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Estimation of the Spin–Lattice Relaxation Time Constants of Low Molecular Weight Solutes in Dilute Complex Aqueous Solutions: Application to Urinary Metabolites

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ABSTRACT: Proton spin–lattice relaxation rate constants (T_1 values) of some selected urinary metabolites were analysed extensively by two different least-squares minimization procedures, based on the simplex and the Marquardt–Levenberg algorithms. Comparative multiplicate analysis under different experimental conditions using laboratory solutions of some of the urinary metabolites was employed in addition to direct measurements on human urine samples. The T_1 values estimated for most of the metabolites by the two methods agreed to within 10–15%. © 1998 John Wiley & Sons, Ltd.

KEYWORDS: NMR; ^{1}H NMR; spin–lattice relaxation; simplex algorithm; Marquardt–Levenberg algorithm; $T_{1\mathrm{null}}$ values

INTRODUCTION

High-resolution pulsed nuclear magnetic resonance (NMR) spectroscopy of protons (^{1}H) may be used to obtain quantitative measurements of solute concentration in complex mixtures, for example, in body fluids such as urine. However, sufficient time must be allowed to elapse between the successive pulses used to sample the nuclear spins to enable them to recover fully from the effects of these pulses; it is therefore essential that the nuclear relaxation times of the spin system be measured before quantitative experiments can be undertaken.[1]

The rate at which the magnetization of a given proton approaches its equilibrium value following a perturbation is given by its spin–lattice relaxation time constant, T_1. In dilute solutions the spin–lattice relaxation of most small, low molecular weight molecules is dominated by dipole–dipole interactions.[2,3] Assuming isotropic tumbling within the extreme narrowing region, the dipole–dipole relaxation rate constant for two non-equivalent nuclei, *i* and *j*, is given by

$$1/T_1(\mathrm{dd}) \propto \sum_i \frac{\gamma_i^2 \gamma_j^2}{r_{ij}^6} \tau_c(i, j) \qquad (1)$$

where γ_i and γ_j are the magnetogyric ratios of the *i* and *j* nuclei, r_{ij} is their internuclear separation and $\tau_c(i, j)$ is the motional correlation time of the vector between them. The net T_1 value for a nucleus is dependent on the overall tumbling rate of the molecule as a unit, plus the contribution from any additional motion of a substituent group, such as a methyl group, which carries that particular nucleus. Equation (1) implies that, if τ_c decreases, the T_1 of the protons in question will increase.

Metabolites can either have simple structures with equivalent protons, as in glycine, or they can have an ensemble of magnetically non-equivalent protons, as in hippurate; the protons in the latter will have different relaxation times, related to the molecular environments of the protons. T_1 values of methyl groups are expected to be dependent on the barriers to internal rotation of the group, more hindered groups (with longer internal τ_c values) having shorter relaxation times.[4]

If high-resolution NMR spectroscopy is to be used to obtain accurate quantitative measurements of solute concentration, it is necessary to use sufficiently long relaxation delays to ensure complete spin–lattice (T_1) relaxation of the nuclear spins of all solute molecules.[5,6] Since all of the spins which are of interest are excited simultaneously in time-averaged pulsed NMR studies, an insufficiently long relaxation delay will lead to a differential saturation of the nuclear energy levels of the different molecules and hence a greater loss of signal intensity for the spins which have the longer T_1 values. Although the signal intensities of such partially saturated spin systems can be analysed, for solutions which have large numbers of solutes, each containing one or more magnetically non-equivalent protons, such analysis is cumbersome. In practice, a relaxation delay of about five times the longest T_1 value is usually used

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since this results in more than 99% recovery of the total magnetization.[7]

T_1 values of protons (^{1}H) have been reported for a number of solutes in different solutions.[8,9] Numerous T_1 studies of other nuclei, particularly ^{13}C, have been reported.[10] Colebrook and co-workers[2,3] and Valensin and co-workers[11,12] have reported extensive studies on the applications of T_1 values to elucidating the stereochemistry of natural products and other organic compounds. T_1 values of metabolites in human muscle extracts have been reported.[1] Although T_1 values of water in urine have been used to study different renal conditions,[13,14] to our knowledge studies on the T_1 values of urine metabolites have not been reported previously. Such information became necessary during a quantitative investigation in this laboratory into human urine metabolites.

