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Raised transaminase activity of blood plasma from rats with experimentally-induced kidney damage detected by spin-echo ^{1}H-NMR spectroscopy

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Abstract: We report the application of spin-echo ^{1}H-NMR spectroscopy to the detection of raised plasma transaminase activity in rats treated with the nephrotoxic cephalosporin antibiotic cephaloridine (CPH). Spin-echo ^{1}H-NMR analysis of lyophilized plasma, reconstituted in H_2O reveals a doublet at δ 1.48 for alanine. However when samples were reconstituted with ^2H_2O we noted that in samples from CPH-treated rats (but not in control samples) there was a variable degree of appearance of a singlet at δ 1.47 together with a reduction in the doublet at δ 1.48. We suggest that this is due to the release of transaminases from damaged tissue which, via a reversible conversion of alanine to pyruvate, causes selective deuteration of alanine at the α-hydrogen (α-CH) position. This observation suggests that these ^{1}H-NMR spectral patterns are dependent on the level of plasma transaminases and this may provide a novel indicator of tissue damage.

Keywords: *Alanine; alanine aminotransferase activity; blood plasma; cephaloridine; glutamine; nephrotoxicity; proton deuterium exchange; spin-echo ^{1}H-NMR spectroscopy.*

Introduction

High resolution nuclear magnetic resonance (NMR) spectroscopy can be effectively applied to the analysis of biological fluids, cells and tissue extracts [1–3]. Key advantages of NMR as a biochemical probe include its non-invasive and non-destructive nature which allows the simultaneous detection of a wide range of xenobiotic and endogenous low molecular weight (MW) metabolites (enabling the exploration of perturbed biochemical pathways). In recent years the utility of ^{1}H-NMR spectroscopy of biofluids as a method in biochemical toxicology has been proven, as changes in the characteristic ^{1}H-NMR profiles of each biological matrix can be related to alterations in intermediary metabolism as a result of toxic insult [3–5]. Furthermore, NMR spectroscopy of biofluids has successfully uncovered a number of novel low MW metabolite markers which have been used to characterize experimental toxicity states [6]. Conventional enzymatic and chromatographic methods are often more sensitive than ^{1}H-NMR spectroscopy for detecting low levels of metabolites; however in toxicological assessments NMR is believed to be equally sensitive to the detection of lesions as the more traditional methods, and more revealing in terms of information on both the site and biochemical nature of the lesion [1]. In addition to the analytical potential of NMR for studying metabolite composition, NMR spectroscopic methods can also provide information on dynamic molecular interaction and reactions of metabolites in the complex biofluid matrix [1]. Such observable phenomena include enzymatic transformations of metabolites, metal complexation reactions and macromolecular binding [1].

As part of a series of recent NMR investigations on altered intermediary metabolism following toxic insult by the nephrotoxic xenobiotics uranyl nitrate (UN) and cephaloridine (CPH), we measured ^{1}H-NMR spectra of blood plasma [7, 8]. Single pulse NMR spectra of plasma are dominated by broad lines from lipids and lipoproteins, which can be edited out by use of spin-echo pulse sequences such as the Hahn spin-echo (HSE) experiment [9]. When a delay of 1/2J is used, there is phase-inversion of proton-coupled doublets and quartets whereas

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uncoupled singlets and triplets appear phased upright [9]. Thus the methyl resonance of the amino acid alanine normally appears as a phase-inverted doublet because of its coupling to the α-hydrogen (α-CH). However in HSE spectra of plasma from Fischer 344 (F344) rats treated with UN and CPH we noted a change to the appearance of the alanine methyl resonance from a phase-inverted doublet to a phase upright singlet, in the case where the plasma had been freeze-dried and reconstituted in ^2H_2O [7, 8]. We have investigated this phenomenon and tested the hypothesis that the cause is due to the elevated level of plasma transaminase enzymes released from damaged renal tissue.

Transaminases are found in all cell types and have a major role in amino acid metabolism, however they have particularly high activities in the liver and kidney because of the extensive use of amino acids for gluconeogenic substrates in these tissues [10]. In cases of toxic insult, or liver or kidney disease, plasma transaminases may be raised very significantly. Quantitative determinations of transaminases are used extensively in medicine to detect cellular dysfunction, and the development of simplified laboratory procedures for transaminase determinations has stimulated increased use of such tests in the study of animal diseases [11]. The purpose of the present work was therefore to investigate further the alanine phase-inversion phenomenon in spin-echo spectra of freeze-dried plasma reconstituted in ^2H_2O, and to prove that the phenomenon originates from raised transaminase levels in treated rats. This study has been exemplified using plasma from F344 rats following administration of CPH.

