molecular interactions in biofluids [6, 7] and hence sample preparation is particularly important for spectral assignment and interpretation. These studies form part of a wider program designed to investigate the effects of sample preparation and choice of NMR experiment on $^1$H-NMR spectra of body fluids. In this study we have chosen to evaluate samples from patients in end stage renal failure.

## Experimental

Blood was collected from patients (eight males and seven females, mean age 46.5 years, range 22–73 years) undergoing treatment for chronic renal failure, e.g. haemodialysis (HD), continuous ambulatory peritoneal dialysis (CAPD), or dietary restriction alone. Blood samples were collected into lithium heparinized tubes, centrifuged at 3000 rpm (10 min; 4°C), the plasma separated and stored frozen until required. Control samples from normal healthy volunteers were treated similarly.

### *$^1$H-NMR analysis*

$^1$H-NMR measurements were made on a JEOL GSX500 spectrometer operating at 11.7 Tesla (500 MHz proton resonance frequency) at ambient probe temperature. To 0.6 ml plasma, 0.1 ml $^2H_2O$ (field/frequency lock) was added and run using the single pulse and Hahn spin–echo (HSE) pulse sequence. The pulse sequence for the Hahn spin–echo experiment [8] is: D-[$90°_x$–$\tau$–$180°_y$–$\tau$–collect FID], where $\tau$ = 60 ms and D = 3 s.

The HSE experiment produces phase modulation of signals with singlet and triplet resonances phased upright and 3 bond $^1$H–$^1$H doublets and quartets phase inverted with this value of $\tau$. The samples were also lyophilized, redissolved in an equal volume of $^2H_2O$ and rerun using the HSE experiment. Following titration of plasma samples to pH 2 a further HSE experiment was performed. Finally each sample was deproteinized using trichloroacetic

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acid, titrated to pH 7.0 and rerun using the single pulse experiment. For each sample 128 free induction decays were collected into 32K points using a pulse width of 45° for the single pulse experiment, an acquisition time of 2.7 s and a pulse delay of 2.2 s in order to achieve fully $T_1$ relaxation. An exponential line broadening function of 0.5 Hz was applied prior to Fourier transformation and chemical shifts were referenced externally to sodium trimethylsilyl [2,2,3,3-$^2H_4$]-1-propionate (TSP, $\delta = 0$ ppm). Water suppression was achieved using gated irradiation (decoupler off during acquisition). Resonance assignments were made using chemical shift, spin–spin coupling patterns, pH dependencies of chemical shifts and ultimately by standard addition.

## Results and Discussion

Plasma samples were prepared for analysis in several different ways in order to obtain maximum information and achieve "mobility-edited" spectra and "chemically-edited" spectra. A single pulse spectrum (Fig. 1A) of the plasma from a patient in end-stage renal failure shows a broad featureless envelope due to proteins and lipoproteins, on top of which can be seen resonances derived from slower tumbling molecules. The HSE spectrum (Fig. 1B) eliminates these broad lines, thus revealing the sharper low molecular weight/high molecular mobility resonances of interest which are also phase-modulated. Lyophilization of the sample brings about changes in the spectrum (Fig. 1C), although the pH of the sample remained at 7·0. The observed increases in the intensities of threonine and glutamine resonances indicates that these amino acids are partially protein-bound (in non-lyophilized plasma) and that significant structural changes have occurred in the tertiary structure of the binding protein on lyophilization. Deuteration of the $CH_2$ group of the creatinine ($\delta = 3.8$ ppm) resulted in its disappearance from the spectrum. Acidification of the sample (Fig. 1D) revealed a number of chemical shift differences, e.g. acetate. Resonances from citrate became observable indicating that citrate was partially protein-bound (electrostatically) in plasma or in fast exchange between bound and free states on an intermediate NMR-time scale.