Standard methods for calculating T_1 values utilize various forms of peak-picking routines from which the respective T_1 values are subsequently calculated for one or more selected sets of peaks. In this work, we used two different non-linear least-squares minimization algorithms to analyse data from inversion–recovery experiments[5] to obtain the T_1 values of low molecular weight metabolite molecules, both in test solutions and in human urine. The first technique, based on the Marquardt–Levenberg algorithm,[15] sequentially fits all of the peaks in the absorption part of a complex spectrum without operator intervention (we have coined the term 'pix-fit' for this method, since it stems from the fitting of data to each pixel in an NMR image). The second technique employs a simplex algorithm (referred to as 'sim-fit') where fitting is restricted to peaks specified manually. A comprehensive evaluation of the pix-fit routine, which has also been used to obtain quantitative T_1 maps in NMR imaging, is reported elsewhere.[16]

EXPERIMENTAL

Eight standardized solutions were used to test the accuracy of the T_1 values calculated using the pix-fit and sim-fit techniques: (A) 4.00 mM and (B) 10 mM aqueous glycine (Aldrich Chemicals, Gillingham, Dorset, UK), (C) 4.00 mM aqueous alanine (BDH Chemicals, Poole, Dorset, UK), (D) 1 mM and (E) 4.41 mM aqueous lactic acid (BDH Chemicals), (F) 0.98 mM and (G) 4.02 mM aqueous sodium hippurate (Sigma Chemicals, St Louis, MO, USA) and a 4.00 mM solution of the sodium salt of trimethylsilylpropionate (TSP) (Aldrich Chemicals, Milwaukee, WI, USA) in D_2O. These test solutions (600 μl) were placed separately in 5 mm NMR tubes and 200 μl of TSP/D_2O were added to each. The same test compounds were used to make two separate sets of mixtures. The first (M1), third (M3), fourth (M4), fifth (M5) and sixth (M6) identical mixtures were made by mixing, by volume, alanine (C), glycine (A) and TSP (4 mM) in proportions 2:2:1; this mixture was then diluted so that the concentrations of the test metabolites would be similar to that of mixture M2, which was made by mixing equal volumes of alanine (C), glycine (A), hippurate (G) lactate (E) and TSP (4.00 mM). Two aliquots of mixture M3 were degassed by passing nitrogen gas for 15 min and the two NMR sample tubes which contained aliquots of mixture M4 were placed in a vacuum for 10 min; it was assumed that the above procedures did not achieve complete degassing of mixtures M3 and M4. Mixtures M5 and M6 were subjected to a freeze–thaw cycle under vacuum and the NMR tubes were sealed.

The urine samples were prepared by adding 200 μl of TSP/D_2O to 600 μl of fresh urine samples obtained from the same volunteer on two different occasions. D_2O was used as a field/frequency lock, and TSP, in addition to being used as a test compound for T_1 calculations, was used as the chemical shift reference compound. pH values of solutions were measured using a Corning Delta II series pH meter (Model 2220, accuracy ±0.01 pH unit) at 23 °C. Specific gravity was measured using Multistix S.G. (AMES reagent strips, Ames Division, Miles Laboratories, slough, UK) used in urinalysis.

Acquisition and pulse sequence

Experiments were performed using a Bruker AMX spectrometer operating at 500 MHz at a probe temperature of 310 °K. The samples were not spun during acquisition. The 90° pulse length was determined for each sample after tuning the probe and locking the field on the D_2O signal; 32 or 64 signal averages were collected with a sweep width of 16.125 ppm and 0.49 Hz per point digital resolution. Spectra were baseline corrected.

The water signal was suppressed using a presaturation sequence[17] which, with the selected delays, gave excellent suppression. The same transmitter was used both to produce the selective irradiation of the water protons using low-power pulses prior to the beginning of acquisition and during the relaxation and recovery delays, and also the high-power excitation pulses; this eliminated any phase incoherence which might have arisen had a second decoupler channel been used. The excitation pulses[18,19] were applied as a train of three 90° pulses with 3 μs and 5 ms delays between the first–second and second–third pulses, respectively. These pulses were phase cycled using the same phase cycle as for the first increment of a NOESY experiment.[17,18] This cycle selects just the first magnetization that is aligned along the *z*-axis during the 5 ms delay, and also eliminates contributions not sensitive to the phase of the first pulse. The sequence with the selected delays has been used extensively in this laboratory, and results in excellent water suppression (Figs 1 and 2).

T_1 measurements and fitting the data

T_1 values were measured using the solvent suppression pulse sequence mentioned above incorporated into the

