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ORIGINAL ARTICLE

Improved quantification from ^{1}H-NMR spectra using reduced repetition times

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Abstract A method of choosing the correction factor based on Bloch equation for the quantitative estimation of metabolite from ^{1}H NMR spectra recorded with reduced recycle delay is prescribed. The procedure reduces the experimental time without substantially compromising the accuracy of quantitative estimation. It is based on choosing the correction factors, which depend on T_1 and T_2 of the metabolite and recycle delay used for recording the spectra. It is validated by studying a mixture of amino acids with known concentration of constituents and human serum sample and it provides accuracy of quantitative estimation to 95–96%.

Keywords T_1 and T_2 relaxation · CPMG · NMR spectroscopy · Body fluids · Metabolites · Quantitation

Abbreviations

CPMG	Carr–Purcell–Meiboom–Gill
FID	Free induction decay
qNMR	Quantitative NMR
TSP	Trimethyl silane propionic acid sodium salt

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1 Introduction

NMR spectroscopy has tremendous potential for the metabonomic studies of the biological fluids as it provides both qualitative as well as quantitative information for the small molecular weight metabolites present in the sample (Bell and Sadler 1999; Malz and Jancke 2005; Gang et al. 2007). The added advantage is the wealth of information for a number of metabolites that can be obtained in a single step analysis of biological fluids. Considerable progress has been achieved in this field over last few years. The technique has been applied to identify biomarkers for various diseases (Gang et al. 2007; Lindon et al. 2000, 2004; Viant et al. 2003) such as diagnosis of Malabsorption syndrome (Bala et al. 2004, 2006), Meningitis (Subramanian et al. 2005), Tyrosinemia (Bell et al. 1989), Alkaptonuria (Shuchi et al. 1989), Dicarboxylic aciduria (Daviesa et al. 1992), and identification of organs dysfunction like chronic Renal failure (Bell et al. 1991), Hepatic failure (Saxena et al. 2006), monitoring Liver graft dysfunction (Singh et al. 2006) etc. However, there are some experimental factors associated with quantitative determination from NMR which need careful consideration (Pauli et al. 2006). For the accurate quantitative analysis from NMR spectra, all experimental parameters should be carefully optimized to achieve acceptable accuracy and precision. Recycle delay is one of the most important parameters for quantitative analysis. Many research articles have reported different recycle delay times of 50 s, 60 s etc. for complete relaxation of magnetization (Saxena et al. 2006; Gang et al. 2007; Griffiths and Irving 1998; Larive et al. 1997) for recording the NMR spectra. It is recommended that recycle delay should ideally be five times the longest longitudinal relaxation time (T_1) of a particular metabolite present in the sample (Evilia 2001). For a large number of samples,

such allowance of complete relaxation leads to increase in overall measurement time for each sample under study. Metabonomics study requires analysis of hundreds of samples for statistical significance and this requirement harnesses the limitation on experimental time (Saude et al. 2006). Experimental time should be as low as possible with an acceptable accuracy for quantification. Although the difference of errors due to incomplete relaxation of various metabolites associated in any metabonomic/metabolomic studies remains constant when compared with control subjects but quantitative variation when compared with other analytical methods result in significant ambiguities. The effect of incomplete relaxation due to shorter repetition time can be removed by using relaxation agents (Griffiths and Irving 1998; Caytana et al. 2007). But this method has limitation since choice of unique relaxation agent for different metabolites is difficult. Standard addition method followed by re-measurement of spectra (Gang et al. 2007) can also be used to provide relative intensities which are not affected by T_1 relaxation. This method requires measurement of a single sample twice and hence it also increases the overall experimental time. In this article, we report a method based on Bloch Equation (Bloch 1946) for improved quantitative estimation by one dimensional ^{1}H NMR experiment recorded with incomplete relaxation delay, usually much shorter than five times longest spin–lattice relaxation (T_1) delay.

Quantitative analysis of small metabolites in biofluids such as serum, plasma, ascitic fluids etc. cannot be carried out by single pulse NMR spectroscopy due to broad resonances from high molecular weight lipids and lipoproteins. There are several methodologies like de-proteinization (Voet and Voet 1990; Wevers et al. 1994) ultracentrifugation (Voet and Voet 1990; Daykin et al. 2002) and diffusion sensitized technique (de Graaf and Behar 2003) etc. to overcome the problem. Hahn-echo (Kriat et al. 1992) and CPMG (Van et al. 2003) have also been used for such a purpose. All these methods have advantages and disadvantages related to quantitative accuracy. Retaining the noninvasive nature and minimal sample preparation required for NMR spectroscopy, CPMG and Hahn-echo are widely used methods for analyses of bio-fluids where concentrations of macromolecules are high (Viant et al. 2003; Saxena et al. 2006; Singh et al. 2006; de Graaf and Behar 2003; Van et al. 2003; Nishijima et al. 1997; Lucas et al. 2005; Shiyan et al. 1992). NMR parameter which affects the quantitative accuracy is spin–spin relaxation (T_2) when CPMG and Hahn-echo pulse sequences are used. The quantitative accuracy is greatly affected by spin-spin relaxation (T_2) times for different metabolites and total echo time used in CPMG pulse sequence to filter broad signals. In this article, we propose a T_2 correction factor to improve the accuracy of quantification of small molecular weight metabolites. We further demonstrate that CPMG measurement, using shorter recycle time along with implementation of T_1 and T_2 correction factors can provide quantitative results with 95–96% accuracy.

2 Materials and methods

2.1 Solution preparation

We used standard solutions of mixtures of amino acids and other metabolites for evaluation of T_1 and T_2 as well as for quantitative estimation. Four groups of sample consisting of mixtures of 14 amino acids, lactate, acetate and succinate were employed. All compounds were purchased from Merck Chemicals and Sigma–Aldrich with a minimum purity of 99% (AR grade). Therefore, no further purification was carried out. Deuterium oxide (99.8% D atom, Sigma–Aldrich) was used for solution preparation. All samples were divided into groups so as to avoid overlap of the signals. Mixture #1 contained Alanine, Leucine, Methionine, Glycine, Histidine, and Tryptophan, Mixture #2 contained Valine, Arginine, Glutamine, and Aspartic acid. Mixture #3 contained Isoleucine, Proline, Asparagines, and Phenylalanine. And Mixture #4 contained Lactate, Alanine, Acetate, Succinate and Glycine. The one dimensional ^{1}H NMR spectra with the resonance assignments are shown in Fig. 1. For verification of the results some of the metabolites e.g. Alanine, Glycine were taken in two groups in two different concentrations. The concentrations of all metabolites in different samples varied in the range of 60–200 mg/dl. T_1 values of different resonances from standard samples were measured by inversion recovery method. The distribution of T_1 values are shown in Fig. 2a. It can be seen we have wide variation in T_1 values ranging from 0.8 to 7.5 s. Similarly T_1 distribution of some of the unassigned resonances in human urine sample are shown in Fig. 2b.

