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Glutathione in whole blood: a novel determination using double quantum coherence transfer proton NMR spectroscopy

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Double quantum selective coherence transfer proton NMR spectroscopy has been used to observe glutathione in whole blood. The efficient water suppression of this technique avoids the need to resuspend the cells in D_2O, hence avoiding equilibrium and kinetic isotope effects. Using this method we estimate the concentration of glutathione in fresh whole rabbit blood at ~1.7 mM.

Glutathione; Proton NMR; Rabbit blood

1. INTRODUCTION

Glutathione plays a key role in many cellular processes including detoxification of xenobiotica [1–3] and homeostasis of redox potential [1]. Given the importance of reduced glutathione (gsh) in many biochemical reactions there is active interest in measuring cellular concentrations [4–6]. Many techniques have been developed to measure the concentrations of glutathione in cells [4,7]. Whilst these methods produce reliable and reproducible results, they nonetheless require biopsy and destructive biochemical analysis. ^{1}H NMR has previously been used to measure glutathione in blood and indeed both reduced (gsh) and oxidized forms (gssg) were observed [5,8]. However, the intense water signal interfered strongly with measurements using traditional spin-echo ^{1}H NMR spectroscopy and thus, cells were usually packed and resuspended in isotonic D_2O [5,6,8–12].

Double quantum selective coherence transfer (DQCT) ^{1}H NMR spectroscopy selects coupled resonances and exhibits exceptional water suppression. This technique has been called metabolite specific spectroscopy [13] and it was previously used to measure lactate in tumors [13,14] and the perfused heart [15]. We now demonstrate the observation and measurement of glutathione using DQCT ^{1}H NMR in whole rabbit blood and estimate the concentration at ~1.7 mM.

2. MATERIALS AND METHODS

Fresh whole blood was drawn from the lateral ear vein of New Zealand White rabbits and stored chilled in heparinized containers prior to NMR observation. In some cases the blood was centrifuged producing blood with 2–3× normal hematocrit.

NMR experiments were performed at 9.4 T (^{1}H at 400 MHz) using a GE Omega spectrometer with a 9 cm bore magnet and self-shielded gradients. Samples in 5 mm NMR tubes were placed in a home-built 3 cm birdcage NMR coil and shimmed on the water signal. Typical linewidth 1.6 Hz in solution and ~17 Hz for whole blood. GSH was selectively observed using DQCT ^{1}H NMR with the sequence shown in Fig. 1 [14]. Multiple quantum coherence was generated using two 90° pulses ($\pi/2_{(a\,b)}$) followed by a third selective binomial $1\bar{3}3\bar{1}$ $\pi/2_{(c)}$ pulse to bring the signal into the single quantum domain. Additional π pulses removed the chemical shift dependence of the multiple quantum coherence. All pulses were on resonance for the β-CH_2 protons of gsh at 2.91 ppm. $\pi/2_{(a\,b)}$ were separated by 37.5 ms, which corresponds to approximately 1/2J, the AX_2 coupling constant of the cysteinyl residue of gsh. To achieve optimal coherence transfer a selective $\pi/2_{(c)}$ $1\bar{3}3\bar{1}$ pulse was used with interpulse delay 780 μs corresponding to $1/2\Delta\delta$, the separation of gsh-cysteinyl CH and CH_2 protons ($\Delta\delta$: 1.6 ppm = 640 Hz at 9.4 T). The delay τ was optimized at 20 ms to maximize signal in the DQ phase. Typical $\pi/2$ pulse = 56 μs and repetition time (TR) 0.8 s. Gradients α and β (2 ms crusher) removed pulse imperfections and γ, 2γ selected the double quantum coherence. Generally 2,000 acquisitions were collected (~25 min) with 2k data points across a spectral width of 4,000 Hz.

In order to quantify gsh an external standard capillary of alanine (50 mM) in water was used, which provided resonances at 1.4 and 3.7 ppm. The technique was optimized using standard solutions of gsh

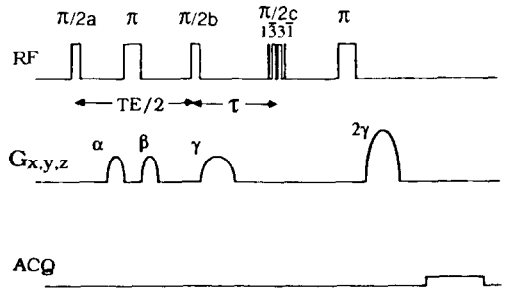

Fig. 1. DQCT ^{1}H NMR pulse sequence [14].