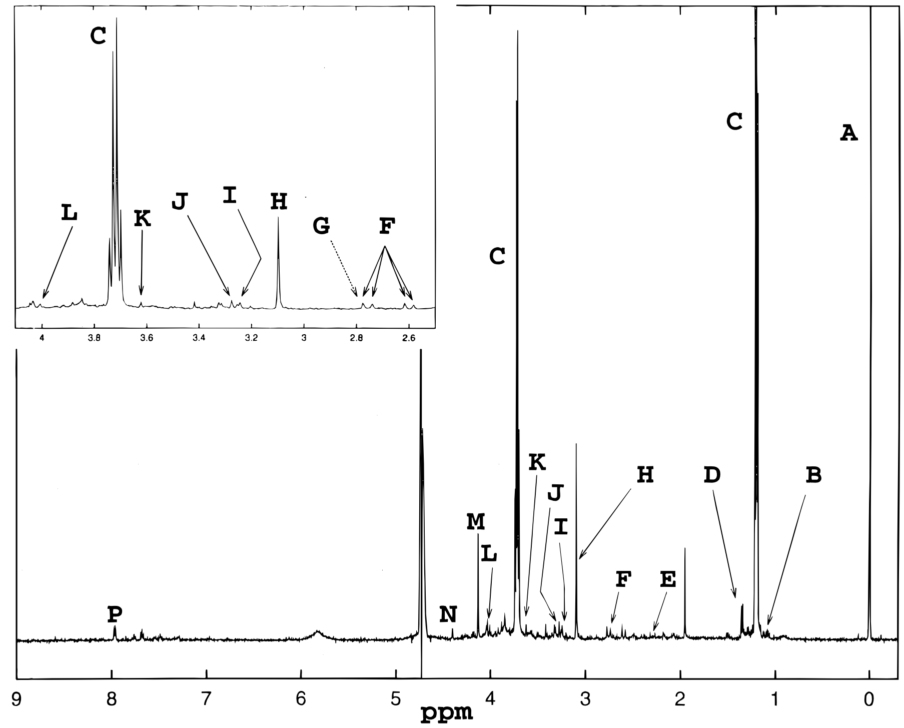

Figure 1. 500 MHz ^{1}H spectrum of urine-I (80 : 20 H_2O–D_2O). Peaks: A, TSP; B, 3-hydroxybutyrate; C, lactate; D, alanine; E, acetoacetate; F, citrate; G, sarcosine; H, creatinine-I; I, GPC; J, betaine; K, glycine; L, hippurate-I; M, creatinine-II; N, dihydroxyacetone; P, hippurate-II.

inversion–recovery sequence.[5] Variable relaxation delays (τ values) were chosen in a series of pilot scans; for some individual test solutions 16 variable delays were used, whereas for other solutions 24 such variable delays were used.

The resultant data were Fourier transformed without apodization, followed by phase correction. Chemical shift assignments were carried out with reference to the TSP peak, using previous literature assignments.[20,21] The spectral points corresponding to the respective

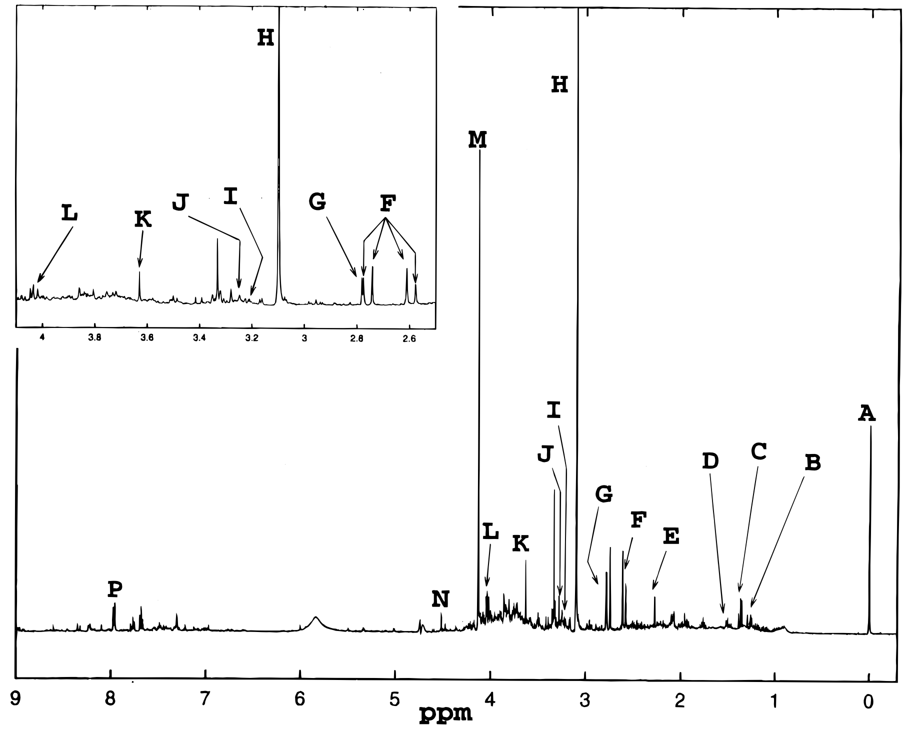

Figure 2. 500 MHz ^{1}H spectrum of urine-II (80 : 20 H_2O–D_2O). Peaks as in Fig. 1.

peaks (chemical shifts) in a spectrum were determined using the Bruker software. Each processed inversion–recovery data set was fitted using both the pix-fit routine installed on a UNIX-based DECstation 3100 computer, and also the sim-fit routine on a microcomputer.

For the pix-fit analysis, the absorption mode part of the processed complex data was first stripped from its dispersion mode counterpart. This process yielded 2K, 8K or 16K data points per spectrum and the resultant absorption data were analysed by using a three-parameter fit to the following equation:

$$M_t = M_0(1 - 2\rho e^{-t/T_1}) \tag{2}$$

where M_t is the magnetization detected after a recovery time t, M_0 is the equilibrium magnetization of the spins with a relaxation time constant of T_1, the offset, ρ, is used to compensate for imperfect inversion, if any, of the spins and the so-called null point T_1 ($T_{1\text{null}}$) is equal[22] to t/ln 2. The approximate $T_{1\text{null}}$ times for each set of test solutions and for some selected peaks in urine were calculated by plotting the intensities of the peaks of interest for each variable delay. The approximate T_1 values so obtained provided useful comparison values during the estimation of the T_1 value using the non-linear regression algorithms.