For validation of the results, a known concentration of Formate and Uracil (40 mg/dl) was mixed with 400 µl serum sample with ammonium chloride. NH_4Cl was used (final concentration of 0.8 M) to release the bindings of small metabolites and serum proteins and for achieving better water suppression (Yamaguchi et al. 1989).

2.2 NMR experimental acquisition

All NMR experiments were performed on Bruker Biospin Avance 400 MHz wide bore (89 mm) NMR spectrometer with 5 mm Broad Band Inverse probe at 300 K temperature. Wilmad NMR tubes (5 mm) and coaxial insert containing reference standard TSP dissolved in D_2O, were used (Griffiths and Irving 1998; Henderson 2002). For each spectrum, manual shimming was done and optimized so

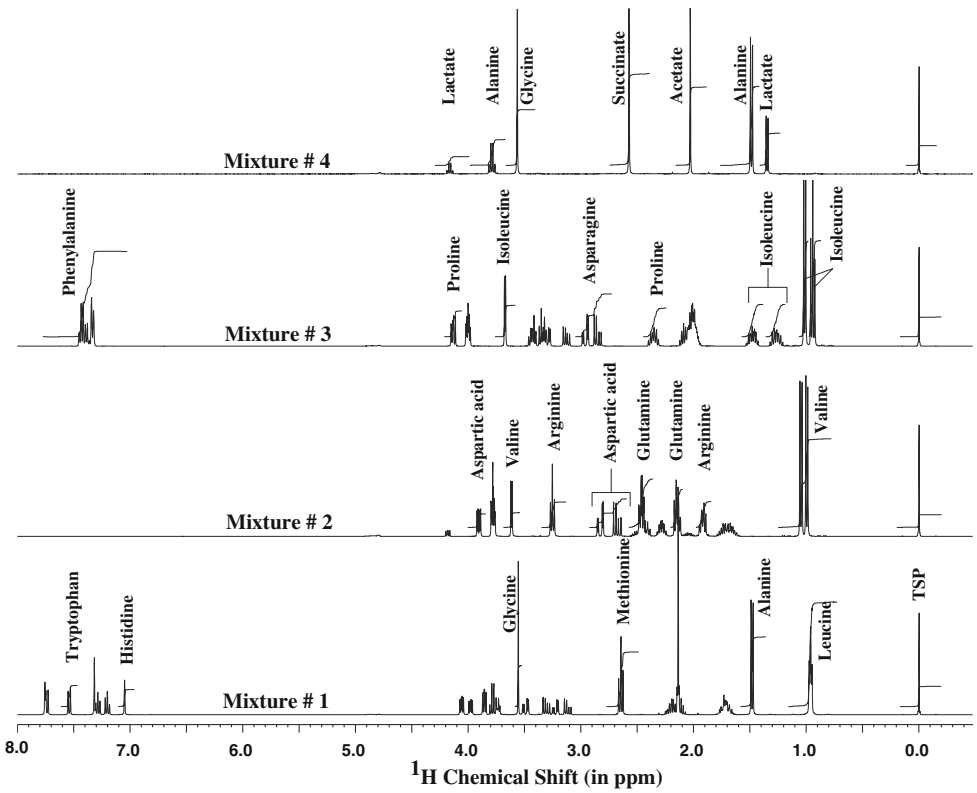

Fig. 1 ^{1}H NMR spectrum of all resonances taken for the evaluation of T_1 and T_2 correction factor. All metabolites divided into four mixture groups to avoid overlap of the resonances

that TSP line widths were in the range of 0.9–1.1 Hz. All spectra were acquired with an acquisition time of 2.044 s, relaxation delay of 1–50 s, 32 transients scans and 4 steady state scans prior to acquisition, 32K data points, spectral width of 8012.82 Hz, 90° flip angle (10.6 μs) and optimum low power level for water presaturation during re-cycle delay. The temperature of the sample was continuously monitored and no significant change in temperature was observed due to long irradiation for water presaturation. A single pulse sequence and Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence with water presaturation were performed for quantitative analyses on standard samples. A single echo time of 200 μs was used in CPMG pulse sequence. For incorporation of T_1 correction factor a repetition time of 6.04 s was chosen (Acquisition Time 2.04 s, Relaxation Delay 4.0 s) which was sufficient for water suppression. CPMG pulse sequence was used for recording the NMR spectra of serum with a total echo time of 200 and 400 ms for effective suppression of macromolecules in both the echo time along with quantitative estimation of small molecular weight metabolites.

Standard inversion recovery experiments were performed for measuring the longitudinal relaxation time for all resonances. For the measurement of T_1 in serum, the inversion recovery pulse sequence was modified as [180°–τ–90° –(δ–180–δ)$_n$–aq.] (Kriat et al. 1992) with presaturation of water signal. Transverse relaxation rate (T_2) was determined by CPMG pulse sequence with variable echo-time.

2.3 NMR quantitation

XWINNMR software 3.5 was used for processing of raw data. All FIDs were zero-filled to 16K points before performing Fourier transformation, with multiplication and decaying exponential function corresponding to 0.3 Hz line broadening. Manual phase correction was performed followed by automatic base line correction. Signals which are well separated were selected for quantification. All peaks in the spectra were manually integrated and calibrated with respect to TSP signal. Concentrations of metabolites were calculated from the spectra recorded at different recycle delay time varying from 1.0 to 50.0 s and region of peak integration was kept constant. The concentration measured from the spectra recorded with 50.0 s recycle delay time was compared with value measured from spectra recorded with shorter recycle delay by incorporating T_1 correction factor.