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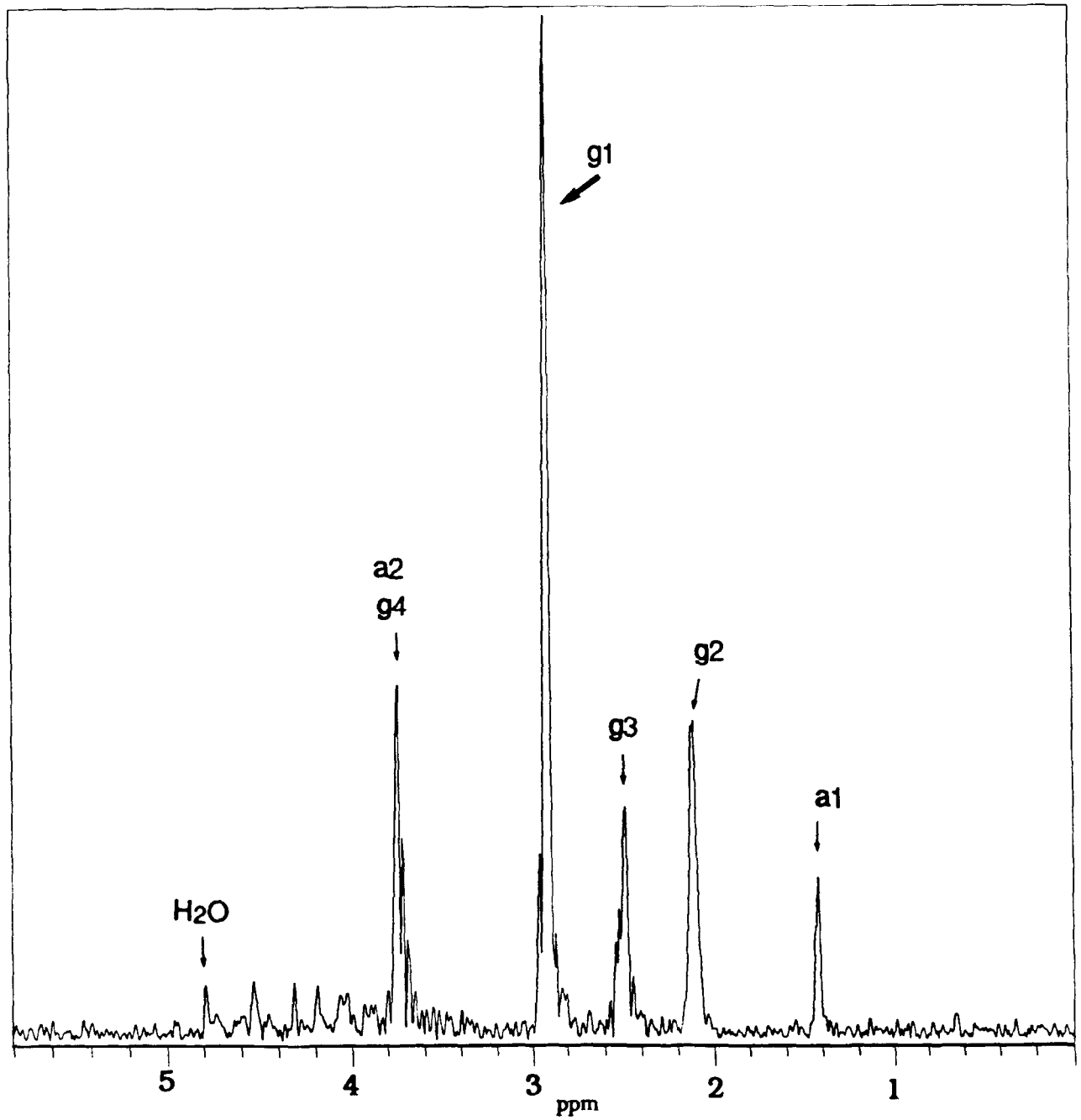

Fig. 2. DQCT ^{1}H NMR spectrum of 50 mM glutathione in water pH 7 with alanine (50 mM) external standard. Dominant peaks correspond to glutathione: β-CH_2 Cys (g1, 2.91 ppm), CH_2-Glu (g2, 2.49 ppm; g3, 2.10 ppm) and Gly (g4, 3.74 ppm). Alanine resonances occur at a1 (1.42 ppm, CH_3) and a2 (3.7 ppm, CH). Water suppression exceeded $\times 10^4$.