The 90° pulse for the test solutions was approximately 6 μs and for urine it was approximately 8 μs; these pulse widths corresponded to excitation bandwiths, at half-height, of *ca.* 166 and *ca.* 128 kHz, respectively, well beyond the *ca.* 16 kHz experimental frequency range. A computer-simulated excitation profile for an average 180° pulse of 15 µs showed at 12% deviation from maximum excitation at the extremes of this range; the approximate offset (ρ) values which compensate for incomplete inversion were, therefore, substituted when the data were fitted to Eqn (2).

The second T_1 calculation process (sim-fit) used a modified version of the non-linear simplex algorithm[23] in which the intensities of the peaks of interest were fitted independently against the relaxation delay by Eqn (2). All of the results for both the pix-fit and the sim-fit techniques are presented here with 95% confidence interval values.

Spectra were measured at 37 °C because cooling of urine specimens often yielded a colloidal suspension which dissolved as the temperature was raised. The longer pulse lengths for urine than for the test samples are probably due to the high ionic strength of urine leading to inductive losses in the sample, requiring more power to produce a 90° pulse. Paramagnetic dipolar relaxation contributions to spin–lattice relaxation from the dissolved oxygen were considered to be constant for all of the components in each sample.[22] This was verified by demonstrating that degassed solutions of the mixtures (mixtures M3, M4, M5 and M6) gave the same relative T_1 values as the normal solutions.

T_1 values (300 MHz, 37 °C) were also measured for 50 mM solutions of alanine in D_2O and in D_2O/buffered saline solution (pH 7.2). The average T_1 values (alanine and reference signals) were about 10% shorter in the saline solution.

Molecular modelling

Barriers to internal rotation of methyl groups in the lowest energy conformation of some of the metabolites were calculated using the dihedral driver option of PCMODEL 4.0 (Serena Software, Bloomington, IN, USA). PCMODEL is a molecular mechanics program that uses the MMX force field.

DISCUSSION

The reference samples selected are metabolites commonly found in urine in sub-millimolar or millimolar concentrations.[21] TSP was used as the internal reference standard in each solution for calculating peak areas, and hence the concentrations of the metabolites. The proportions of the metabolites in mixture M1 were adjusted to give similar peak intensities, based on the nine protons of TSP and two or three protons in glycine and alanine. The second mixture (M2) had twice as much TSP as mixture M1, and the hippurate (G) and lactate (E) solutions had approximately four times (see Table 2) the concentrations of the hippurate (F) and the lactate (D) solutions; these were used to check whether any variation in concentrations, at the level found in urine, had any effect on T_1 values. Previous pilot experiments showed that most of the metabolites in urine had T_1 values less than 5 s; therefore, a 30 s relaxation delay was considered sufficient to allow for complete relaxation recovery.

Maximum τ values chosen for both the pix-fit and the sim-fit methods were at least $3T_1$ to ensure that the relaxation curve was well defined with little weighting from possible errors in the inverting 180° pulse angle. The sensitivity of the measurements to the range of τ values employed was investigated by repeating the experiments on mixture M1, mixture M2, urine-I and urine-II under identical conditions except that the maximum variable delay (τ) used was 5 s, which is less than $3T_1$ for some components. Under those circumstances, the standard errors calculated for each metabolite were significantly larger for the slower than for the faster relaxing components. For example, the peaks which had relatively short T_1 values (e.g. the doublet of alanine at 1.48 ppm) yielded a more satisfactory fit than species with larger T_1 values, such as TSP.

The pix-fit method carries out a fit for every spectral point, rather than for a selected peak intensity (sim-fit method). Hence it is important that the spectral points bear a constant relationship to the frequencies in the spectra. The spectral point for a given peak and a given block size (data size) varied only within ± 1 point. This was verified by analysing the data for a single solution and also data from different samples (± 0.5 unit pH variation, Table 1) and 12 samples of urine spectra

Table 1. T_1 values of reference samples in individual solutions, mixtures and urine, calculated using the $T_{1\mathrm{null}}$, pix-fit and sim-fit methods (± values denote the 95% confidence intervals)