3 Results and discussion

3.1 Quantification of metabolites

Two commonly used NMR pulse sequences, single pulse and CPMG with water signal pre-saturation, were chosen in this study. The accurate quantitation of metabolites is complicated in biofluids such as serum having high concentration of proteins contents. The CPMG pulse train

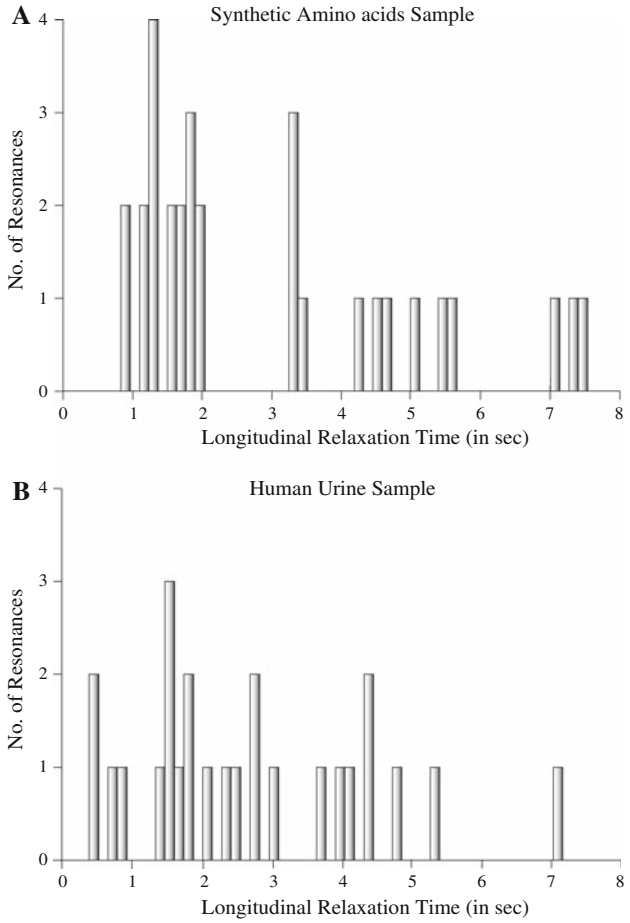

Fig. 2 Distribution of T_1 (longitudinal relaxation) values of different resonances in (**a**) synthetic mixture of amino acids solution and (**b**) lyophilized human urine sample reconstituted in D_2O

exploits the shorter transverse relaxation rate of macromolecules. This train of fast spin-echo is based on the original Hahn spin echo (Hahn 1950) modified by Carr and Purcell (Carr and Purcell 1954) to reduce attenuation effect due to molecular diffusion, and further improved by Meiboom and Gill (1958) to reduce the cumulative error resulting from imperfection of 180° pulse. For example, the one dimensional spectrum shown in Fig. 3 where broad signals are filtered by using echo time of 200 ms and only sharp signals are retained which arise from low molecular weight metabolites. Therefore, quantitative analysis of serum can be carried out by CPMG pulse sequence rather than Hahn-echo pulse sequence.

3.2 Effect of spin-lattice relaxation (T_1) on quantitative NMR (qNMR)

Experimental time and quantitative accuracy both are critical parameters for quantitative analysis from NMR spectroscopy. One has to optimize the all acquisition experimental parameters for an optimum quantitative accuracy in minimum experimental time. To study the effect of relaxation a series of experiments were performed using single pulse and CPMG sequence at different repetition times varying from 3.04 to 52.04 s (Fig. 4). Concentration of the metabolites were calculated through integration of the resonance referencing with known concentration of TSP. Spectra recorded with incomplete relaxation provides substantial quantitative inaccuracy depending upon the T_1 of corresponding resonance. Quantitative results of metabolites having lower T_1 values than

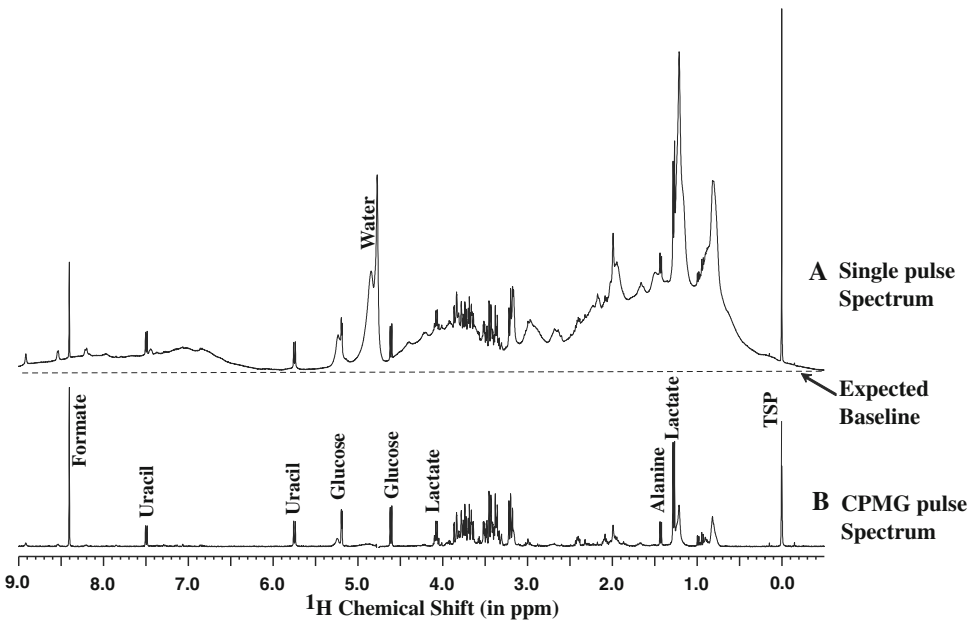

Fig. 3 (A) Single pulse ^1H NMR spectrum of human serum full of broad resonances and (B) CPMG spectrum of same sample provides a better baseline along with suppression of broad signals