(1–50 mM) in water at pH 7. Chemicals were obtained from Sigma, St. Louis, MO.

3. RESULTS

3.1. *Calibration*

DQCT ^{1}H NMR essentially eliminated the water signal and we routinely achieved water suppression ~10^5-fold, as seen in Fig. 2. On the basis of previous reports we assign the dominant resonance at 2.9 ppm to the β-CH_2 protons of gsh [2,8,9,16]. Signals at 1.4 ppm and 3.7 ppm correspond respectively to the CH_3 and CH protons of the external quantitation standard alanine. Additional signals at 2.1 ppm and 2.5 ppm are attributed to the CH_2's in the glutamyl residue in gsh [9]. In addition the CH_2 protons of the glycinyl residue occur at 3.7 ppm overlapping the methine proton of alanine. Spectra were obtained with various concentrations of

gsh (1–50 mM) in water at pH 7 and a linear relationship was determined between the intensity of the signal at 2.9 ppm as referenced to alanine (CH_3) and the concentration of gsh (Fig. 3).

3.2. *Blood*

Gsh was readily detected in fresh whole rabbit blood in 20 min. Fig. 4 shows a typical spectrum obtained from a sample of rabbit blood (packed ×2). In addition to β-Cys CH_2, faint signals were observed for gsh-Glu. Intense signals were provided by the external standard alanine. In addition, signals occurred at 1.3 and 4.1 ppm which were assigned to the CH_3 and CH protons of lactate in the blood. We estimate gsh to be ~1.7 mM in fresh whole rabbit blood.

3.3. *Verification of gsh*

When gsh was added to a sample of blood the signal at 2.9 ppm increased. The increase in signal provided a second means of quantitation and again indicated a blood gsh concentration ~1.6 mM. To investigate interference from other common biomolecules, which have been reported to resonate close to 2.9 ppm, we added samples of creatine and gssg to solutions of gsh. NCH_3 of creatine resonated very close to β-CH_2(Cys) of gsh (3.0 ppm), but did not appear in the DQCT spectrum. GSSG gave rise to two resonances for β-CH_2(Cys) at 2.9 and 3.1 ppm.

4. DISCUSSION

We have observed gsh in whole blood using DQCT ^{1}H NMR and estimate the concentration ~1.7 mM. Unlike most biochemical assays [4,7] this technique is non-destructive. In contrast to previous NMR studies, DQCT specifically selects coupled resonances effectively eliminating the water signal and avoiding the need to repeatedly wash cells and resuspend in D_2O [5,6,8,10].

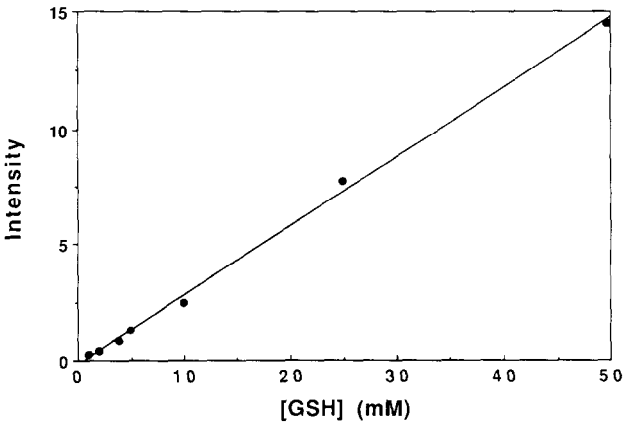

Fig. 3. Variation of signal intensity of β-CH_2 Cys was linear with gsh concentration ($r > 0.998$).