Compound	Peak (ppm)[a]	$T_{1\mathrm{null}}$ (s)	$T_{1\text{pix-fit}}$ (s)	$T_{1\text{sim-fit}}$ (s)
(C) Alanine (pH = 7.54)	1.48 (d)	2.17	2.39 ± 0.02	2.36 ± 0.02
	3.79 (q)	4.06	3.16 ± 0.02	4.20 ± 0.03
(A) Glycine-I (pH = 7.53)	3.57 (s)	2.82	2.72 ± 0.01	2.71 ± 0.02
(B) Glycine-B1	3.57 (s)	3.62	3.58 ± 0.03	3.54 ± 0.02
(B) Glycine-B2 (pH = 7.71)		3.76	3.60 ± 0.01	3.58 ± 0.02
(F) Hippurate-I (pH-7.57)	3.97 (d)	2.68	4.05 ± 0.01	NC[b]
	7.56 (t)	NC[b]	NC[b]	NC[b]
	7.65 (t)	NC[b]	NC[b]	NC[b]
	7.83 (d)	3.62	3.55 ± 0.03	NC[b]
(G) Hippurate-II (pH = 7.33)	3.97 (d)	1.95	1.86 ± 0.01	2.25 ± 0.06
	7.56 (t)	3.83	4.03 ± 0.05	4.06 ± 0.10
	7.65 (t)	4.98	6.37 ± 0.06	4.77 ± 0.08
	7.83 (d)	3.84	4.29 ± 0.02	4.34 ± 0.02
(D) Lactate-I (pH = 7.40)	1.33 (d)	2.53	2.17 ± 0.01	2.50 ± 0.02
	4.11 (q)	4.30	5.25 ± 0.03	6.63 ± 0.11
(E) Lactate-II (pH = 7.22)	1.33 (d)	2.46	2.39 ± 0.01	2.51 ± 0.02
	4.11 (q)	4.15	4.29 ± 0.04	4.66 ± 0.03
(M1) Mixture 1 (pH = 7.80)				
Alanine	1.48 (d)	1.99	2.43 ± 0.03	2.24 ± 0.02
	3.79 (q)	3.24	3.96 ± 0.02	3.39 ± 0.05
Glycine	3.57 (s)	2.38	3.07 ± 0.01	2.28 ± 0.02
(M2) Mixture 2 (pH = 7.35)				
Alanine	1.48 (d)	2.11	2.39 ± 0.01	2.32 ± 0.02
	3.79 (q)	3.72	4.76 ± 0.03	3.99 ± 0.05
Glycine	3.57 (s)	2.98	3.01 ± 0.01	2.75 ± 0.01
Hippurate	3.97 (d)	1.85	1.87 ± 0.01	2.48 ± 0.02
	7.56 (t)	3.81	3.94 ± 0.03	4.34 ± 0.04
	7.65 (t)	4.82	6.25 ± 0.04	6.09 ± 0.07
	7.83 (d)	3.95	4.21 ± 0.01	4.34 ± 0.03
Lactate	1.33 (d)	2.43	2.78 ± 0.02	2.93 ± 0.02
	4.11 (q)	4.01	4.18 ± 0.05	4.32 ± 0.08
(M3′) Mixture 3A[c] (pH = 7.14)				
Alanine	1.48 (d)	2.12	2.69 ± 0.01	2.67 ± 0.01
	3.79 (q)	3.20	5.18 ± 0.02	4.55 ± 0.02
Glycine	3.57 (s)	3.19	3.42 ± 0.01	3.42 ± 0.02
(M3″) Mixture 3B[c] (pH = 7.14)				
Alanine	1.48 (d)	1.91	2.69 ± 0.01	2.67 ± 0.01
	3.79 (q)	3.63	5.18 ± .0.02	4.44 ± 0.02
Glycine	3.57 (s)	3.30	3.42 ± 0.01	3.42 ± 0.02
(M4′) Mixture 4A[d] (pH = 7.21)				
Alanine	1.48 (d)	2.13	2.69 ± 0.01	2.67 ± 0.01
	3.79 (q)	2.51	5.18 ± 0.02	4.55 ± 0.02
Glycine	3.57 (s)	2.10	3.42 ± 0.01	3.42 ± 0.02
(M4″) Mixture 4B[d] (pH = 7.22)				
Alanine	1.48 (d)	2.36	2.69 ± 0.01	2.67 ± 0.01
	3.79 (q)	3.69	5.18 ± 0.02	4.55 ± 0.02
Glycine	3.57 (s)	3.10	3.42 ± 0.01	3.42 ± 0.02
(M5) Mixture 5[d] (pH = 7.29)				
Alanine	1.48 (d)	2.33	2.21 ± 0.01	2.14 ± 0.02
	3.79 (q)	4.16	3.13 ± 0.02	3.61 ± 0.02
Glycine	3.56 (s)	3.51	2.98 ± 0.01	3.27 ± 0.02
(M6) Mixture 6 (pH = 7.29)				
Alanine	1.48 (d)	2.30	2.68 ± 0.01	2.57 ± 0.01
	3.79 (q)	4.20	4.44 ± 0.01	4.62 ± 0.01
Glycine	3.56 (s)	3.55	4.17 ± 0.01	3.76 ± 0.02
Urine-I (pH = 6.82)				
Alanine	1.48 (d)	1.57	1.42 ± 0.11	1.46 ± 0.02
Glycine	3.57 (s)	2.74	2.75 ± 0.01	3.38 ± 0.02
Hippurate	3.87 (d)	1.75	1.78 ± 0.02	1.65 ± 0.03
	7.83 (d)	4.07	4.00 ± 0.03	4.30 ± 0.07
Lactate	1.33 (d)	2.36	2.21 ± 0.01	2.58 ± 0.03
Urine-II (pH = 7.81)				
Alanine	1.48 (d)	1.55	1.89 ± 0.01	1.85 ± 0.03
Glycine	3.57 (s)	3.19	3.16 ± 0.01	3.75 ± 0.02
Hippurate	3.97 (d)	1.87	1.94 ± 0.02	2.12 ± 0.04
	7.83 (d)	4.59	4.61 ± 0.04	4.76 ± 0.10
Lactate	1.33 (d)	2.01	2.04 ± 0.02	2.12 ± 0.06

[a] s, Singlet; d, doublet; q, quartet; t, triplet.