Experimental

Animals and treatments

Twelve male F344 rats (Harlan Olac; 200–250 g) were divided into four groups of three rats each. Animals were housed individually in plastic metabolism cages, in a well ventilated room with regular light cycles (12 h: 0700–1900 h). Food and tap water were provided *ad libitum*. Following an acclimatization period of 2 days, rats received a single i.p. injection of 0.9% aqueous NaCl (control), or a 250 mg ml^{-1} solution of CPH (Sigma) in 0.9% NaCl, equivalent to doses of 375, 750 or 1500 mg kg^{-1} CPH.

Sample collection and preparation

Whilst the animals were under terminal deuteriochloroform anaesthesia 48 h following CPH, blood was removed from the inferior *vena cava* into heparinized tubes. The plasma was separated immediately from the erythrocytes by centrifugation (5000 rpm; 2 min; at room temperature) and stored at −20°C prior to NMR measurements. In selected cases plasma samples obtained from control animals were incubated with an ammonium sulphate suspension of the enzyme alanine aminotransferase (ALT; E.C.2.6.1.2.; Sigma; 100 units per sample) at 37°C for 4 h. Plasma samples were analysed by spin-echo ^{1}H-NMR spectroscopy (see below) both before and after lyophilization. For non-lyophilized samples, a volume of 0.65 ml plasma was diluted with 0.1 ml ^2H_2O prior to ^{1}H-NMR measurement, the ^2H_2O providing an internal field-frequency lock. All lyophilized samples were reconstituted in an equivalent volume of ^2H_2O (0.75 ml) prior to ^{1}H-NMR measurement.

^{1}H-NMR measurements

NMR measurements were made on a Bruker WH400 spectrometer operating at 400.13 MHz at ambient probe temperature (298 ± 1 K). Plasma was analysed using the HSE pulse sequence:

$$D - (90 - \tau - 180 - \tau - \text{acquire FID})$$

repeated 128 times where $\tau = 68$ ms and D = a relaxation delay of 3 s between cycles. In selected cases sequential ^{1}H-NMR spectra were collected at various time intervals over a 4 h period. The primary use of this pulse sequence in the present context was to attenuate broad resonances from plasma proteins with short T_2 relaxation times so that they do not contribute to the spin-echo spectrum. In this way only signals from mobile low MW metabolites are observed. However, the HSE experiment also results in a phase-modulation of signals that is dependent on their spin–spin coupling constants and coupling multiplicity; phase inversion is achieved for doublets and quartets (relative to singlets and triplets) where $\tau = 1/2J$ [9, 12]. The delay τ value of 68 ms employed in the present experiments was chosen so that doublets and quartets with $^3J_{H-H}$ coupling constants of approximately 8 Hz appear inverted. Suppression of the water signal was achieved by applying a gated

(irradiation channel off during acquisition) secondary irradiation field at the water resonance frequency. An exponential apodization function corresponding to a line broadening of 1.0 Hz was applied to each free induction decay (FID) prior to Fourier transformation (FT) to improve signal-to-noise ratios. Chemical shifts were referenced to the centre of the doublet methyl resonance for endogenous lactate at δ 1.33. Resonance assignments were confirmed by a consideration of chemical shift, spin–spin coupling patterns, coupling constants, and the literature [1].

Computer simulations of the effects of proton/deuterium exchange on the spin-echo spectra of alanine

Computer simulations of the appearance of the alanine methyl signals were made by calculating the algebraic sum of the methyl doublet and singlet signals using a program written in BASIC for an IBM-PC. A coupling constant of 7 Hz was assumed, together with a Lorentzian lineshape with a linewidth at half height of 2 Hz. The signal from the alanine with α-CD was taken to be 4 Hz to low frequency of that from alanine with α-CH.