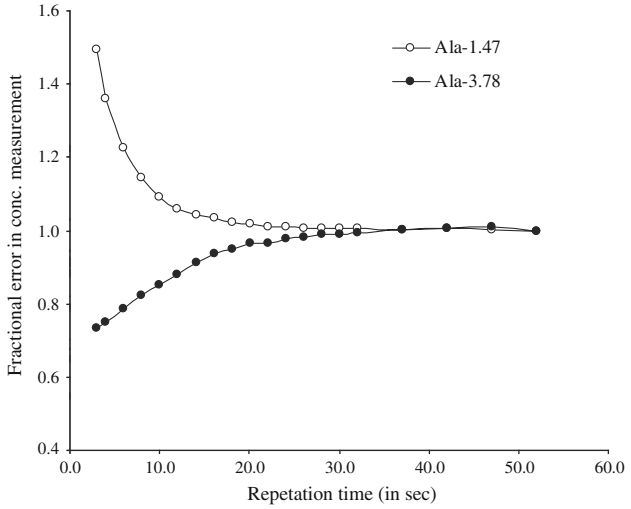

Fig. 4 Erroneous values of concentration measured for Alanine at different repetition time for (**a**) –CH_3 resonance having T_1 lower than TSP and (**b**) –CH resonance having T_1 higher than TSP resonance. Fractional error is defined as ratio of measured concentration at shorter relaxation delay with concentration measured at full relaxation delay

TSP provide higher values than those measured from fully relaxed spectra, because rate of relaxation of metabolite resonance is faster than TSP. Therefore relative integral value will be higher for metabolite resonance. Whereas metabolite resonances having T_1 longer than TSP provide lower values than those measured from fully relaxed spectra. In case of metabolites having T_1 equal or nearly equal to TSP, there is no effect of repetition time on the measured concentration. The overall intensities of resonances increase with increase in repetition time while relative intensities with respect to TSP vary differently depending upon the difference in the T_1 values of respective resonances and TSP. Therefore, quantitative estimation of particular metabolites using its various resonances provides different erroneous values. For example in Fig. 4, the –CH_3 signal (1.47 ppm, doublet, $T_1 = 1.93$ s) and –CH signal (3.78 ppm, quartet, $T_1 = 7.11$ s) of the Alanine show different concentrations at variable repetition time when calculated with respect to the reference. (TSP, 0.0 ppm, $T_1 = 4.40$ s).

Using shorter repetition time a T_1 correction factor was incorporated in order to reduce the experimental time and inaccuracy appearing due to T_1 effect. Equation for correction factor was derived from solution of Bloch equation.

$$\mathrm{Conc}_{(\infty)} = \mathrm{Conc}_{(t)} \frac{1 - \exp^{-t/T_1^S}}{1 - \exp^{-t/T_1^M}} \quad (1)$$

Here 't' is the repetition time and T_1^S and T_1^M are the T_1 of TSP and test metabolite respectively. $\mathrm{Conc}_{(\infty)}$ and $\mathrm{Conc}_{(t)}$ are the concentrations at fully relaxed experiment and at 't' repetition time experiment respectively.

Above equation can be implemented for calculation of actual concentration (value at full relaxation) using concentration at any shorter repetition time. A theoretical calculation was carried out using above equation and T_1 of metabolites used in quantitation and the reference standard having T_1 of 4.40 s at different repetition times (Fig. 5a). A similar experimental curve was also observed for 30 resonances selected for quantitation (Fig. 5b). Concentration measured at shorter repetition time e.g. at 3.04 s provides maximum inaccuracy up to 80% depending upon T_1 of metabolites and TSP resonances and decreases with increase in repetition time and becomes much less near full relaxation. Recording spectrum at shorter repetition time will not affect the final result of principle component analysis (PCA) because the effect of T_1 relaxation would be same for both control and disease samples used in a study. But cut off value for a metabolic marker may change when compare with quantitative analysis by NMR spectroscopy at full relaxation or with other techniques like HPCL, UV etc. T_1 correction factor was incorporated for quantitative estimation of all resonances belonging to different metabolites. Concentration for each metabolite was measured at two points; one at shorter relaxation and the other at full relaxation. Concentration measured at shorter repetition time was multiplied by T_1 correction factor and

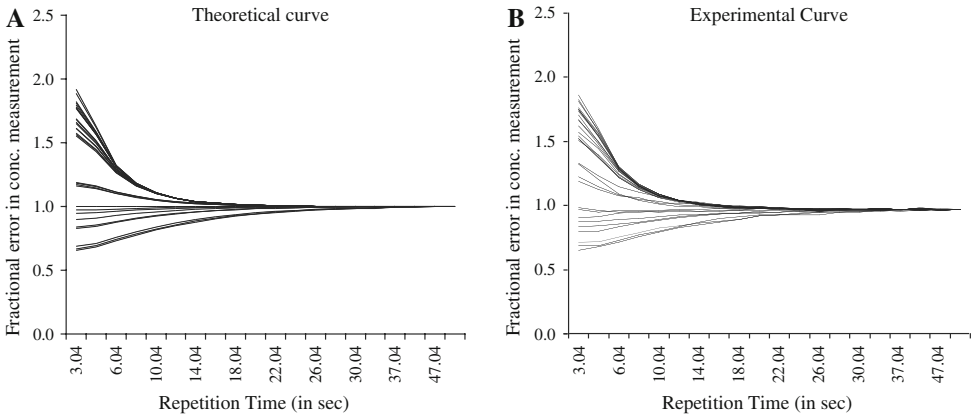

Fig. 5 Variation of erroneous concentration due to incomplete relaxation. (**a**) Theoretical calculation and (**b**) experimental calculation of concentration at different repetition time using T_1-correction equation

then compared with concentration measured from fully relaxed spectrum. From (Fig. 7a, blue bar) it is clearly shown that quantitative estimation without T_1 correction factor provides inaccuracy distribution up to 30% i.e. maximum metabolites fall in the inaccurate region. The histogram in Fig. 7 shows the distribution in fraction error (ratio of concentration measured in an experiment with concentration measured from fully relaxed spectrum) in concentration measurement. Metabolites falling in the fraction error region of 1.2–1.3 have T_1 lower than TSP and those metabolites having T_1 longer fall in error region lower than 1.0. Metabolites having T_1 equal or nearly equal to TSP were not affected by incomplete relaxation. Incorporation of T_1 correction factor improved in the quantitative estimation and provided an average accuracy of 97.5% (Fig. 7a, red bar). It is interesting to note that the metabolites having T_1 around one second (e.g. Arginine, 1.91 ppm, δ–CH2–, T_1 0.83 s) should ideally relax within 6.04 s of repetition time and give correct value of concentration measurement, but practically this is not found

Table 1 Summary of quantitative accuracy of metabolites in synthetic sample with and without T_1 and T_2 correction factor