[b] Not calculated because of very low intensity or overlapping by an unsuppressed peak.

[c] 0.24 Hz per point digital resolution. [d] 1.96 Hz per point digital resolution.

obtained under identical experimental conditions (±0.80 unit pH variation). Similar pH values were mandatory in this study because protonation or deprotonation of some metabolites will have a dual effect. First, it can lead to a significant change in the chemical shift, thereby changing the peak point in the data set; second, and less important, the intramolecular dipolar interactions could change, thereby altering the relaxation rate. Stirring the solutions was necessary to minimize the instability of the pH reading. However, buffered solutions were not used because the pH measured for all test solutions did not vary beyond a range of 0.5 pH units (Table 1). The specific gravities of all test solutions were equal to unity, while those of the two urine samples were 1.002 and 1.001. Experimental conditions similar to those in the urine study were maintained at all times.

Variation in the T_1 values of methyl groups between metabolites was investigated using barriers to internal rotation calculated by molecular mechanics procedures. Correlations between methyl group rotational barriers and relaxation rates have been established previously.[4,24] Since interproton distances within a methyl group are short, dipolar relaxation within the group is efficient and significant intramolecular dipolar relaxation contributions from outside the group are unlikely.[3] Hence differences in the relaxation times of methyl groups in different compounds arise largely from the combined effects of different overall tumbling rates and different rates of internal methyl group rotation. Calculated rotational barriers (kcal mol^{-1}) decrease in the order 3-hydroxybutyric acid (3.07), sarcosine (3.02), alanine (2.65), lactic acid (2.64), TSP (2.00), betaine (1.97), acetoacetic acid (0.75) and creatinine (0.75). Alanine and lactate, with similar size, polarity and rotational barriers, have similar observed relaxation rates (Table 3), shorter than that of TSP (with faster rotating methyl groups) (Table 2), suggesting that methyl rotation is a significant factor in the overall relaxation rate of those compounds. However, there is an absence of an overall correlation between methyl internal rotational barriers and T_1 values of the compounds investigated, indicating that factors external to the molecules are largely responsible for determining relaxation rates. For example, betaine, which has an internal methyl rotational barrier almost identical with that of TSP, has a much shorter T_1. Relaxation may take place to water molecules and paramagnetic species in solution, while overall tumbling rates, in addition to depending on molecular size and geometry, may be affected by the polarity of the molecule and the extent of solvation.

The complete set of TSP T_1 values for all of the solutions examined is given in Table 2. The average T_1 value obtained for TSP using the null method is 3.95 ± 0.12 s; the T_1 values calculated by the pix-fit and sim-fit methods were 4.39 ± 0.26 and 4.39 ± 0.43 s, respectively, showing excellent agreement for the two algorithms. However, it was of interest to address the variation of T_1 values observed for TSP in test samples and urine; since appropriate corrections had been made to compensate for imperfect 180° pulses and variations in signal intensities, the variations observed are assumed to be related to the relaxation mechanisms of the protons in TSP. Spin–lattice relaxation of the methyl protons in TSP has contributions to τ_c from both internal methyl group rotation and overall molecular tumbling. Since the calculated methyl group rotational barrier (2.00 kcal mol^{-1}) is relatively low, methyl rotation is expected to have a significant influence on the overall relaxation rate. TSP exhibits the slowest relaxation of all the species investigated in this study; it is also the least polar species and so is the least likely

Table 2. T_1 values calculated for the methyl protons of TSP using the $T_{1\text{null}}$, pix-fit and sim-fit methods for test solutions and urine (± values denote 95% confidence intervals)