Results and Discussion

400 MHz HSE ^{1}H-NMR spectra of control plasma and non-lyophilized samples revealed the expected inverted alanine doublet at δ 1.48 and characteristic signals for methyl groups of lactate at δ 1.33, 3-D-HB (3-D-hydroxybutyrate) at δ 1.21 and valine at δ 0.99 and 1.04 as illustrated in Fig. 1. By eliminating the broad resonances from macromolecular plasma constituents with short T_2 relaxation times using spin-echo Fourier transform methods, Fig. 1 shows that it is possible to obtain useful biochemical information on mobile protons, mostly of low MW, non protein-bound metabolites present in plasma. The majority of the resonance assignments in HSE spectra of plasma have been documented previously however [1, 13, 14] and will not be discussed further here.

^{1}H-HSE NMR analysis of lyophilized plasma (reconstituted in ^2H_2O) from CPH-treated rats however, revealed a variable phase-modulation of the alanine signal as illustrated for rats which had received doses of 375 and 1500 mg kg^{-1} CPH (Fig. 1). A complete replacement of the alanine doublet with an

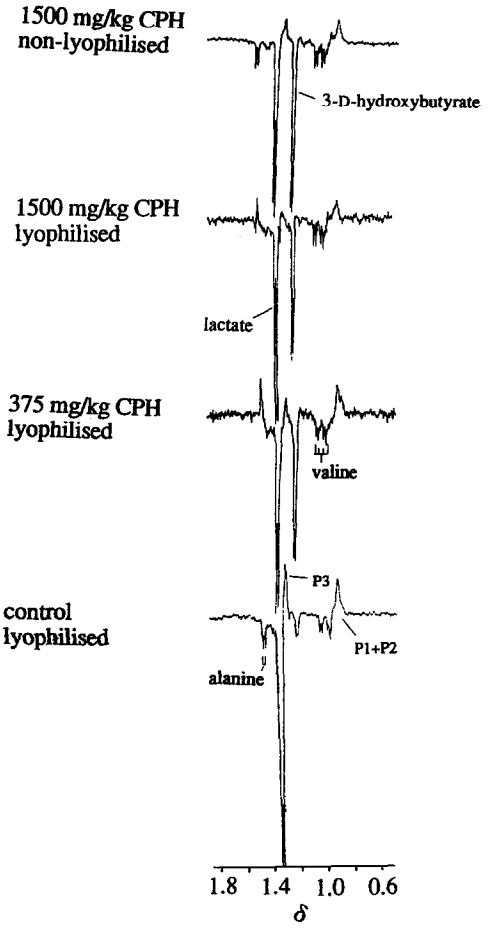

Figure 1
Partial 400 MHz HSE spectra (δ 0.6–1.8) of plasma samples obtained 48 h after dosing from a control rat and following CPH treatment. Lyophilized samples were reconstituted in D_2O. Peaks P1–P3 arise from the CH_3 (P1, P2) and CH_2 (P3) groups of lipoprotein components.

oppositely phased, i.e. upright, singlet was evident in the HSE spectra obtained from rats treated with 375 mg kg^{-1} CPH. We hypothesized that the abnormal phase modulation behaviour of the alanine signal in HSE spectra of CPH-treated rats was due to the release of ALT to the plasma from damaged renal tissue. ALT catalyses the reversible transamination reaction between alanine and glutamate, depicted in Fig. 2. The reaction scheme illustrates the ALT-catalysed transfer of the amino group from an amino acid to a keto acid to form the corresponding amino acid and to produce the keto acid of the original amino donor that is characteristic of transamination [10]. As a consequence, we proposed that in CPH-treated rats the α-CH of alanine in ^2H_2O reconstituted plasma is progressively lost to the

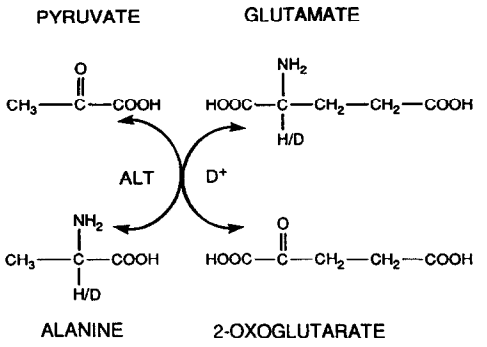

Figure 2
Reaction scheme of reversible ALT-catalysed transamination reactions.

solvent (Fig. 2) and thus in the presence of ^2H_2O this becomes α-CD on reformation of the alanine, and thereby eliminates the three bond J-coupling of the α-CH to the methyl protons. A computer simulation of the resultant signals caused by mixing deuterio and proteo-alanine was therefore applied to examine the effect of varying the proportions of α-CH and α-CD on the alanine signal appearance (Fig. 3). It can be clearly seen that there is a continuous transition between the limits of 100% protonation of the α-CH of alanine (inverted doublet) and 100% deuteration (upright singlet of intensity twice that of undeuterated doublet components). The variable extent of the phase-inversion of the alanine doublet noted between the CPH-treated samples (Fig. 1) indicated that the degree of deuteration varies. This variation could arise from the time elapsed between NMR analysis and sample reconstitution with ^2H_2O, and from variations in ALT levels.