Compounds	Chem. shift (ppm)	Proton type	T_1 (s)	T_2 (s)	$RWTT_1$[a]	RWT_1[b]	$RWTT_2$[c]	RWT_2[d]	$RPWT_1$[e]	RWT_1T_2[f]
Leucine	0.95	CH_3	1.21	1.07	1.28	0.99	0.74	0.96	0.88	0.99
Alanine	1.47	CH_3	1.96	1.71	1.24	1.01	0.84	0.96	0.93	0.98
Methionine	2.63	CH_2	1.59	1.30	1.27	1.01	0.81	0.98	0.91	1.01
Glycine	3.56	CH_2	4.65	3.25	0.97	1.00	0.98	0.99	1.03	1.01
Histidine	7.02	CH	7.54	2.22	0.78	1.01	0.88	0.96	1.01	1.05
Tryptophan	7.55	Ar-CH	5.00	2.68	0.93	1.05	0.95	0.98	1.11	1.12
Valine	1.05	CH_3	1.31	1.22	1.28	1.00	0.90	0.99	0.88	0.99
	3.59	CH	4.48	2.81	0.99	0.99	1.10	1.00	1.00	1.02
Arginine	3.25	CH_2	0.96	0.71	1.28	0.98	0.85	0.98	0.83	0.99
	1.91	CH_2	0.83	0.69	1.29	0.97	0.86	0.99	0.79	0.99
Glutamine	2.46	CH_2	1.78	1.40	1.25	1.01	0.91	1.02	0.91	0.97
	2.15	CH_2	1.26	1.27	1.29	1.00	1.07	1.03	0.88	1.00
Aspartic acid	2.60	CH_2	1.57	1.56	1.27	1.00	0.89	0.99	0.90	0.99
	3.89	CH	5.49	2.96	0.90	1.00	0.92	1.02	1.11	1.10
Isoleucine	0.90	CH_3	1.68	1.21	1.27	1.01	0.82	1.02	0.90	1.01
	1.00	CH_3	1.18	0.93	1.30	1.02	0.76	1.05	0.87	1.02
	1.26	CH_2	1.33	1.15	1.29	1.01	0.80	1.01	0.89	1.00
	1.46	CH	1.32	1.14	1.30	1.00	0.80	1.01	0.89	1.01
	3.65	CH	3.41	2.46	1.11	1.01	1.01	1.06	1.01	1.02
Proline	2.40	CH	3.29	2.63	1.10	1.01	0.97	1.00	0.91	1.00
	4.15	CH	7.36	3.93	0.91	1.02	1.10	1.05	1.27	1.13
Asparagine	2.90	CH_2	1.65	1.25	1.28	1.00	0.85	0.99	0.90	1.02
Phenylalanine	7.45	Ph-CH	3.37	2.45	1.01	1.03	0.96	1.00	0.98	1.01
Lactate	1.33	CH_3	1.79	1.21	1.22	0.99	0.86	1.06	0.88	0.95
	4.12	CH	3.31	1.32	1.16	1.05	0.94	1.05	0.92	0.99
Alanine	1.47	CH_3	1.93	1.62	1.23	1.00	0.91	1.03	0.97	0.99
	3.78	CH	7.11	3.71	0.79	0.96	1.11	1.05	1.11	1.08
Acetate	1.95	CH_3	5.60	3.20	0.88	0.99	1.03	1.04	1.03	1.00
Succinate	2.46	CH_2	1.89	1.45	1.22	0.99	0.90	1.06	0.96	1.01
Glycine	3.56	CH_2	4.31	3.02	0.99	0.98	1.07	1.06	1.05	1.03

[a] Ratio of measured concentration at shorter relaxation time with concentration measured with full relaxation time without incorporating T_1 correction factor

[b] Ratio of observed concentration at shorter repetition time and full relaxation with T_1 correction

[c] Ratio of observed concentration by single pulse and CPMG pulse sequence at full relaxation without T_2 correction

[d] Ratio of observed concentration by single pulse and CPMG pulse sequence at full relaxation with T_2 correction

[e] Ratio of estimated concentration by incorporating T_1 correction factor in the CPMG experiment measured at shorter repetition time with concentration measured by single pulse experiment at full relaxation

[f] Ratio of observed concentration by single pulse at full relaxation and CPMG at shorter repetition time with both T_1 and T_2 correction

due to incomplete relaxation of the standard reference TSP. It is therefore obvious that T_1 of both reference and metabolites resonances play important roles in quantitative accuracy. Detailed analysis of concentration measurement for each metabolite is presented in Table 1. In case of ERETIC method used for quantitation, the electronically generated reference signal intensity is not affected by repetition time (Akoka et al. 1999). But quantitative accuracy will also be affected when ERETIC method is used at shorter repetition time, because of metabolites resonances having longer T_1 values e.g formate having longer T_1 value is 7.65 s which needs a repetition time of approximately 38.0 s (5 * T_1), consequently increases the experimental time. Therefore ERETIC method cannot be helpful in reduction of the experimental time because of wide distribution of T_1 in a mixture (see Fig. 2). For quantitative analysis by ERETIC method using shorter repetition time, a T_1 correction factor due to metabolites resonance should be incorporated. ERETIC method would be performed in new version instrument without any additional electronic device to generate reference signal (Silvestre et al. 2001) whereas in old spectrometers it is not possible without adding an external electronic device for synthesizing electronic reference signal.

Quantitative analysis using overlapping signals can also be performed with help deconvolution if the T_1 of that particular resonance is known or can be evaluated.

3.3 Effect of spin–spin relaxation (T_2) on qNMR

A detailed study has been carried out by earlier worker (Van et al. 2003) for analysis of body fluids containing macromolecules using CPMG pulse sequence along with WET pulse sequence for suppression of water signal. Suppression of broad signal depends upon the T_2 of corresponding signal and total echo time used. But along with suppression of broad signal most of the sharp signal is also suppressed depending upon the T_2 of corresponding signal. For study of T_2 effect a series of experiments were performed using single pulse sequence and CPMG pulse sequence with a fix echo time and recycle delays. A constant difference was observed at any delays time using a fixed echo time (Fig. 6). The δ-CH_2-proton of Arginine observed at 1.91 ppm was taken to describe the effect of spin–spin relaxation (Fig. 6). It was observed that the concentration variations due to T_1 effect at different repetition times were found to be similar for single pulse and CPMG pulse sequence analysis. Concentration difference of 35 and 65 mg/dl were observed for total echo time of 200 and 400 ms, respectively.