Solution	Compound	$T_{1\text{null}}$ (s)	$T_{1\text{pix-fit}}$ (s)	$T_{1\text{sim-fit}}$ (s)
C	Alanine	3.89	4.39 ± 0.01	5.01 ± 0.01
A	Glycine	3.75	3.87 ± 0.04	5.10 ± 0.01
B	Glycine-B1[a]	3.80	4.41 ± 0.02	4.40 ± 0.02
B	Glycine-B2[a]	3.86	4.13 ± 0.02	4.09 ± 0.02
F	Hippurate-I	4.17	3.69 ± 0.01	3.52 ± 0.02
G	Hippurate-II	3.72	5.51 ± 0.01	2.99 ± 0.01
D	Lactate-I	3.81	4.91 ± 0.01	4.56 ± 0.02
E	Lactate-II	3.96	3.67 ± 0.01	3.28 ± 0.01
M1	Mixture 1	3.92	4.08 ± 0.01	2.90 ± 0.01
M2	Mixture 2	3.95	3.92 ± 0.01	5.89 ± 0.02
M3′	Mixture 3A	4.34	4.24 ± 0.01	4.22 ± 0.01
M3″	Mixture 3B	4.06	3.86 ± 0.02	4.33 ± 0.02
M4′	Mixture 4A	4.49	4.61 ± 0.02	4.66 ± 0.05
M4″	Mixture 4B	4.57	4.51 ± 0.01	5.07 ± 0.01
M5	Mixture 5	4.68	4.72 ± 0.01	3.86 ± 0.02
M6	Mixture 6	4.78	4.43 ± 0.01	4.66 ± 0.03
	Urine-I	4.25	4.67 ± 0.02	4.68 ± 0.04
	Urine-II	4.26	5.37 ± 0.02	5.83 ± 0.03

[a] The experiments were repeated on the same solution under identical conditions.

Table 3. Average T_1 values (from Tables 1 and 2) calculated for some metabolites using the $T_{1\text{null}}$, pix-fit and sim-fit methods (± values denote 95% confidence intervals)

Compound	Peak (ppm)	$T_{1\text{null}}$ (s)	$T_{1\text{pix-fit}}$ (s)	Error (%)	$T_{1\text{siM-fit}}$ (s)	Error (%)
Alanine	1.48	1.88 ± 0.37	2.33 ± 0.26	11.01	2.27 ± 0.24	10.52
	3.79	3.67 ± 1.02	4.05 ± 0.66	16.24	3.91 ± 0.47	11.99
Glycine	3.57	2.82 ± 0.37	3.19 ± 0.28	8.76	3.19 ± 0.31	9.82
Hippurate	3.97	1.86 ± 0.10	1.86 ± 0.08	4.30	2.13 ± 0.56	26.29
	7.56	3.82 ± 0.13	3.98 ± 0.56	14.10	4.20 ± 1.78	42.38
	7.65	4.90 ± 1.18	6.30 ± 0.76	12.10	5.43 ± 8.39	—
	7.83	4.01 ± 0.45	4.13 ± 0.48	11.62	4.44 ± 0.34	7.65
Lactate	1.33	2.36 ± 0.25	2.32 ± 0.36	15.52	2.53 ± 0.35	13.83
	4.11	4.08 ± 0.89	4.54 ± 1.46	—	5.72 ± 11.56	—
TSP	0.0	3.97 ± 0.14	4.39 ± 0.26	5.96	4.39 ± 0.43	9.73

compound to have its overall rotational rate influenced by solvation. Since a significant variation of T_1 with pH was not observed in any of the solutions investigated, differing levels of protonation of the carboxylate anion are unlikely to be responsible for the variation in T_1 values observed. That the greatest variation in observed T_1 values is observed for the slowest relaxing species is consistent with a recent report that thermal convection in non-spinning NMR sample tubes may influence the time evolutions of magnetization during an inversion–recovery pulse sequence.[25]

The T_1 values obtained for the hippurate peak at 3.97 ppm in solutions F and G (Table 2) showed a variation of up to 34.1%. This is because the signal was partly obscured by a poorly suppressed water signal; since that signal does not show a true recovery, the intensity of nearby peaks can vary randomly. This residual water signal also interferes with other low-intensity peaks (Table 1), e.g. the anomeric protons of glucose near 4.6 ppm. Nevertheless, the T_1 values calculated for the aromatic proton peaks of hippurate, which are shifted from the water signal, agree well with each other; those protons have relatively long relaxation times, 3.98 ± 0.56 and 4.13 ± 0.48 s for the doublets at 7.56 and 7.83 ppm, respectively, because dipolar relaxation is relatively inefficient between aromatic ring protons (Table 2). In contrast, the methylene protons of the side-chain of hippurate (3.97 ppm) have a shorter T_1 value ($T_1 = 1.86 \pm 0.08$ s), reflecting efficient relaxation because of the close proximity of those protons.

In human urine, the low-frequency region where the methyl signals of alanine appear is relatively crowded with the signals of other metabolites;[20] interference from such signals, especially when the metabolites of interest are present in low concentrations, is probably the main reason for differences in T_1 values observed for urine. The presence of any urinary paramagnetic ions, which would contribute to a reduction in T_1 values, was discounted in the present study since global reductions in metabolite T_1 values were not observed. Urine-I (Fig. 1) was relatively dilute, so that detection of some metabolite signals, such as the acetoacetate peak, was not possible (Table 4). Pix-fit calculated T_1 values for known acetoacetate peaks agreed well with those calculated for urine-II (Fig. 2) by the other methods. However, the T_1 value calculated for dihydroxyacetone in urine-I by the pix-fit method differed from the corresponding value for urine-II (the large error returned by the fitting procedure perhaps indicates an unsuccessful fitting to this very low-intensity signal). Nevertheless, the T_1 values