In vitro application of this NMR technique was previously reported to observe lactate in tumor cells and tissue and indeed maps of lactate were presented using MRI [13]. DQCT selects coupled resonances and the CH_3CH (AX_3) system of lactate provides an ideal system. Furthermore, lactate may occur at relatively high concentrations in tissues (~10 mM), particularly under hypoxic conditions [13]. We have detected gsh by exploiting the AX_2 system (CH_2CH) in the cysteinyl residue of gsh. This is particularly attractive because the thiol group is involved in many biochemical reactions and the proton chemical shifts of the β-CH_2 Cys are sensitive to conjugation or oxidation [2,8,10,17]. At physiological pH both the methylene protons (Cys gsh) resonate at 2.91 ppm. Formation of adducts or oxidation makes these protons non-equivalent and the new resonances are readily detected. Chemical shift may also change with pH, but this is only observed above the $pK_a \sim 8$ of the thiol [16,17].

Metabolic studies by ^{1}H NMR often suffer interference from biological water. In essence this NMR pulse sequence acts as a filter allowing the specific observation of coupled signals from molecules with non-equivalent protons. Pulses $\pi/2_{(a,b)}$ generate multiple quantum coherence, a property of coupled resonances only, but this cannot be observed directly by NMR [14]. Thus, the third $\pi/2$ pulse is used to produce an NMR visible signal. The use of gradients γ, 2γ further refines the selection to double quantum coherence only. Other pulses and gradients enhance the efficiency of the selection process to maximize the observed signal.

This NMR technique is highly selective [13] and non-coupled resonances such as water and creatine are eliminated. In traditional spin echo ^{1}H NMR creatine could interfere with gsh determination since the resonances are very close [12]. Various metabolites with coupled resonances may be observed simultaneously. We have optimized the sequence to detect β-CH_2 in gsh, but additional resonances from gsh are also observed (Glu, Gly) together with other molecules. The observation of additional resonances from gsh adds confidence to the identification of gsh. The observation of other molecules indicates that this technique could be used to follow additional biochemical processes (e.g. glycolysis) as well as determining [gsh]. GSSG could interfere with the determination of gsh; however, the cellular concentration is typically ~1% of gsh [1] and the presence of gssg would be immediately apparent from a signal at 3.1 ppm. Both resonances from lactate are observed and this provides an indication of cellular oxygenation and the prevalence of glycolysis. The observation of alanine is important as a quantitation standard in this work. The concentration of gsh we estimate corresponds with values previously reported by others in blood (~2 mM) using biochemical assay [18] or NMR [8].

Traditional biochemical assays based on fluorescence or HPLC may detect much lower concentrations of gsh