A series of experiments were performed on standard solution of amino acids using CPMG pulse sequence at different echo time. Concentrations measured by CPMG

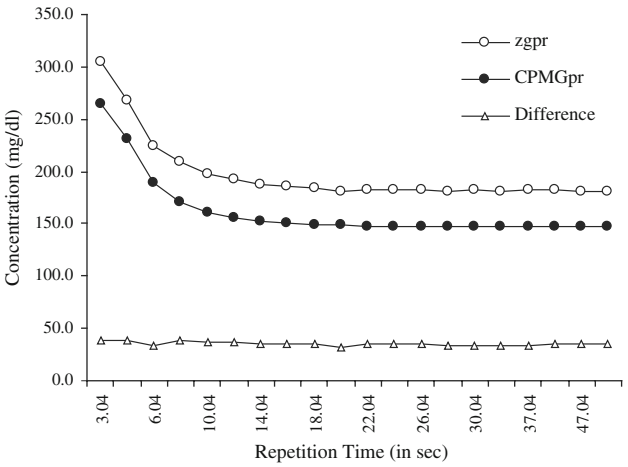

Fig. 6 Variation of erroneous concentration due to T_1 and T_2 at different delays and a fix echo time. Concentration measured by (**a**) single pulse sequence and (**b**) CPMG pulse sequence. (**c**) The concentration difference between single pulse and CPMG

sequence with short echo time are equal to the concentrations measured from the spectrum recorded with single pulse sequence. We have been able to compare the quantitative estimation of concentration measured from the spectrum recorded with a longer echo time with incorporation of T_2 correction factor. It is not always true that concentration calculated by CPMG pulse is lower than the actual concentration. If the T_2 of metabolites resonance is longer than TSP the provided conc. will be higher than actual concentration i.e. calculated by single pulse sequence. For example, T_2 of Alanine (resonance at 3.78 ppm) and Proline (resonance at 4.15 ppm) have T_2 values greater than T_2 of TSP (Table 1). The values of measured concentration by CPMG for these resonances are higher than measured by one pulse experiment. For estimating the error in concentration measurement by CPMG sequence, we derived the following expression based on Bloch equation.

$$\mathrm{Conc}_{(zg)} = \mathrm{Conc}_{(cpmg)} \frac{\exp^{-t_s/T_2^S}}{\exp^{-t_s/T_2^M}} \quad (2)$$

Here t_s is the echo time and T_2^S and T_2^M are the T_2 of TSP and test metabolite respectively. $\mathrm{Conc}_{(zg)}$ and $\mathrm{Conc}_{(cpmg)}$ is the measured concentration by single pulse and by CPMG experiments respectively.

For validation of T_2 correction factor, quantitative estimation of actual concentration was carried out from the CPMG analysis at different echo times (10 ms, 50 ms, 100 ms, 200 ms, 400 ms, 2.0 s etc.) and compared with concentrations measured by single pulse sequence at full relaxation. Distributions in fractional errors (ratio of concentration measurement in CPMG sequence and single pulse experiment) in concentration measurements with and

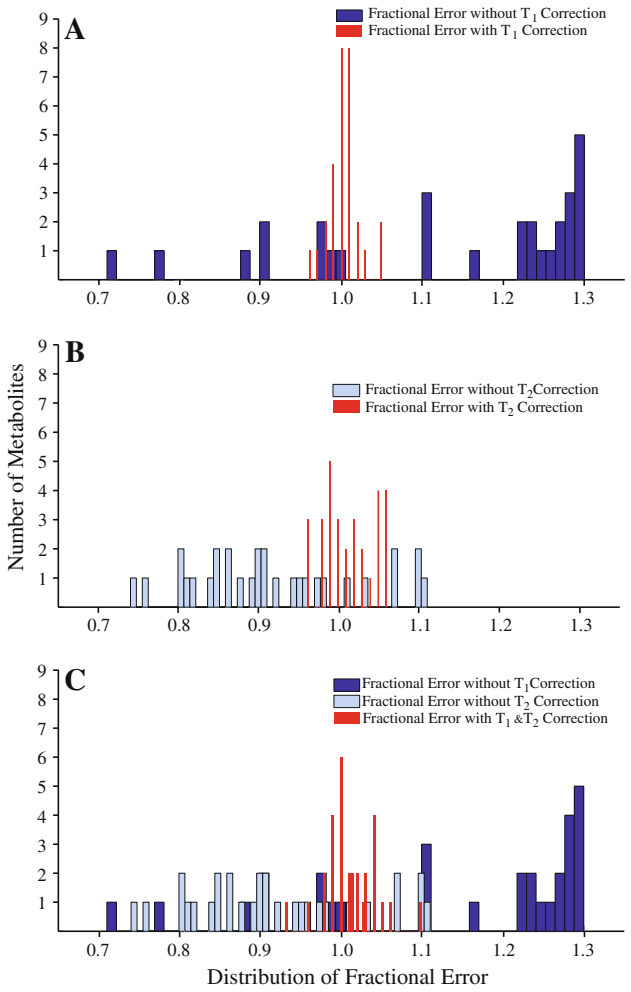

Fig. 7 Graphical representation of quantitative inaccuracy of metabolites resonances with and without T_1 and T_2 correction factor. Fractional error is defined as ratio of observed and actual concentration. (**a**) Distribution of fractional error with and without T_1 correction factor. (**b**) Distribution of fractional error with and without T_2 correction factor. (**c**) Distribution of fractional error with and without T_1 and T_2 correction factor when quantitative analysis was carried out by CPMG sequence using shorter repetition time

without incorporation of T_2 correction factor are shown in Fig. 7b. We can clearly see that the distribution of errors in concentration measured by CPMG (Fig. 7b, sky blue bar) sequence reduces from 75% to 96% by incorporating T_2 correction factor (Fig. 7b, red bar). Quantitative analyses of serum samples using CPMG pulse and shorter relaxation delays introduce inaccuracy in the measured concentration due to incomplete T_1 and T_2 relaxation. Therefore, for improved quantitative analyses one has to either increase the relaxation delay in order to remove the effect of T_1 followed by implementation of T_2 correction factor or to incorporate T_1 along with T_2 correction factor in shorter repetition time measurements.