The time-dependence of the alanine signal appearance was investigated in lyophilized plasma from an animal treated with 375 mg kg^{-1} CPH. The alanine deuteration reaction was initiated by ^2H_2O reconstitution and HSE spectra were accumulated and collected (in 'blocks' of 128 scans) at various time intervals over a 4 h period. The results illustrated in Fig. 4 demonstrate the gradual disappearance, with time, of the inverted doublet from alanine with α-CH and its replacement with an upright singlet representing the formation of alanine with the α position fully deuterated. Comparison of the simulated lineshape expected for different molar proportions of the CH and CD forms of alanine (Fig. 3) suggests that, in this plasma sample with this level of ALT, the time-

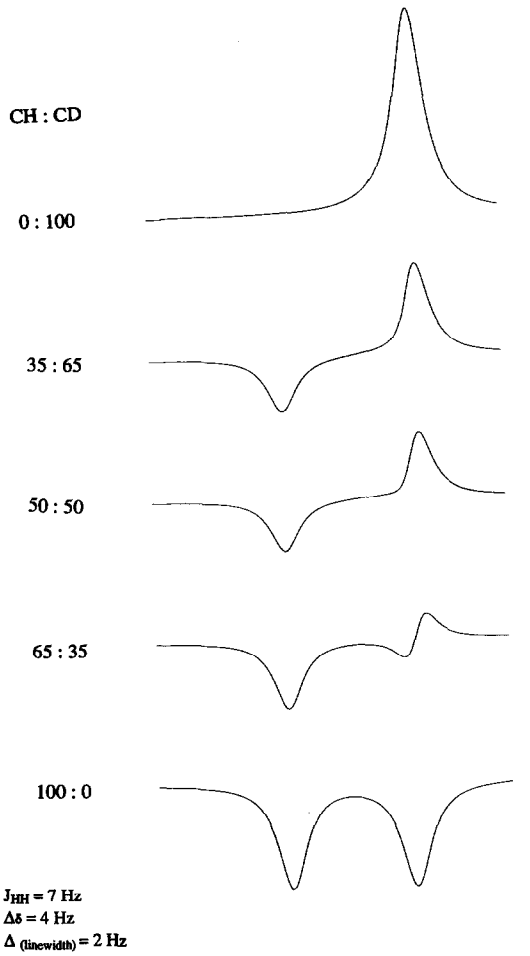

Figure 3
Simulated alanine methyl resonance as observed in a Hahn spin-echo spectrum as a function of degree of deuteration of the α-CH.

dependence of the deuteration of the α-CH of alanine appears to be linear and completed by approximately 100 min. Additional confirmatory evidence that the deuteration of alanine was due to increased ALT was provided by the measurement of a spectrum of control plasma to which a purified preparation of ALT, in excess, had been added in the presence of ^2H_2O. This spectrum also revealed that the usual inverted doublet is converted to the upright singlet expected for deuteration at the α position (Fig. 5).

Although ALT is generally recognized as a biochemical marker of liver damage, elevated ALT levels following CPH administration to F344 rats have been reported previously in the absence of hepatotoxicity. Kuo and Hook demonstrated that CPH induced significant elevations in ALT activity at doses above