Table 4. T_1 values of selected metabolites of urine-I and -II calculated using the $T_{1\text{null}}$, pix-fit and sim-fit methods (± values denote 95% confidence intervals)

		Urine-I			Urine-II		
Compound	Peak (ppm)[a]	$T_{1\text{null}}$ (s)	$T_{1\text{pix-fit}}$ (s)	$T_{1\text{sim-fit}}$ (s)	$T_{1\text{null}}$ (s)	$T_{1\text{pix-fit}}$ (s)	$T_{1\text{sim-fit}}$ (s)
3-Hydroxybutyrate	1.22 (d)	1.34	1.33 ± 0.15	NC[b]	1.55	1.45 ± 0.00	1.94 ± 0.20
Acetoacetate	2.34 (s)	NC[b]	1.64 ± 0.01	NC[b]	1.61	1.78 ± 0.01	NC[b]
Citrate	2.65 (d)	0.61	0.54 ± 0.02	0.54 ± 0.02	0.71	0.65 ± 0.03	0.88 ± 0.05
Sarcosine	2.73 (s)	3.38	3.47 ± 0.02	NC[b]	6.01	6.29 ± 0.19	6.20 ± 0.23
Creatinine-I	3.06 (s)	3.81	3.91 ± 0.01	3.79 ± 0.05	3.82	3.85 ± 0.03	3.96 ± 0.07
GPC	3.22 (s)	1.11	1.12 ± 0.05	1.14 ± 0.09	1.24	1.31 ± 0.03	1.32 ± 0.11
Betaine	3.25 (s)	1.83	1.77 ± 0.11	NC[b]	2.27	2.44 ± 0.01	2.78 ± 0.04
Creatinine-II	3.94 (s)	3.03	3.08 ± 0.02	3.07 ± 0.08	2.99	2.97 ± 0.01	3.07 ± 0.02
Dihydroxyacetone	4.43 (s)	NC[b]	5.51 ± 0.22	NC[b]	2.62	2.61 ± 0.01	4.02 ± 0.03

[a] s, Singlet; d, doublet.

[b] Not calculated because of very low intensity or overlap by another peak.

calculated for most of the other metabolites in urine-II were close to the corresponding values for urine-I (Table 4).

The T_1 values obtained from urine-I for the 1.33 ppm methyl signal of lactate (Table 3), 2.21 ± 0.01 s, the methyl signal (3.06 ppm) of creatinine (3.91 ± 0.01 s) (Table 4), the hippurate peaks at 3.97 ppm (1.86 ± 0.08 s) and 7.83 ppm (4.13 ± 0.48 s) (Table 3), and for the methylene group of glycine (3.57 ppm, 3.19 ± 0.28 s) (Table 3) represent good fitting; a standard error of less than 10% is considered satisfactory for T_1 values.[26]

The $N(CH_3)_3$ groups in glycerylphosphorylcholine (GPC) (1.12 ± 0.05 s, pix-fit) and betaine (1.77 ± 0.11 s, pix-fit) have much shorter T_1 values than the $C(CH_3)_3$ groups in TSP, despite the similar calculated methyl group internal rotational barriers in betaine and TSP. The chemical shift of the trimethylamine peak in betaine and GPC solutions did not change over a pH range of 6.5–8.4, so the differences observed between the T_1 values of the methyl signal of betaine and of GPC in urine-I and urine-II (Table 4) were not due to the difference in pH (0.99 pH units). It is unlikely that the highly basic trimethylamine units in those compounds will change their degree of protonation over the limited pH range of the urine samples sufficiently to affect relaxation rates.

The largest apparent difference in T_1 between the two urine samples was observed for sarcosine. Studies on urine showed that there was a noticeable chemical shift dependence of the methyl signal of sarcosine over the range of physiological pH (no measurements were made on the methylene signal). Since the sarcosine signal is pH dependent and was sometimes overlapped by part of the citrate quartet, sarcosine T_1 values could be determined accurately only when there was no overlap. The pH dependence, if any, of the T_1 values of sarcosine in test solutions was not investigated.

CONCLUSIONS

The T_1 values of metabolites found in urine were estimated using two different optimization algorithms. T_1 values of standardized laboratory solutions gave an estimate of the statistical variations in the experimental determination of T_1 values under conditions comparable to those used for the urine samples. The pix-fit application of the Marquardt–Levenberg algorithm implemented in this study offers the advantage of speed, since the whole spectral range (all of the spectral points) is fitted, without intervention, in about 4 min, the T_1 value for any peak in the spectrum was subsequently extracted. In contrast, the peak picking routine implemented in the simplex method to calculate the peak height requires operator selection of peaks to be analysed; typically it requires several minutes for complete processing of a single T_1 value. The T_1 values of most of the metabolites in urine measured by the above procedures have a maximum error limit of 15%.

To our knowledge, this is the most extensive study of the T_1 values of urinary metabolites and simple metabolic solutions performed with two different non-linear regression algorithms. The work indicates the experimental conditions necessary to make accurate intensity measurements; it is not necessary to adjust the pH of urine.

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