$$\mathrm{Conc}_{(\mathrm{zg})} = \left[\mathrm{Conc}_{(\mathrm{cpmg})}\frac{\exp^{-t_s/T_2^S}}{\exp^{-t_s/T_2^M}}\right] \times \left[\frac{1-\exp^{-t/T_1^S}}{1-\exp^{-t/T_1^M}}\right] \quad (3)$$

Here $\mathrm{Conc}_{(\mathrm{zg})}$ is estimated concentration at full relaxation by single pulse program. Equation 3 can be directly used for concentration measurement by CPMG pulse program at shorter repetition time which includes correction for both T_1 and T_2 relaxation Incorporation of both T_1 and T_2 correction factors increase accuracy. Figure 7c shows the distribution of fractional errors in the concentration measurement from such experiment. It can be clearly seen that the fractional error can be reduced from 70–130% (Fig. 7c, blue and sky blue bar) to 95–96% (Fig. 7c, red bar) by incorporating simultaneously T_1 and T_2 correction factors.

3.4 Validation of method in serum sample

For validation of correction factors, known concentrations of formate and uracil were externally added to the serum samples. All serum samples were mixed with NH_4Cl (final conc. 0.8 M) to release the bindings and better water suppression. The single pulse ^1H NMR and CPMG spectra

Table 2 Summary of validation results in serum

Metabolite	T_1	T_2	$RWTT_1$[a]	RWT_1[b]	$RWTT_2$[c]	RWT_2[d]	$RPWT_1$[e]	RWT_1T_2[f]
Formate	7.65	1.39	0.72	1.01	0.86	0.94	0.87	0.95
			0.74	1.04	0.85	0.93	0.88	0.96
			0.72	1.01	0.96	1.05	0.97	1.06
			0.75	1.05	0.85	0.93	0.89	0.97
			0.74	1.03	0.86	0.94	0.89	0.97
Uracil	1.97	0.59	1.28	1.02	0.70	0.92	0.71	0.95
			1.26	1.01	0.65	0.87	0.66	0.87
			1.19	0.95	0.86	1.15	0.82	1.09
			1.23	0.99	0.77	1.02	0.76	1.01
			1.25	1.00	0.87	1.15	0.87	1.16

[a,b,c,d,e,f] Same as Table 1

of serum sample are shown in Fig. 3a and b respectively. The concentration of formate and uracil were measured at both shorter repetition time and full relaxation. Incorporation of T_1 correction factor provides an average inaccuracy of 3% for formate ($n = 5$) and 0.5% for uracil ($n = 5$). Similarly incorporation of T_1 and T_2 correction factors at shorter repetition time provides an average inaccuracy of 2% for formate and uracil (Table 2).

4 Conclusions

The method for the fast metabonomic studies of biological fluid based on accurate quantification of metabolite from the one dimensional spectra recorded with smaller recycle delay is presented. Utility of the application of T_1 and T_2 correction factors for the improved quantitation of metabolites is demonstrated and validated.

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References

Akoka, S., Barantin, L., & Trierweiler, M. (1999). Concentration measurement by proton NMR using the ERETIC method. *Analytical Chemistry, 71*, 2554–2557. doi:10.1021/ac981422i.

Bala, L., Ghoshal, U. C., Ghoshal, U., et al. (2006). Malabsorption syndrome with and without small intestinal bacterial overgrowth: A study on upper-gut aspirate using ^{1}H NMR spectroscopy. *Magnetic Resonance in Medicine, 56*, 738–744. doi:10.1002/mrm.21041.

Bala, L., Gowda Nagana, G. A., Ghoshal, U. C., Misra, A., Bhandari, M., & Khetrapal, C. L. (2004). ^{1}H NMR spectroscopic method for diagnosis of malabsorption syndrome: A pilot study. *NMR in Biomedicine, 17*, 69–75. doi:10.1002/nbm.866.

Bell, J. D., Brown Judith, C. C., & Sadler, P. J. (1989). NMR studies of body fluids. *NMR in Biomedicine, 2*, 246–256. doi:10.1002/nbm.1940020513.

Bell, J. D., Lee, J. A., Lee, H. A., Sadler, P. J., Wilkie, D. R., & Woodham, R. H. (1991). Nuclear magnetic resonance studies of blood plasma and urine from subjects with chronic renal failure: Identification of trimethylamine-N-oxide. *Biochimica et Biophysica Acta, 1096*, 101–107.

Bell, J. D., & Sadler, P. J. (1999). Body fluids. In *Encyclopedia of magnetic resonance* (vol. 2, pp. 989–1001). New York: Wiley.

Bloch, F. (1946). Nuclear induction. *Physical Review, 70*, 460–474. doi:10.1103/PhysRev.70.460.

Carr, H. Y., & Purcell, E. M. (1954). Effect on diffusion on free precision in nuclear magnetic resonance experiments. *Physical Review, 94*, 630–638. doi:10.1103/PhysRev.94.630.

Caytana, E., Remaud, G. S., Tenailleaua, E., & Akoka, S. (2007). Precise and accurate quantitative ^{13}C NMR with reduced experimental time. *Talanta, 71*(3), 1016–1021. doi:10.1016/j.talanta.2006.05.075.

Daviesa, S. E. C., Woolf, D. A., Chalmers, R. A., Raftera, J. E. M., & Iles, R. A. (1992). Proton nmr studies of betaine excretion in the human neonate: consequences for choline and methyl group supply. *The Journal of Nutritional Biochemistry, 3*(10), 523–530. doi:10.1016/0955-2863(92)90074-S.

Daykin, C. A., Foxall, P. J. D., Connor, S. C., Lindon, J. C., & Nicholson, J. K. (2002). The comparison of plasma deproteinization methods for the detection of low-molecular-weight metabolites by ^{1}H nuclear magnetic resonance spectroscopy. *Analytical Biochemistry, 304*, 220–230. doi:10.1006/abio.2002.5637.

de Graaf, R. A., & Behar, K. L. (2003). Quantitative ^{1}H NMR spectroscopy of blood plasma metabolites. *Analytical Chemistry, 75*(9), 2100–2104. doi:10.1021/ac020782+.

Evilia, R. F. (2001). Quantitative NMR spectroscopy. *Analytical Letters, 34*(13), 2227–2236. doi:10.1081/AL-100107290.