The value of sample acidification in the separation of trimethylamine-*N*-oxide

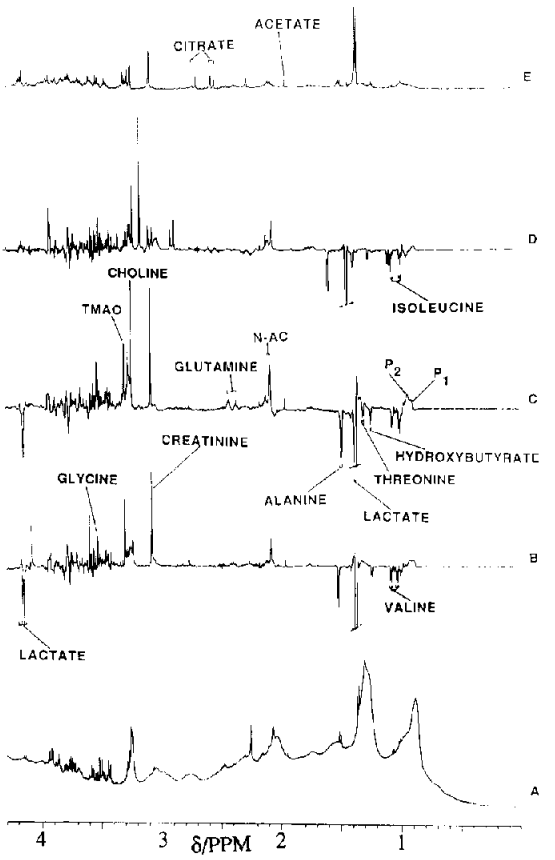


**Figure 1**
$^1$H-NMR spectra 500 MHz (region to low frequency of water) of plasma from a patient in end stage renal failure: (A) single pulse experiment of untreated plasma, (B) HSE spectrum of untreated plasma, (C) HSE specrum of lyophilized plasma reconstituted in $^2H_2O$, (D) HSE spectrum of lyophilized, acidified plasma (pH 2.5) reconstituted in $^2H_2O$, (E) single pulse spectrum of deproteinized plasma titrated back to pH 7.0. See text for experimental conditions. N-ac, *N*-acetyl glycoprotein resonances. $P_1$ and $P_2$ are the $CH_3$ and $(CH_2)_n$ resonances of mobile fatty acids.

(TMAO) and betaine overlapped resonances is shown in Fig. 2 where a model solution of osmolytes (methylamine, dimethylamine (DMA), trimethylamine (TMA), TMAO and betaine) was analysed over a range of pHs. These metabolites show different pH-dependent shifts. This form of chemical editing highlights the necessity for careful sample preparation in order to maximize spectral information and to avoid misinterpretation. The procedure of lyophilization and reconstitution in $^2H_2O$ appears to be the most "cost-effective" in terms of spectral information and for additional suppression of the HOD resonance.

When compared to normal plasma, the spectra from renal failure patients showed

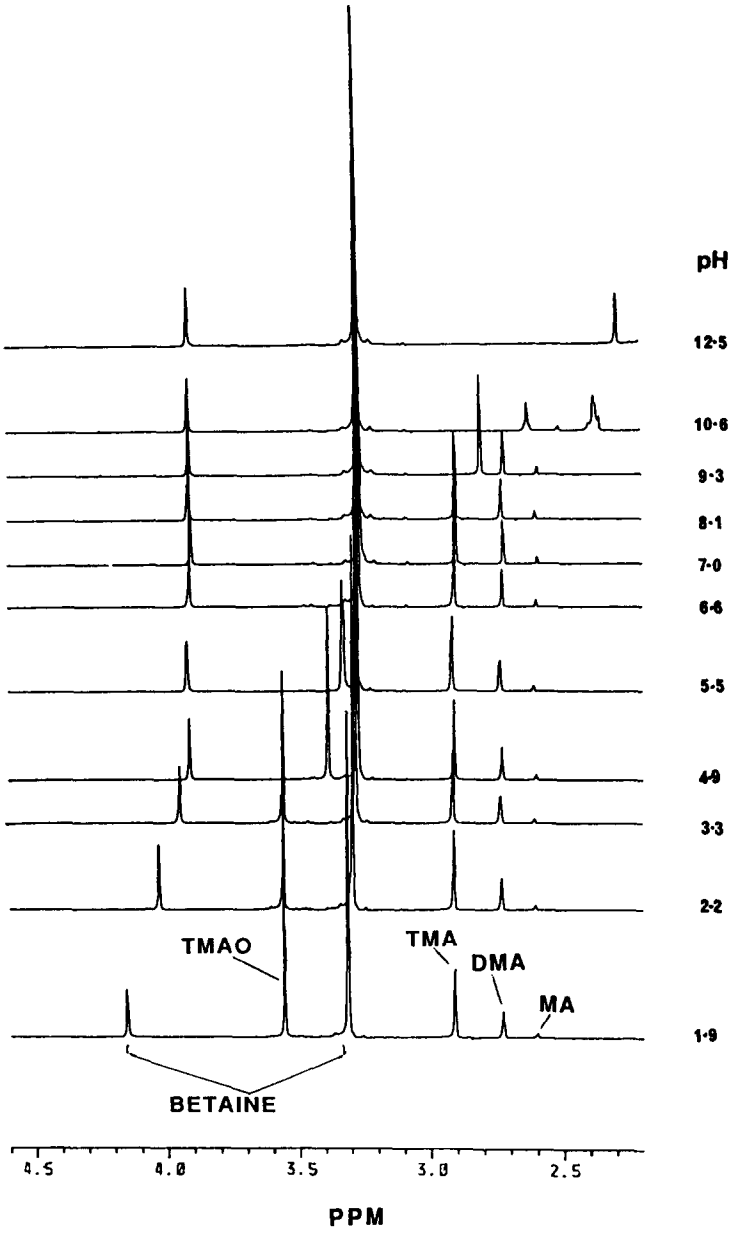


**Figure 2**
$^1$H-NMR spectra (400 MHz) of a solution containing model osmolytes under a range of different pHs. See text for experimental conditions. DMA, dimethylamine; MA, methylamine; TMA, trimethylamine; TMAO, trimethylamine-*N*-oxide.