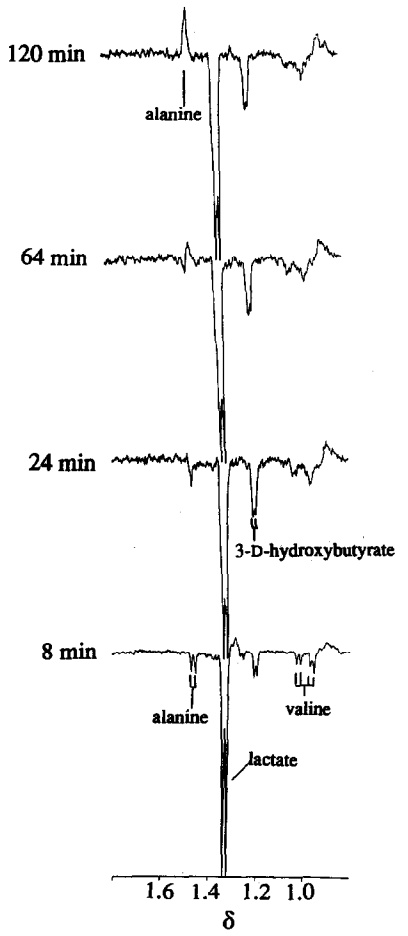

Figure 4
Partial 400 MHz HSE spectra (δ 0.8–1.8) acquired over a 2 h period of a lyophilized plasma sample obtained from a rat 48 h after administration of 375 mg kg^{-1} CPH.

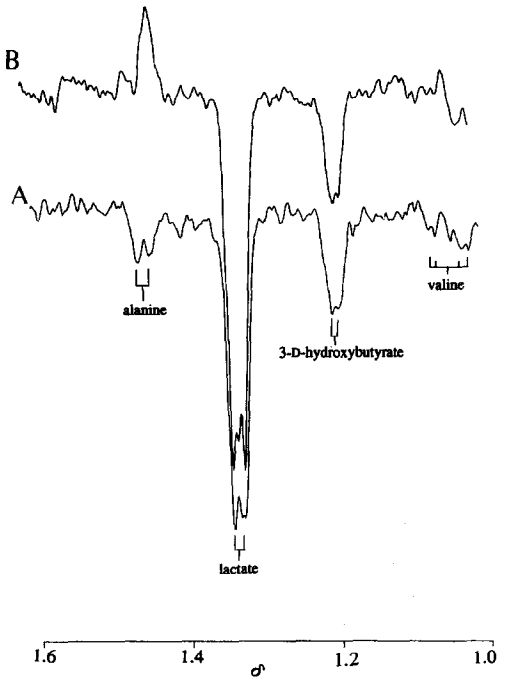

Figure 5
Partial 400 MHz HSE spectra (δ 1.0–1.6) of lyophilized control rat plasma: (A) before and (B) after incubation with a purified preparation of ALT.

1000 mg kg^{-1} when severe renal injury only was apparent [15]. Reports of increased ALT activity following UN administration also exist, although in the presence of UN-induced nephrotoxicity and hepatotoxicity [16]. In addition, ^{1}H-NMR studies using model solutions containing L-alanine have previously been used to determine the specific properties of one half of a transamination reaction independently of the other half. Babu and Johnston, using deuterium-decoupled ^{1}H-NMR spectroscopy, demonstrated that purified preparations of ALT in ^2H_2O catalysed the rapid exchange of both the α and β hydrogens of L-alanine [17]. In their studies, the α-CH spectra diminished throughout the course of the reaction due to the formation of α-CD and the presence of exchange intermediates such as -$CHCDH_2$, -$CHCD_2H$ and -$CHCD_3$ was also demonstrated. The multiple exchange occurred by a single interaction of the substrate with the enzyme and it was suggested that the proton was exchanged from the amino acid onto the active site of the enzyme and back to the substrate at one position, while the other position was exchanging with ^2H_2O [17]. Babu and Johnston noted that the CH_3 deuterated at a comparable rate to the CH [17] and hence evidence of this may have been expected in the present study. A CH_2D group would appear as an inverted doublet signal from coupling to the CH and also a $^2J_{HD}$ splitting (resulting in a triplet of intensity 1:1:1) of approximately 2 Hz which would produce some line broadening. A CHD_2 group would be expected to appear as a 7 Hz doublet with a superimposed quintet of 1:2:3:2:1 intensity and again some line broadening would result. However we did not observe such line broadening in the present study. Similarly, the appearance of the glutamate multiplet was not altered here (data not shown).

Conclusions

The altered alanine signal appearance after lyophilization of plasma and reconstitution into

^2H_2O observed in the present study, and the possible link with altered ALT activity, may potentially be a direct and novel assay of plasma enzyme activity and therefore tissue damage. ALT is widely used in toxicology and clinical biochemistry as a quantitative marker of hepatotoxicity (and nephrotoxicity), and hence the unique application of ^{1}H-NMR spectroscopy to ALT detection further exemplifies its use as an adjunct to the conventional techniques for monitoring toxicity.

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