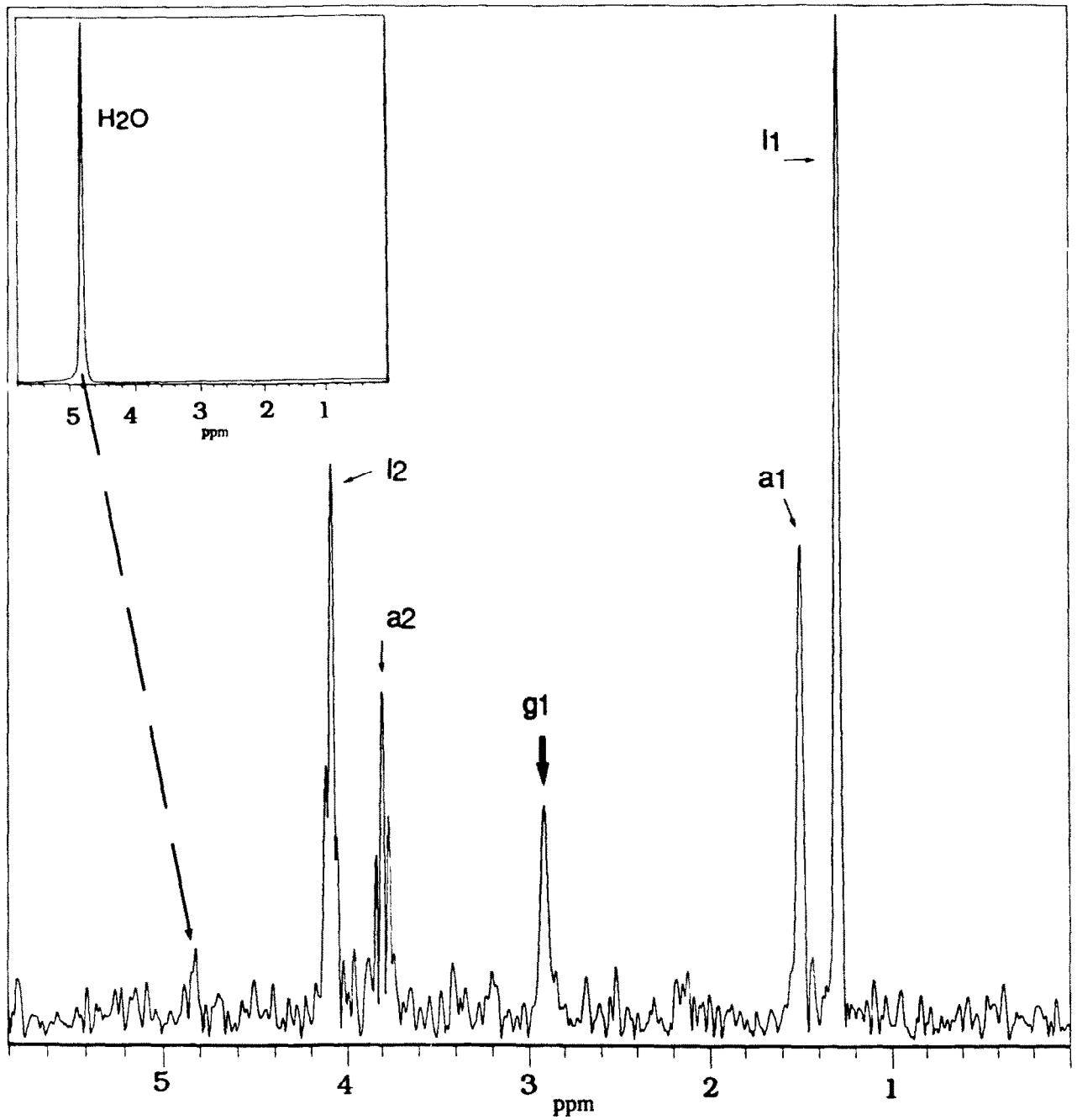

Fig. 4. Top (inset): ^{1}H NMR spectrum of whole rabbit blood (packed ×2). Water is observed at 4.8 ppm. Bottom: DQCT spectrum of same sample acquired in 25 min. GSH (β-CH_2 Cys) is observed at 2.9 ppm together with signals from lactate (1.3 and 4.1 ppm) and external standard alanine (1.47 and 3.7 ppm). Water is essentially eliminated and reference to the standard indicates a cellular gsh concentration ~1.7 mM.

(μM), but they are destructive requiring both biopsy and derivatization [4,7]. Although gsh has a thiol chromophore the weak absorption would be masked in vivo. Previous NMR investigations have used the spin-echo technique; however, this produced weaker water suppression and required the exchange of cellular water by D_2O [5,6,8,10–12]. Although cells tolerate D_2O at low concentrations, high levels may produce significant equilibrium and kinetic isotope effects [19] precluding long-term physiological studies. Indeed, it has been shown that deuterons are incorporated into lactate during the metabolism of glucose by erythrocytes in D_2O and this causes a substantial isotope effect [9]. Cellular gsh has also been demonstrated using ^{13}C NMR spectroscopy [2,20,21]. Direct observation of ^{13}C natural abundance gsh was recently reported without the need for isotopic enrichment in specific tumor cell lines, which exhibit elevated gsh [20]. Alternative approaches

include the administration of ^{13}C-labelled precursors of gsh to enhance the signal as demonstrated in the lens [21] or the use of a molecular amplifier reporter molecule. [^{13}C]Formaldehyde undergoes an equilibrium reaction with gsh to form *S*-(hydroxymethyl)glutathione, which has a characteristic signal and has been used to estimate gsh in bacteria [2].

Gsh is implicated in MDR (multiple-drug resistance) and resistance to radiotherapy in tumor cells [1,22,23] and substantial increases in gsh have been reported in drug resistant cell lines [1,3]. We are currently evaluating the technique described here to examine tumor cells.

Gsh is involved in many cellular processes particularly involving drug detoxification and quenching free radicals [1,22,23]. There is some indication that the concentration of gsh in blood may be a useful prognostic indicator for disease therapy, e.g. the response of rheumatoid arthritis to penicillamine therapy [6,12]. The technique demonstrated here may simplify the analysis of gsh for routine quantitation in the future.

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