markedly elevated plasma creatinine levels (Fig. 3). All patients also showed a significant increase in plasma lactate and TMAO. The patients undergoing H/D treatment ($n$ = 6) were further differentiated by the presence of DMA and an alteration in the choline–TMAO ratio. Glycine was predominantly raised in the CAPD patients but not significantly in the others. Plasma creatinine measurements are used clinically to assess the glomerular filtration rate, although the concentration ratio shows little absolute change until, functionally, the patient has lost one kidney [9]. The normal range for plasma creatinine is 45–120 mmol $l^{-1}$. Patients with chronic renal failure can have levels of over 1000 mmol $l^{-1}$, (see Fig. 3). The raised plasma lactate levels seen in these patients reflects the metabolic disturbances (principally acidosis) that occur with diminished renal function. The metabolic acidosis is due to either decreased tubular reabsorption of bicarbonate or to

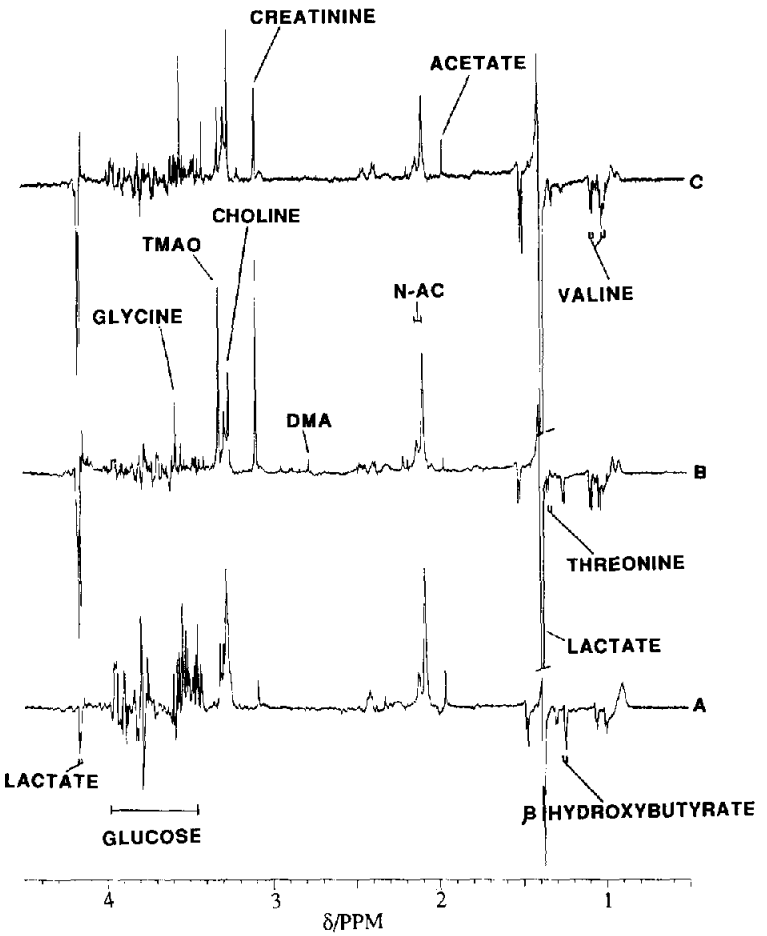


**Figure 3**
$^1$H-NMR spectra (500 MHz) of human plasma (A) control plasma, (B) plasma from a patient undergoing H/D treatment, (C) plasma from a patient undergoing CAPD treatment. See text for experimental conditions. DMA, dimethylamine; TMAO, trimethylamine-*N*-oxide; N-ac, *N*-acetyl resonances of glycoproteins.

retention of acids other than carbonic. Lactic acidosis due to severe anorexia is often an unpleasant side-effect of chronic renal failure. TMAO and choline have recently been reported as important protective osmolytes in the renal inner medulla [10], the significance of elevated plasma TMAO levels has yet to be evaluated but may prove to be a marker of renal damage.

## Conclusion

These studies have demonstrated the necessity of evaluating methods of sample preparation and NMR experimental techniques in order to arrive at an optimized strategy for maximizing the molecular information from $^1$H-NMR spectra of plasma. Furthermore, we have shown that certain patients with chronic renal failure show abnormalities in plasma composition as revealed in NMR spectra which may have clinical significance.

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