Gang, S., Kautz, R., Shiqi, P., Guohui, C., & Giese, R. (2007). Calibration by NMR for quantitative analysis: P-Toluenesulfonic acid as a reference substance. *Journal of Chromatography A, 1138*, 305–308. doi:10.1016/j.chroma.2006.10.010.

Griffiths, L., & Irving, A. M. (1998). Assay by nuclear magnetic resonance spectroscopy: Quantification limits. *Analyst (London), 123*, 1061–1068. doi:10.1039/a800625c.

Hahn, E. L. (1950). Spin echoes. *Physical Review, 80*, 580–594. doi:10.1103/PhysRev.80.580.

Henderson, T. J. (2002). Quantitative NMR spectroscopy using coaxial inserts containing a reference standard: Purity determinations for Military nerve agents. *Analytical Chemistry, 74*, 191–198. doi:10.1021/ac010809+.

Kriat, M., Gouny, S. C., Dury, J. V., Sciaky, M., Viout, P., & Cozzone, J. P. (1992). Quantitation of metabolites in human blood serum by proton magnetic resonance. A comparative study of the use of Formate and TSP as concentration standards. *NMR in Biomedicine, 5*, 179–184. doi:10.1002/nbm.1940050404.

Larive, C. K., Jayawickrama, D., & Orfi, L. (1997). Quantitative analysis of peptides with NMR spectroscopy. *Applied Spectroscopy, 51*, 1531–1536. doi:10.1366/0003702971939055.

Lindon, J. C., Holmes, E., Bollard, M. E., Stanley, E. G., & Nicholson, J. K. (2004). Metabonomics technologies and their applications in physiological monitoring, drug safety assessment and disease diagnosis. *Biomarkers, 9*(1), 1–31.

Lindon, J. C., Nicholson, J. K., Holmes, E., & Everett, J. R. (2000). Metabonomics: Metabolic processes studied by NMR spectroscopy of biofluids. *Concepts in Magnetic Resonance, 12*(5), 289–320. doi:10.1002/1099-0534(2000)12:5<289::AID-CMR3>3.0.CO;2-W.

Lucas, L. H., Larive, C. K., Wilkinson, P. S., & Huhn, S. (2005). Progress toward automated metabolic profiling of human serum: Comparison of CPMG and gradient-filtered NMR analytical methods. *Journal of Pharmaceutical and Biomedical Analysis, 39*, 156–163. doi:10.1016/j.jpba.2004.09.060.

Malz, F., & Jancke, H. (2005). Validation of quantitative NMR. *Journal of Pharmaceutical and Biomedical Analysis, 38*(5), 813–823. doi:10.1016/j.jpba.2005.01.043.

Meiboom, S., & Gill, D. (1958). Modified spin echo method for measuring nuclear magnetic relaxation times. *The Review of Scientific Instruments, 29*, 688–691. doi:10.1063/1.1716296.

Nishijima, T., Nishina, M., & Fujiwara, K. (1997). Measurement of lactate levels in serum and bile using proton nuclear magnetic resonance in patients with hepatobiliary diseases: Its utility in detection of malignancies. *Japanese Journal of Clinical Oncology, 27*(1), 13–17. doi:10.1093/jjco/27.1.13.

Pauli, G. F., Jaki, B. U., & Lankin, D. C. (2006). Quantitative ^{1}H NMR: Development and potential of a method for natural products analysis. *Journal of Natural Products, 68*, 133–149. doi:10.1021/np0497301.

Saude, E. J., Slupsky, C. M., & Sykes, B. D. (2006). Optimization of NMR analysis of biological fluids for quantitative accuracy. *Metabolomics, 2*, 113–123. doi:10.1007/s11306-006-0023-5.

Saxena, V., Gupta, A., Gupta Nagana, G. A., Saxena, R., Yachha, S. K., & Khetrapal, C. L. (2006). ^{1}H NMR spectroscopy for the prediction of therapeutic outcome in patients with fulminant hepatic failure. *NMR in Biomedicine, 19*(5), 521–526. doi:10.1002/nbm.1034.

Shiyan, F., Choy, W. Y., Lam, S. L., & Au-Yeung, S. C. F. (1992). Quantitative determination of glucose in blood plasma and in fruit juices by combined WATR-CPMG ^{1}H NMR spectroscopy. *Analytical Chemistry, 64*, 2570–2574. doi:10.1021/ac00045a018.

Silvestre, V., Goupry, S., Trierweiler, M., Robins, R., & Akoka, S. (2001). Determination of substrate and product concentration in lactic acid bacterial fermentations by proton NMR using the ERETIC method. *Analytical Chemistry, 73*, 1862–1868. doi:10.1021/ac0013204.

Singh, H. K., Yachha, S. K., Saxena, R., et al. (2006). New dimension of ^{1}H-NMR spectroscopy in assessment of liver graft dysfunction. *NMR in Biomedicine, 16*(4), 185–188. doi:10.1002/nbm.829.

Subramanian, A., Gupta, A., Saxena, S., et al. (2005). Proton MR CSF analysis and a new software as predictors for the differentiation of meningitis in children. *NMR in Biomedicine, 18*, 213–225. doi:10.1002/nbm.944.

Van, Q. N., Chmurny, G. N., & Veenstra, T. D. (2003). The depletion of protein signals in metabonomics analysis with the WET-CPMG pulse sequence. *Biochemical and Biophysical Research Communications, 301*, 952–959. doi:10.1016/S0006-291X(03)00079-2.

Viant, M., Rosenblum, E., & Tjeerdema, R. (2003). NMR-based metabolomics: A powerful approach for characterizing the effects of environmental stressors on organism health. *Environmental Science & Technology, 37*, 4982–4989. doi:10.1021/es034281x.

Voet, D., & Voet, J. G. (1990). *Biochemistry*. Canada: Wiley.

Wevers, R. A., Engelke, U., & Heerschap, A. (1994). High-resolution ^{1}HNMR spectroscopy of blood plasma for metabolic studies. *Clinical Chemistry, 40*(7), 1245–1250.

Yamaguchi, S., Koda, N., & Yamamoto, H. (1989). Analysis for homogentisic acid by NMR spectrometry, to aid diagnosis of Alkaptonurla. *Clinical Chemistry, 35*(8), 1806–1807.